

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS QUÍMICAS



TESIS DOCTORAL

**Plataformas electroquímicas para el diseño de biosensores de
afinidad aplicados a la detección de biomarcadores de
enfermedades autoinmunes**

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid

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Summary

Introduction
Aims of this work
Research results
Milestones

ELECTROCHEMICAL PLATFORMS FOR THE DESIGN OF AFFINITY BIOSENSORS APPLIED TO THE DETECTION OF BIOMARKERS OF AUTOIMMUNE DISEASES

1.1. INTRODUCTION

Autoimmunity is the self-destructive effector response of the immune system in which different levels of manifestation with a variety of nuances can be found. Autoimmune diseases emerge when the immune system does not distinguish between foreign and its own elements and produces antibodies that attack body cells. This condition can be initiated by an infection, but the immune activation persists in the absence of the antigen. There are at least eighty different autoimmune diseases which can be subtle in onset and present a variety of clinical manifestations. This situation makes hard to establish a proper diagnosis which depends upon careful clinical evaluation.

Usually, autoimmune diseases are classified into two types according to the number of organs damaged. Firstly, organ-specific autoimmune diseases which can be endocrine, dermatological or hematological, are characterized by the involvement of a particular tissue. Furthermore, systemic autoimmune diseases occur when antibodies attack non-specific antigens in more than one organ. In this work, we have focused on diseases of the second type, mainly in rheumatoid arthritis (RA) and multiple sclerosis (MS).

RA is a common and chronic inflammatory disease characterized by an excessive immune activation and inflammation which almost involve all body tissues. Typically, RA symptoms appear as symmetric polyarticular joint pain and swelling but also patients may suffer from fatigue, fever, muscle pains, weight loss and, in some cases, depression. There are several genetic and environmental factors highly associated with an increased risk for suffering RA. The most common contributors are female sex, family background, mucosal inflammation and microbial factors. Furthermore, obesity, exposure to tobacco smoke and air pollution, high consumption of sodium, red meat, and low iron and vitamin D levels are well-known risk factors for the development of RA.

Regarding MS, this chronic, inflammatory and neuro-degenerative autoimmune disease results in damage to myelin sheaths in the central nervous system (CNS). Myelin is the most abundant membrane structure within the vertebrate nervous system, allowing the fast conduction of nerve impulses through the nerve fibers, due to its high resistance and low capacitance. MS affects neurons within the brain and spinal cord that are involved with sensory and motor control functions. It is characterized by relapses, remissions and progression of disability over time, which lead to inhibited muscle coordination and loss of cognitive functions among other neurological effects.

Besides the importance of establishing new effective disease-suppressing therapies, sensitive detection methods in order to prevent the occurrence of undesirable consequences of autoimmune diseases, which are associated with this persistent and erosive illness, are required. Faced with the time consuming and expensive methods commonly used, clinical tests based on biomarkers monitoring should be carried out to facilitate the diagnosis and evaluate the severity of these diseases.

1.2. AIMS OF THIS WORK

The main objective of this work is the development of electrochemical bioplatfroms for the determination of autoimmune diseases biomarkers specific for autoimmune pathologies with inflammatory nature, which present a difficult diagnosis in initial stages. Thus, several types of biomolecules related to RA and MS, recognized as biomarkers of these diseases have been chosen as target compounds, and detection methods based on electrochemical biosensors with sufficient sensitivity and accuracy to be used in clinical trials have been developed.

For this purpose, several configurations involving the use of screen-printed electrodes (SPEs) for implementation of both integrated immunosensors and magnetically assisted bioplatfroms were implemented. In the first case, the electrode surface was modified with materials able to immobilize biomolecules also improving the electrochemical responses, and the second type employed functionalized magnetic microbeads as support for immunoreagents immobilization. Furthermore, all the developed designs implied the establishment of immunoassay schemes based on antibody-antigen reactions to quantify the unknown concentration of analytes.

As it will be seen, all this research work was carried out by application of the usual methodology of bioelectroanalysis, which involves the design of the electrochemical bioplatfrom and the immunosensing strategy, the optimization of experimental variables, characterization studies, construction of the calibration plots and establishment of the analytical characteristics: sensitivity, selectivity and stability, among others. Moreover, in all cases, the analytical usefulness of the methods was demonstrated by application to samples of clinical interest.

Last but not least, the results obtained by each of the methodologies applied throughout the different research works, were validated by comparison with those results provided by reference commercial methodologies, such as ELISA immunoassays.

1.3. RESEARCH RESULTS

In this work, gold SPEs (SPAuEs) modified by oxidative grafting with *p*-aminobenzoic acid (*p*-ABA) for covalent immobilization of specific antibodies for Transforming Growth Factor β 1 (TGF- β 1) or Peptide YY (PYY) were used for developing electrochemical immunosensors applied to plasma, serum and saliva.

Moreover, carbon SPEs (SPCEs) modified with multiwalled carbon nanotubes (MWCNTs) for immobilization of antibodies for Chemokine (C-X-C motif) Ligand 7 (CXCL7) assisted by click chemistry, and Interleukin 1 beta (IL-1 β) cytokine using electro-click methodology were also used, and the prepared immunosensors were also applied to serum and saliva.

Furthermore, dual and single SPCEs were also utilized as electrochemical substrates for implementation of magnetically assisted bioplatforms, applied to the simultaneous determination of Rheumatoid Factor (RF) and Anti-Cyclic Citrullinated Peptide (CCPA), or Matrix Metalloproteinase-3 (MMP3) and CXCL7, and individually for anti-MBP and Chemokine (C-C motif) ligand 5 (CCL5). All these configurations were applied to serum samples of healthy individuals or patients diagnosed with RA or MS.

1.4. MILESTONES

The work carried out in this Doctoral Thesis has allowed the development of four electrochemical immunosensors involving novel immobilization strategies as well as four bioplatfroms for implementing magnetically assisted immunoassays, two of them dual. All these designs were applied to the determination of biomarkers related to autoimmune diseases, focusing on systemic inflammatory diseases, especially rheumatoid arthritis (RA) and multiple sclerosis (MS).

It should be noted the implementation of novel immobilization schemes for immunoreagents involving the functionalization of screen-printed gold electrodes by oxidative grafting of arylamines were prepared for the first time and applied to the determination of the cytokine TGF- β 1 and peptide PPY, demonstrating the usefulness of this strategy against the preparation of monolayers of thiol derivatives, being demonstrated.

Another noteworthy contribution is the use of azide-functionalized carbon nanotubes linked to ethynylated immunoglobulin, by Cu(I)-catalyzed azide-alkyne cycloaddition reaction ("click"), as supports for antibody immobilization in the preparation of immunosensors for CXCL7 chemokine and interleukin IL-1 β . These two configurations are relevant due to the scarcity of applications of "click chemistry" to the preparation of biosensors. In addition, in this work, the advantage of the electrochemical synthesis of the catalyst ("electro-click") by applying a reduction potential in the presence of a Cu(II) salt was also demonstrated.

Furthermore, with the aim of preparing multiple schemes for the simultaneous detection of various biomarkers, two dual electrochemical immunoplatfroms involving functionalized magnetic microparticles (MBs) were developed and applied to the simultaneous determination of two auto-antibodies: factor rheumatoid (RF) and anti-cyclic citrullinated peptide (CCPA), as well as two proteins, the chemokine CXCL7 and the enzyme matrix metalloproteinase-3 (MMP-3), all of them relevant biomarkers of rheumatoid arthritis.

A similar strategy was used for the preparation of two individual immunoplatfroms for the determination of biomarkers of multiple sclerosis: the auto-antibody anti-myelin basic protein (anti-MBP) and the chemokine CCL5.

The particular advantages of the methods developed for these biomarkers using the proposed immunodetection devices in comparison with other methods involving electrochemical transduction, if they exist, and with respect to ELISA immunoassay procedures, have been described throughout the Thesis.

Finally, it should be noted that both immunosensors and detection immunoplatfroms demonstrated their analytical utility through the application and validation of results on real samples of serum or saliva, being the results obtained in the analysis of sera samples from patients with diagnosed RA or MS being especially important.

As a result of the research work described in this Doctoral Thesis, the following scientific articles were published:

1. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Screen-printed gold electrodes functionalized with grafted p-aminobenzoic acid for the construction of electrochemical immunosensors. Determination of TGF- β 1 cytokine in human plasma, *Electroanalysis* 30 (2018) 1327 – 1335.

2. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Oxidative grafting vs. monolayers self-assembling on gold surface for the preparation of electrochemical immunosensors. Application to the determination of peptide YY, *Talanta* 193 (2019) 139 – 145.

3. S. Guerrero, D. Cadano, L. Agüí, R. Barderas, S. Campuzano, P. Yáñez-Sedeño, J.M. Pingarrón, Click chemistry-assisted antibodies immobilization for immunosensing of CXCL7 chemokine in serum, *Journal of Electroanalytical Chemistry* 837 (2019) 246 – 253.

4. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Design of electrochemical immunosensors using electro-click chemistry. Application to the detection of IL-1 β cytokine in saliva, *Bioelectrochemistry* 133 (2020) 107484.

5. S. Guerrero, E. Sánchez-Tirado, G. Martínez-García, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Electrochemical biosensor for the simultaneous determination of rheumatoid factor and anti-cyclic citrullinated peptide antibodies in human serum, *Analyst* 145 (2020) 4680 – 4687.

6. S. Guerrero, E. Sánchez-Tirado, L. Agüí, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Simultaneous determination of CXCL7 chemokine and MMP3 metalloproteinase as biomarkers for rheumatoid arthritis, *Talanta* 234 (2021) 122705.

7. S. Guerrero, E. Sánchez-Tirado, L. Agüí, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Monitoring autoimmune diseases by bioelectrochemical detection of autoantibodies. Application to the determination of anti-myelin basic protein autoantibodies in serum of multiple sclerosis patients, *Talanta* 243 (2022) 123304.

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Resumen

Introducción
Objetivos del trabajo
Resultados
Logros

PLATAFORMAS ELECTROQUÍMICAS PARA EL DISEÑO DE BIOSENSORES DE AFINIDAD APLICADOS A LA DETECCIÓN DE BIOMARCADORES DE ENFERMEDADES AUTOINMUNES

2.1. INTRODUCCIÓN

La autoinmunidad es la respuesta efectora autodestructiva del sistema inmunitario en la que se pueden encontrar diferentes niveles de manifestación con una variedad de matices. Las enfermedades autoinmunes surgen cuando el sistema inmunológico no distingue entre elementos extraños y propios, y produce anticuerpos que atacan a las células del cuerpo. Esta condición puede ser iniciada por una infección, pero la activación inmune persiste en ausencia del antígeno. Hay al menos ochenta enfermedades autoinmunes diferentes que pueden tener un inicio sutil y presentar una variedad de manifestaciones clínicas. Esta situación dificulta establecer un diagnóstico adecuado que depende de una cuidadosa evaluación clínica.

Por lo general, las enfermedades autoinmunes se clasifican en dos tipos según la cantidad de órganos dañados. En primer lugar, las enfermedades autoinmunes órgano-específicas que pueden ser endocrinas, dermatológicas o hematológicas, se caracterizan por la afectación de un determinado tejido. Por otro lado, las enfermedades autoinmunes sistémicas ocurren cuando los anticuerpos atacan antígenos no específicos en más de un órgano. En este trabajo nos hemos centrado en las enfermedades del segundo tipo, principalmente en la artritis reumatoide (RA) y la esclerosis múltiple (MS).

La RA es una enfermedad inflamatoria común y crónica caracterizada por una excesiva activación inmune e inflamación que involucra a casi todos los tejidos del cuerpo. Por lo general, los síntomas aparecen en forma de dolor e inflamación de las articulaciones poliarticulares simétricas, pero los pacientes también pueden sufrir fatiga, fiebre, dolores musculares, pérdida de peso y, en algunos casos, depresión. Existen varios factores genéticos y ambientales altamente asociados con un mayor riesgo de padecer RA. Los más comunes son el sexo femenino, los antecedentes familiares, la inflamación de las mucosas y los factores microbianos. Además, la obesidad, la exposición al humo del tabaco y a la contaminación del aire, el alto consumo de sodio,

carnes rojas, así como los niveles bajos de hierro y vitamina D, son conocidos factores de riesgo para el desarrollo de la RA.

En cuanto a la MS, esta enfermedad autoinmune crónica, inflamatoria y neurodegenerativa provoca daños en las vainas de mielina del sistema nervioso central (CNS). La mielina es la estructura de membrana más abundante dentro del sistema nervioso de los vertebrados, y permite la rápida conducción de los impulsos nerviosos a través de las fibras nerviosas debido a su alta resistencia y baja capacitancia. La MS afecta las neuronas dentro del cerebro y la médula espinal que están involucradas con las funciones de control sensorial y motor. Se caracteriza por recaídas, remisiones y progresión de la discapacidad a lo largo del tiempo que conducen a la inhibición de la coordinación muscular y la pérdida de las funciones cognitivas, entre otros efectos neurológicos.

Además de la importancia de establecer nuevas terapias eficaces supresoras de estas enfermedades, se requieren métodos de detección sensibles para prevenir la aparición de consecuencias indeseables de enfermedades autoinmunes, que están asociadas con estas enfermedades persistentes y erosivas. Ante los métodos lentos y costosos comúnmente utilizados, se deben realizar pruebas clínicas basadas en el seguimiento de biomarcadores que faciliten el diagnóstico y evaluación de la gravedad de la enfermedad.

2.2. OBJETIVOS DEL TRABAJO

El objetivo principal de este trabajo es el desarrollo de bioplataformas electroquímicas para la determinación de biomarcadores de enfermedades autoinmunes específicos para patologías de carácter inflamatorio, que presentan un difícil diagnóstico en etapas iniciales. Así, se han elegido como compuestos diana varios tipos de biomoléculas relacionadas con la RA y la MS, reconocidas como biomarcadores de estas enfermedades, y se han desarrollado métodos de detección basados en biosensores electroquímicos con suficiente sensibilidad y precisión para ser utilizados en ensayos clínicos. Para ello, se implementaron varias configuraciones que involucran el uso de electrodos serigrafiados para la construcción tanto de inmunosensores integrados como de bioplataformas asistidas magnéticamente. En el primer caso, la superficie del

electrodo se modificó con materiales capaces de inmovilizar biomoléculas mejorando además las respuestas electroquímicas, y el segundo tipo empleó micropartículas magnéticas funcionalizadas como soporte para la inmovilización de inmunorreactivos. Además, todos los diseños desarrollados se basaron en el establecimiento de esquemas de inmunoensayo, mediante reacciones anticuerpo-antígeno, para cuantificar la concentración desconocida de los analitos.

Como se verá, todo este trabajo de investigación se llevó a cabo mediante la aplicación de la metodología habitual del bioelectroanálisis que implica el diseño de la bioplataforma electroquímica y la estrategia de inmunodetección, la optimización de las variables experimentales, estudios de caracterización, construcción de los calibrados y establecimiento de las características analíticas: sensibilidad, selectividad y estabilidad, entre otras. Además, en todos los casos, la utilidad analítica de los métodos se demostró mediante la aplicación a muestras de interés clínico.

Por último, pero no por ello menos importante, los resultados obtenidos por cada una de las metodologías, aplicadas a lo largo de los diferentes trabajos de investigación, fueron validados por comparación con los resultados proporcionados por metodologías comerciales de referencia, siendo un ejemplo de ello, los inmunoensayos ELISA.

2.3. RESULTADOS

En este trabajo, se utilizaron electrodos serigrafiados de oro (SPAuEs) modificados mediante "grafting" oxidativo con ácido *p*-aminobenzoico (*p*-ABA) para la inmovilización covalente de anticuerpos específicos para el Factor de Crecimiento Transformante $\beta 1$ (TGF- $\beta 1$) o el Péptido YY (PYY), con el fin de desarrollar inmunosensores electroquímicos aplicados a plasma, suero y saliva.

Además, se modificaron electrodos serigrafiados de carbono (SPCE) con nanotubos de carbono de pared múltiple (MWCNT) funcionalizados con anticuerpos secundarios para la inmovilización de anticuerpos para la quimioquina (C-X-C motif) Ligando 7 (CXCL7) asistida por química "click", y para la citoquina Interleucina 1 beta (IL-1 β) utilizando la metodología de "electro-click". Los inmunosensores preparados también se aplicaron a suero y saliva.

Los SPCE duales y simples también se utilizaron como sustratos electroquímicos para la implementación de bioplataformas asistidas magnéticamente aplicadas a la determinación simultánea del factor reumatoide (RF) y el Péptido Citrulinado Anticíclico (CCPA), así como la enzima Matriz Metaloproteínasa-3 (MMP3) y CXCL7, e individualmente para el anticuerpo anti-Proteína Básica de Mielina (anti-MBP) y la quimioquina (C-C motif) Ligando 5 (CCL5). Todas estas configuraciones se aplicaron a muestras de suero de individuos sanos o pacientes diagnosticados de AR o EM.

2.4. LOGROS

El trabajo realizado en esta Tesis Doctoral ha permitido desarrollar cuatro inmunosensores electroquímicos que implican estrategias de inmovilización novedosas, así como cuatro bioplataformas para la implementación de inmunoensayos asistidos magnéticamente, dos de ellos duales. Todos estos diseños se aplicaron a la determinación de biomarcadores relacionados con enfermedades autoinmunes, centrándose en las enfermedades inflamatorias sistémicas, especialmente la artritis reumatoide (RA) y la esclerosis múltiple (MS).

Cabe destacar que la implementación de novedosos esquemas de inmovilización de inmunoreactivos que involucran la funcionalización de electrodos de oro serigrafiados por grafting oxidativo de arilaminas, fueron preparados por primera vez y aplicados a la determinación de la citoquina TGF- β 1 y el péptido PPY, quedando demostrada la utilidad de esta estrategia frente a la preparación de monocapas de derivados tioles.

Otra contribución destacable es el uso de nanotubos de carbono funcionalizados con azida unidos a una inmunoglobulina etinilada, mediante una reacción de cicloadición azida-alquino catalizada por Cu(I) ("click"), como soporte para la inmovilización de anticuerpos en la preparación de inmunosensores para la quimiocina CXCL7 y la interleucina IL-1 β . Estas dos configuraciones son relevantes debido a la escasez de aplicaciones de la "química click" a la preparación de biosensores. Además, en este trabajo también se demostró las ventajas de la síntesis electroquímica del catalizador ("electro-click") al aplicar un potencial de reducción en presencia de una sal de Cu(II).

Además, con el objetivo de preparar múltiples diseños para la detección simultánea de diversos biomarcadores, se desarrollaron dos inmunoplateformas electroquímicas duales que involucran micropartículas magnéticas (MB) funcionalizadas y se aplicaron a la determinación simultánea de dos autoanticuerpos: factor reumatoide (FR) y anti-péptido cíclico citrulinado (CCPA), así como dos proteínas, la quimiocina CXCL7 y la enzima matriz metaloproteinasa-3 (MMP-3), todos ellos biomarcadores relevantes de la artritis reumatoide.

Una estrategia similar fue utilizada para la preparación de dos inmunoplateformas individuales para la determinación de biomarcadores de esclerosis múltiple: el autoanticuerpo anti-proteína básica de mielina (anti-MBP) y la quimiocina CCL5.

Las ventajas particulares de los métodos desarrollados para estos biomarcadores utilizando los dispositivos de inmunodetección propuestos, en comparación con otros métodos de transducción electroquímica, si existen, y con respecto a los procedimientos de inmunoensayo ELISA, se han descrito a lo largo de la Tesis.

Por último, cabe señalar que tanto los inmunosensores como las inmunoplateformas de detección demostraron su utilidad analítica mediante la aplicación y validación de resultados sobre muestras reales de suero o saliva, siendo especialmente importantes los resultados obtenidos en el análisis de muestras de suero procedentes de pacientes con RA o MS diagnosticada.

Como resultado del trabajo de investigación descrito en esta Tesis Doctoral, se han publicado los siguientes artículos científicos:

1. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Screen-printed gold electrodes functionalized with grafted p-aminobenzoic acid for the construction of electrochemical immunosensors. Determination of TGF- β 1 cytokine in human plasma, *Electroanalysis* 30 (2018) 1327 – 1335.

2. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Oxidative grafting vs. monolayers self-assembling on gold surface for the preparation of electrochemical immunosensors. Application to the determination of peptide YY, *Talanta* 193 (2019) 139 – 145.

3. S. Guerrero, D. Cadano, L. Agüí, R. Barderas, S. Campuzano, P. Yáñez-Sedeño, J.M. Pingarrón, Click chemistry-assisted antibodies immobilization for immunosensing of CXCL7 chemokine in serum, *Journal of Electroanalytical Chemistry* 837 (2019) 246 – 253.
4. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Design of electrochemical immunosensors using electro-click chemistry. Application to the detection of IL-1 β cytokine in saliva, *Bioelectrochemistry* 133 (2020) 107484.
5. S. Guerrero, E. Sánchez-Tirado, G. Martínez-García, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Electrochemical biosensor for the simultaneous determination of rheumatoid factor and anti-cyclic citrullinated peptide antibodies in human serum, *Analyst* 145 (2020) 4680 – 4687.
6. S. Guerrero, E. Sánchez-Tirado, L. Agüí, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Simultaneous determination of CXCL7 chemokine and MMP3 metalloproteinase as biomarkers for rheumatoid arthritis, *Talanta* 234 (2021) 122705.
7. S. Guerrero, E. Sánchez-Tirado, L. Agüí, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Monitoring autoimmune diseases by bioelectrochemical detection of autoantibodies. Application to the determination of anti-myelin basic protein autoantibodies in serum of multiple sclerosis patients, *Talanta* 243 (2022) 123304.

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Introduction

Objectives and work plan

Autoimmune diseases

Analytes and their biological significance

Electrochemical platforms and immunosensors

Reported methods for determination of the target analytes

3.1. OBJECTIVES AND WORK PLAN

The experimental work included in this Doctoral Thesis has been accomplished in the laboratories of the research group of Electroanalysis and Electrochemical (Bio)Sensors, located in the Faculty of Chemical Sciences at the Complutense University of Madrid. The subject fits the objectives of the supported Research Project RTI2018-096135-B-I00, entitled: “Electrochemical immunosensing platforms for autoantibodies and protein antigens of emergent relevance in autoimmune diseases (ELECTRIMMUNE)”, financed by the Spanish Ministry of Science, Innovation and Universities.

The main objective of this work is the development of electrochemical bioplatforms for the determination of autoimmune diseases biomarkers. The idea of this proposal arises from the existence of biomarker proteins specific for autoimmune pathologies with inflammatory nature, which present a difficult diagnosis in initial stages. Some examples are rheumatoid arthritis, multiple sclerosis or lupus. The aspects considered in this research constitute areas of priority importance, and it is to be expected that the results obtained will provide considerable improvements in the detection of such diseases in the coming years. This objective has an applicable nature of great interest for the productive sectors and socioeconomic areas related to the fabrication of biosensors in the pharmaceutical industry and their use in clinical analysis.

Regarding this purpose, the designed configurations have been prepared using different functionalization methodologies by the use of latest-generation nanomaterials, magnetic microparticles, as well as novel strategies for bioelements immobilization and signal amplification. Moreover, despite such specific features, the developed devices combine the advantages of biosensors, particularly the high selectivity, with the benefits of using electrochemical transduction: sensitivity, low cost, shorter assay times, portability and lower sample volume required. Furthermore, all biosensors have been applied to the monitoring of important autoimmune biomarkers, showing a proven usefulness during analysis of biological samples such as saliva, plasma or serum containing physiological concentrations of the species to be determined. In addition, the electrochemical bioplatforms and biosensors were conceived and prepared to determine the proposed biomarkers for the first time or, in some cases, they have shown to improve

the analytical performance of previous methods, including conventional immunoassay techniques involving Enzyme-Linked Immunosorbent Assay (ELISA).

To achieve the scientific-technological goals pursued in this work, the research tasks were carried out following methodologically the successive phases described below:

1. Design of the bioplatfrom configuration and the recognition strategy.
2. Optimization of experimental variables involved in the construction of the electrochemical bioplatfrom and detection.
3. Characterization of the electrode surface and modification steps.
4. Preparation of calibration plots and establishment of analytical characteristics.
5. Assessment of the selectivity of the method and bioelectrode stability.
6. Application to sample analysis and validation.

3.2. AUTOIMMUNE DISEASES

Autoimmunity is the self-destructive effector response of the immune system in which different levels of manifestation with a variety of nuances can be found. Indeed, these go from a low level of self-reactivity which is perfectly common during the maintenance of normal immune system homeostasis, to pathogenic autoimmunity related to immune-mediated dysfunction or injury, passing by an intermediate state with presence of non-harmful autoantibodies in circulation and tissues. The clinical risk starts when an insignificant self-reactivity becomes a pathogenic one [Kono, 2017]. Autoimmune diseases emerge when the immune system does not distinguish between foreign and its own elements, and produces antibodies that attack body cells [Khan, 2019]. This condition, in which tissue injury may be caused by T cell or antibody self-reactivity, can be initiated by an infection, but the immune activation persists in the absence of the antigen. It is known that the regulation of the balance, between a too little response that leads to potential neglect of danger and a too high response that potentially leads to autoreactivity, requires a constant vigilance by the immune system. Apart from this factor, autoimmune diseases require also to be influenced by target organ susceptibility, where genetic predisposition and different environmental exposures play crucial roles [Davidson, 2014]. There are, at least, eighty different autoimmune diseases which can be subtle in onset and present a variety of clinical manifestations. This

situation makes hard to establish a proper diagnosis which depends upon careful clinical evaluation, including a list of agreed characteristic signs and symptoms, a thorough assessment of family, as well as possible environmental exposures [Rose, 2017]. Usually, autoimmune diseases are classified into two types according to the number of organs damaged. Firstly, organ-specific autoimmune diseases which can be endocrine, dermatological or hematological, are characterized by the involvement of a particular tissue. On the other hand, systemic autoimmune diseases occur when antibodies attack non-specific antigens in more than one organ [Caforio, 2017]. Some representative examples of both types are summarized in Table 3.1.

Table 3.1. Classification of autoimmune diseases.

Systemic autoimmune diseases		Organ-specific autoimmune diseases
Connective tissue disease	Mixed connective tissue disease	
Systemic lupus eritematous	Vasculitic syndromes	Hashimoto's thyroiditis
Rheumatoid arthritis	Behcet's syndrome	Multiple sclerosis
Systemic sclerosis	Takayasu's arteritis	Myasthenia gravis
Anklyosing spondylitis	Kawasaki disease	Pernicious and hemolytic anemia
Sjögren's syndrome	Wegener's granulomatosis	Crohn's disease
Reactive arthritis	Sarcoidosis	Phemphigus
Polymyositis	Polyarteritis nodosa	Addison's disease
Inflammatory myopathies	Churg Strauss syndrome	Type 1 diabetes

Rheumatoid arthritis (RA), is a common and chronic inflammatory disease characterized by an excessive immune activation and inflammation which almost involve all body tissues. Indeed, although joints are firstly affected by their inflammation and destruction [Xiao, 2021], other parts of the body, such as the lung, kidney, blood or heart can also be harmed. This complex and heterogeneous disorder shows variable clinical presentation and involves environmental factors that trigger the disease in genetically susceptible individuals. In addition, if RA is not well cared for and properly treated, it can lead to joint damage accumulation and cause irreversible disability, as it is shown in Figure 3.1 [Smolen, 2018]. These effects have a substantial impact on patients in terms of functional limitations, work inability and, overall,

deteriorating quality of life. Typically, RA symptoms appear as symmetric polyarticular joint pain and swelling but also patients may suffer from fatigue, fever, muscle pains, weight loss and, in cases, depression [Littlejohn, 2018]. There are several genetic and environmental factors highly associated with an increased risk for suffering RA. The most common contributors are female sex, family background, mucosal inflammation and microbial factors. Furthermore, obesity, exposure to tobacco smoke and air pollution, high consumption of sodium, red meat, and low iron and vitamin D levels are well-known risk factors for the development of RA [Deane et al., 2017].



Figure 3.1. Stages of rheumatoid arthritis [Jockers, 2021].

Regarding MS, this chronic, inflammatory and neuro-degenerative autoimmune disease results in damage to myelin sheaths in the central nervous system (CNS) (Figure 3.2). Myelin is a complex membrane which forms a sheath around axons in vertebrate species [Tsekenis, 2008]. It is the most abundant membrane structure within the vertebrate nervous system, allowing the fast conduction of nerve impulses through the nerve fibers, due to its high resistance and low capacitance.

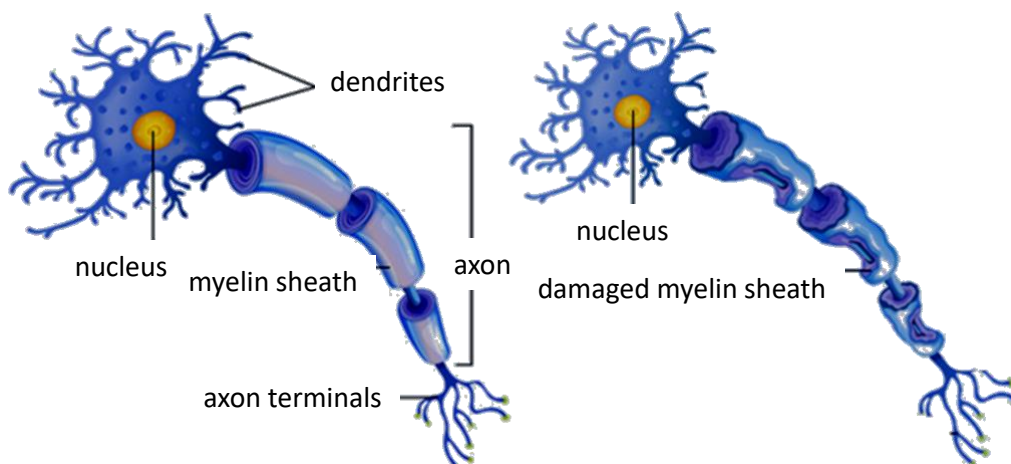


Figure 3.2. Healthy nerve (left) and nerve affected by multiple sclerosis (right).

As an example of demyelinating disease, MS affects neurons within the brain and spinal cord that are involved with sensory and motor control functions. The name multiple sclerosis is derived from the multiple scars (or scleroses) that can be observed on the myelin sheaths. It is characterized by relapses, remissions and progression of disability over time [Oreja-Guevara, 2019] which lead to inhibited muscle coordination and loss of cognitive functions, among other neurological effects [Ottervald, 2010] [Noseworthy, 2000]. There are various identified potential causes of demyelination, including autoimmune reactions, infectious agents, genetic conditions, and also exposure to compounds such as carbamate pesticides. Although it does not have a cure, several treatments are available that may slow the appearance of new symptoms and, fortunately, they can defer the course and symptoms of the disease, if they are used at a nearly stage. Current diagnosis of MS relies on clinical examination supported by laboratory investigations, including magnetic resonance imaging (MRI) to visualize lesions.

Besides the importance of establishing new effective disease-suppressing therapies, sensitive detection methods in order to prevent the occurrence of undesirable consequences of autoimmune diseases, which are associated with this persistent and erosive illness are required [Aletaha, 2010]. Faced with the time consuming and expensive methods commonly used, clinical tests based on biomarkers monitoring should be carried out to facilitate the diagnosis and evaluate the severity of disease. In this work, several types of biomolecules related to RA and MS, recognized as biomarkers of these diseases have been chosen as target compounds, and detection methods based on electrochemical biosensors with sufficient sensitivity and accuracy to be used in clinical trials have been developed.

3.3. ANALYTES AND THEIR BIOLOGICAL SIGNIFICANCE

There is a wide variety of biomarkers whose levels in the body are related to autoimmune diseases. These belong to groups of diverse nature, including cytokines, autoantibodies, peptide hormones and some enzymes. As will be seen, the analytes chosen in this work are included in these families and are signalling as biomarkers of these diseases, although not all of them are specific to a particular illness. Indeed, in

some cases, variations in their concentration can also be used as diagnostic criteria for other related diseases or even cancer.

The characteristics of these biomolecules are described below, highlighting the important predictive value of determining their levels in clinical samples for RA and MS monitoring.

3.3.1. Cytokines

Cytokines are cell-signaling extracellular proteins of molecular weight ranging between 6 and 70 kDa [Stenken, 2015] produced by many different immune cells, including T cells, neutrophils and macrophages which are strongly related to immune response promotion, differentiation processes and regulation activity [Ferreira, 2018]. They act as mediators within highly localized environments, regulating intercellular communication, immunological responses, and inflammation [Lefkowitz, 2001], as well as signalling molecules to control and stimulate the bodydefense system. Usually, these proteins are present at very low concentrations of few $\text{pg}\cdot\text{mL}^{-1}$ and so, increased serum levels of particular cytokines can be used as potential biomarkers of inflammatory responses and disease progression as components of a defense system against foreign pathogens. Furthermore, it is well known that excessive cytokine secretion damages normal cells, prompting an uncontrolled inflammatory response from the immune system [Fajgenbaum, 2020] [Clark, 2007]. This phenomenon, called “cytokine storm”, can kill even healthy people, and its pathogenesis has not yet been elucidated. Recently, it has been reported that this clinical situation plays an important role in the pathogenesis of COVID-19 [Ye, 2020]. Thus, rapid monitoring of cytokine levels is essential to prevent progression to serious diseases and achieve better outcomes for patients.

Several types of cytokines with similar or completely different functions such interferons, lymphokines, tumor necrosis factors (TNFs) transforming growth factors (TGFs) chemokines (CXCLs, CCLs) or interleukins (ILs), may be found [Turner, 2014] [Han, 2012]. The involvement of these proteins in the immune activities and in the course of inflammation processes makes their detection a crucial task in order to predict diseases, characterizing the immune function, or for evaluating the pathogenesis of autoimmune disorders and monitoring their evolution and treatments.

For these studies, not only pro-inflammatory cytokines that enhance the initiation and propagation of autoimmune inflammation but also anti-inflammatory cytokines are important because they facilitate the regression of disease and recovery from its acute phase [Moudgil, 2011].

3.3.1.1. Transforming growth factor beta 1 (TGF- β 1)

The transforming growth factor- β (TGF- β) family is a collection of structurally related multi-functional cytokines involved in a wide range of physiological processes. Three isoforms (TGF- β 1, - β 2, and - β 3) are present in mammals with some differences in biological activities and also in their potencies. They are known as essential regulators of cell-growth, rate of proliferation, differentiation, and production of extracellular matrix proteins [Grainger, 2000], and are related to the pathogenesis of many diseases [Hanna, 2019]. In particular, TGF- β 1 isoform presents a structure formed by interrelated dimeric polypeptide chains [Poniatowski, 2015] consisting of a disulphide-linked 28 kDa dimer of two 112-amino acid peptides (Figure 3.3). There is evidence that it is one of the most abundant cytokines in the bone matrix being ubiquitously distributed throughout different tissues and synthesized by many different cells [Kropf, 1997]. It is involved in immune and inflammatory responses showing a hundred times more potent behaviour than the others. TGF- β 1 concentration levels in plasma from healthy individuals have been reported to range from 0.1 and 25 ng mL⁻¹ [Grainger, 2000], the great variability observed among data depends to some extent on the assay type used in the determination.

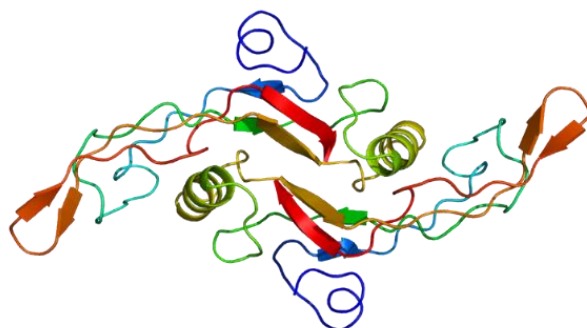


Figure 3.3. Structure of transforming growth factor beta 1 [Hinck, 1996].

This cytokine is produced by several cell lineages such as lymphocytes, macrophages and dendritic cells. Apart from the involvement in inflammatory states [Sanjabi, 2017], increasing evidence also links TGF- β 1 to the progression of renal diseases [Tsapenko, 2013] or cancer [Javle, 2014], and to some autoimmune disorders, such as systemic sclerosis [Dantas, 2016], or systemic lupus erythematosus [Rashad, 2019], among others.

3.3.1.2. Interleukin 1 beta (IL-1 β)

The term “interleukin” (IL) was first coined in 1979 because it was thought these cytokines were expressed by leukocytes. Nowadays, this term has been maintained, even when it has been found to be generated by and affect many other body cells [Secombes, 2011]. Particularly, IL-1 β is produced by a variety of cells including pancreatic cells, adipocytes or macrophages. This is a pro-inflammatory cytokine involved in various autoinflammatory diseases [Lübow, 2020] and playing a specific role in some cases. For instance, the elevation of the IL-1 β concentration is a typical reaction of the brain to acute injury and it can be found in a wide number of chronic neurodegenerative diseases, such as Alzheimer [ShafteI, 2008]. Its structure is shown in Figure 3.4.

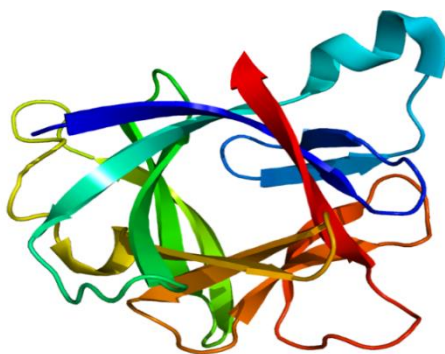


Figure 3.4. Structure of interleukin 1 beta.

The production of IL-1 β is preceded by an inactive 31 kDa precursor, known as pro-IL-1 β , in response to molecular motifs carried by pathogens termed PAMPs - pathogen associated molecular patterns-, then inducing active IL-1 β secretion. Pro-IL-1 β is cleaved directly by the pro-inflammatory protease caspase-1 in the cytosol, whose activation occurs by a multi-protein complex called the inflammasome [López-Castejón, 2011] [Afonina, 2015]. Once activated, IL-1 β acts as potent proinflammatory

cytokine, initiating the cascade of immunoinflammatory responses at the local level, triggering processes such as vasodilatation, and monocytes and neutrophils attraction to sites of tissue damage. It also shows pleiotropic effects on autoimmune diseases, provokes the expression of metalloproteinase genes and induces the secretion of prostaglandin E₂ and proinflammatory mediators, which are involved in joint destruction [Zhao, 2014]. Its decisive mediation in tissue damage by altering cartilage and bone homeostasis, but also the important role of promotion of fever, hyperalgesia and inflammatory atherogenesis should be highlighted [Schett, 2016]. Since uncontrolled IL-1 β activation plays a pivotal role in the pathogenesis of most autoinflammatory disorders, monotherapy blocking IL-1 activity results in a sustained reduction of disease severity [Lopalco, 2015].

This interleukin is predominantly related to rheumatic diseases such as RA, or collagen-induced arthritis (CIA), but also to systemic lupus erythematosus (SLE), or systemic sclerosis (SSc), among others. Besides, it is also associated with other disorders affecting skin, cardiovascular or gastrointestinal systems, and tumors [Galozzi, 2021].

3.3.1.3. Chemokine (C-X-C motif) ligand 7 (CXCL7)

Although chemokines are included in the cytokine family, they are usually considered as a separate group of biomarkers related to autoimmune diseases, due to their high number and importance. These proteins constitute a large group of low-molecular-weight molecules (8–12 kDa) expressed in tissues which play decisive roles in angiogenesis and viral infection processes, acting as inflammatory mediators and immunological modulators. They are known as chemokines or chemotactic cytokines since they induce the migration of immune cells in specific tissues and organs through chemotaxis [Zlotnik, 2012], which implies the creation of an extracellular concentration gradient that is specifically recognized by cells, promoting their movement. So, chemokines regulate leukocyte migration through interactions by cell migration in response to extracellular gradients of diffusible chemicals [Cameron, 2013] [Unver, 2015]. In this way, chemokines fulfil functions such as directing the traffic of various cells to mediate the immune response (facing an infection, for example), as well as promoting homeostasis and development at the cellular level [Jin, 2008].

These peculiar cytokines are characterized by the presence of three or four conserved cysteine residues whose position may be used for classifying them into four families. Particularly, the C–X–C family is characterized by the separation of the two N-terminal cysteines by one variable amino acid residue, while in the C–C subfamily the cysteine residues are adjacent to each other [Turner, 2014]. More than forty different chemokines have been identified in humans that are divided into four large subfamilies: C, CC, CXC, CX3C (Figure 3.5) [Panda, 2016].

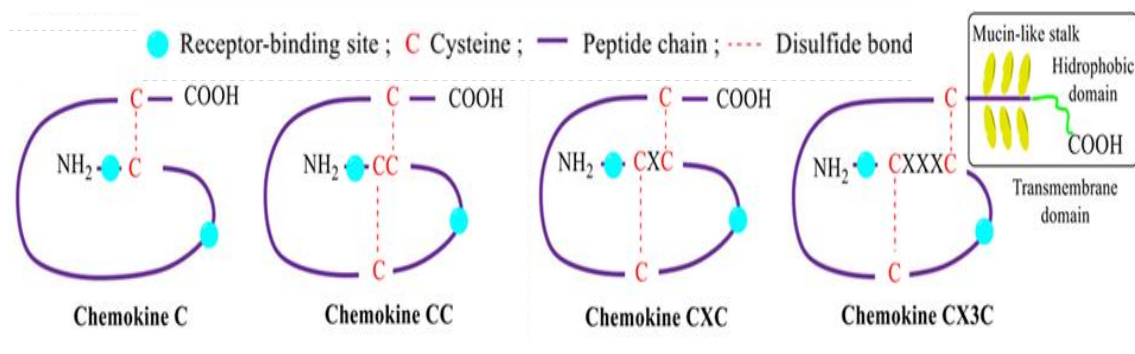


Figure 3.5. Structures of the four major subfamilies of chemokines.

Chemokine C-X-C ligand 7, depicted in Figure 3.6, is a platelet-derived growth factor also designed as Platelet Basic Protein (PBP), Neutrophil-Activating Peptide 2 (NAP-2) or Connective Tissue-Activating Peptide III (CTAP-III). It acts as a potent chemoattractant and activator of neutrophils through binding to its receptor CXCR2, also stimulating a great variety of cellular processes: DNA synthesis, glycolysis, mitosis or prostaglandin E2 secretion, as well as the synthesis of hyaluronic acid. It also shows bactericidal and antifungal activity and plays an important role in tumors growth [Guo, 2017]. Indeed, CXCL7 is highly correlated in the regulation of tumor angiogenesis [Pilatova, 2013] and is considered in many cases as a good biomarker for various types of cancer [Du, 2018] [Li, 2021].

Regarding, autoimmune disorders, CXCL7 was found to be highly expressed in the serum, synovial fluid and synovial tissue of patients who develop RA during the first twelve weeks but at lower levels in longer duration RA. This variation can be useful to reflect local pathological changes occurring during this phase, and study its contribution to RA pathogenesis [Yeo, 2016].

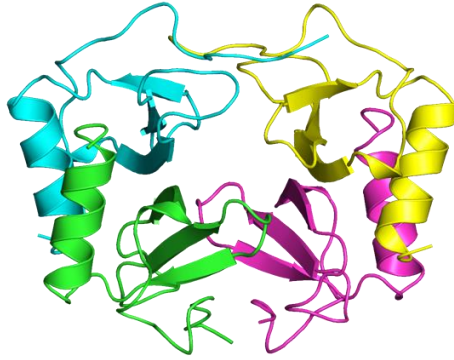


Figure 3.6. Structure of Chemokine (C-X-C motif) ligand 7 [Malkowski, 1995].

3.3.1.4. Chemokine (C-C motif) ligand 5 (CCL5)

The chemokine ligand 5 (CCL5, RANTES) was isolated and characterized in 1990 [Marques, 2013]. As its name indicates, it belongs to the subfamily of CC- or β -chemokines, since it has adjacent cysteine residues near the N-terminus in its structure. The CCL5 molecule (Figure 3.7) with a molecular mass of 8 kDa, consists of 68 amino acids that binds to chemokine receptors (CCR1, CCR3, CCR4, and CCR5) located on immune cells (myeloid cells: leukocytes, etc.; lymphoid cells: T cells, monocytes) promoting their migration by chemotaxis. Finally, CCL5 can act as a biomarker for various predominantly inflammatory pathologies because the expression of this inflammatory chemokine indicates cellular activities related to medical conditions. Among them are atherosclerosis, RA, atopic dermatitis or asthma [Appay, 2001]. Although the overview of inflammatory chemokines is linked to immunity and inflammation, recent studies involve the CCL5/CCR5 chemokine system with the progression of cancer [Aldinucci, 2014].

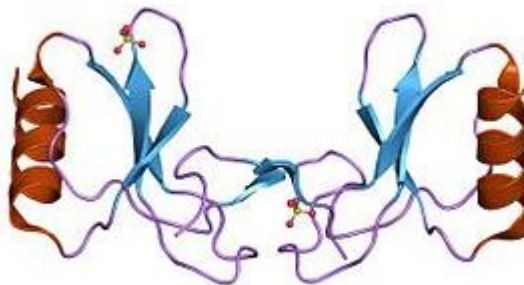


Figure 3.7. Structure of CCL5 [<http://www.ebi.ac.uk>].

3.3.2. Peptide hormones

These are small proteins composed of a low number of amino acids (from some units to a few hundred) [Sanderson, 2009]. Among them, some peptide hormones modulating hunger have been intensively studied, mostly in animals but increasingly also in humans [Schloegl, 2011]. Rheumatoid arthritis has been found to be associated with changes in several hormones and metabolic peptides, being crosstalk between these factors and the immune system relevant for homeostasis during inflammation [Tang, 2017]. A link between the immune system and gastrointestinal endocrine axis may be established through the interaction of proinflammatory cytokines and gut hormones, such as ghrelin and peptide YY (PYY). With the ever-increasing understanding of rheumatoid inflammation and the invention of more biologics to modulate the cytokine network, more attention should be given to the possible immunomodulatory roles of gut hormones in autoimmune inflammatory reactions. Moreover, studies have found a significant correlation between obesity and a higher risk of developing immune-mediated conditions related to RA, MS or psoriatic diseases. In this sense, obesity and peptide hormones regulating hunger appears to be new interesting components of the complex mosaic of autoimmunity [Versini, 2014].

3.3.2.1. Peptide YY (PYY)

First isolated in 1983, Peptide YY is a gastrointestinal hormone consisting of 36 amino acids, whose name derives from the presence of tyrosine residues (Y) at both C and N-terminals (Figure 3.8). This potent anorexigen belongs to the pancreatic polypeptide (PP) family together with PP and neuropeptide Y (NPY) [Erhorn, 2007]. About 70% homology among the three biomolecules exists, highly preserving also the peptide sequences between species.



Figure 3.8. Structure of Peptide YY [Nygaard, 2006].

Due to its anorectic action, PYY has become as an interesting therapeutic target, in order to reduce caloric intake, hunger and thus obesity condition [De Silva, 2012]. Moreover, it has also been reported to exhibit an anti-inflammatory property. As an interesting application, PYY might be a candidate for regulating energy balance during inflammation in patients with RA and other autoimmune diseases [Chen, 2013]. Very recently, PYY has also revealed as a specific plasma biomarker of RA [Balog, 2021].

3.3.3. Autoantibodies

Autoantibodies produced by the immune system act against our own cells or tissues as a first response to infections or as an indication of pathological processes. The presence of antibodies in serum can predict some illnesses well in advance of clinical symptoms or inform on the evolution of diseases. Especially important is the detection of autoantibodies in the field of autoimmune disorders, where the evaluation of their type and concentration is currently used to assess the risk and progression of such disorders and to help in the finding of therapies to prevent or mitigate their impact on patients [Ma, 2017]. Some examples of autoantibodies associated to autoimmune diseases are anti-cyclic citrullinated peptide, rheumatoid factor, anti-aquaporin-4 or anti-nuclear antigens. Although the role of autoantibodies in the appearance and course of these diseases is not thoroughly known, however, their use as serum biomarkers constitutes a minimally invasive and relatively low-cost alternative, compared to other methods of diagnosis that requires invasive protocols, highly qualified personnel and sophisticated instruments [Campuzano, 2019].

Although some autoantibodies collaborate in the maintenance of homeostasis and modulation of innate and adaptive immune responses, conferring protection from rampant autoimmune and inflammatory injuries [Elkon, 2008] [Mannoor, 2013], however, other pathogenic autoantibodies are heavily considered to facilitate the development of various immune-mediated diseases [Dempsey, 2019]. A large number of serum antibodies directed against functional structures of the cell (nucleic acid, nuclear molecules, receptors, or other functional cell components) can be detected in patients with autoimmune diseases, which presence plays a central role in the diagnosis and classification of this type of disorders. Moreover, as these autoantibodies may be present in biological fluids various years before clinical symptoms are manifested, their

detection in clinical samples has been shown to have a strong predictive value [Lleo, 2014]. Furthermore, since serum concentration of certain autoantibodies is associated with therapy response, monitoring these autoantibodies seems to be an additional useful tool for early identification of therapeutic treatments effects during the illness evolution [Eggert, 2010].

3.3.3.1. Rheumatoid factor (RF)

Rheumatoid factor, the first autoantibody to be discovered in RA patients, was described by Waaler [Waaler, 1940]. Contrary to what the name suggests, RF is not specific to RA and it is also observed in other autoimmune illnesses, such as SLE and Sjogren's syndrome [Sang, 2014]. Moreover, this autoantibody has also been related to other non-autoimmune diseases such as infectious illnesses [Ingegnoli, 2013]. Various rheumatoid factors exist, they consist of immunoglobulins, being IgM the most common that shows affinity directed against the Fc portion of IgG autoantigens forming immune complexes (Figure 3.9) [Corper, 1997] subsequently phagocytosed by the inflammatory cells. An increased avidity to antigens during the immune response is shown that originates the characteristic inflammation of RA in such way that higher concentrations cause more severe disease.

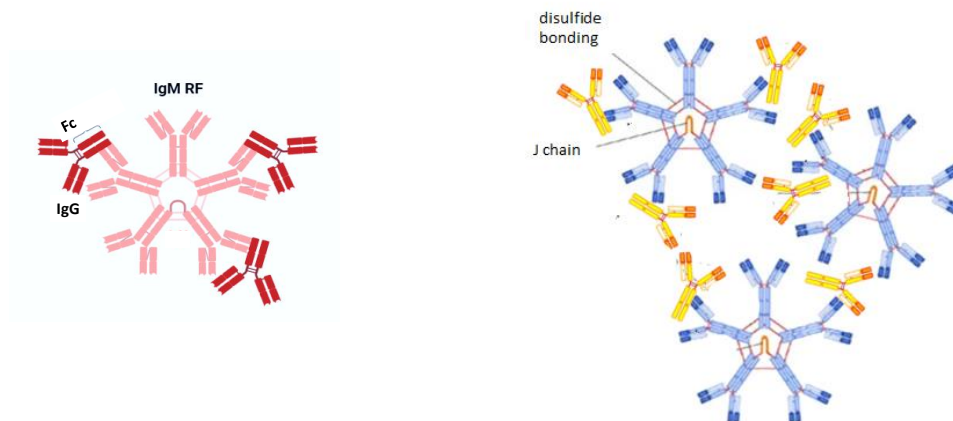


Figure 3.9. Structure of an IgM RF pentamer (left) interacting with IgG (red). Various IgM monomers (blue) showing disulfide bonding to each other and to Fc fragments of IgG (yellow) (right).

There is evidence that RF is a pathogenic autoantibody with a key role in the pathophysiology of RA. Serum of RA patients shows frequent presence of autoantibodies, with IgM-RF as the most prevalent, targeting the Fc fragment of the patient's own IgG molecules. In normal conditions, transient production of low-affinity IgM RF is regularly induced by immune complexes and polyclonal B-cell activators. The physiological role of RF under normal conditions includes promoting stability of IgG bound to solid surfaces, such as bacterial walls [Song, 2010b]. However, high-level RF in RA is believed to exert such functions in a pathogenic manner and thus to potentiate inflammation and antigen trapping in the joints. For instance, in RA, RF may induce the formation of immune complexes at the sites of synovial inflammation, located in the cartilage matrix, ensuring the activation of complement and leukocyte infiltration. B cells with RF specificity migrate into the synovium of RA patients, presenting a variety of antigens to T cells and this may contribute to the perpetuation of local inflammatory responses and amplification of RF production in the synovium. Thus, RF may prolong B cell survival and hence maintain its own production [Brito Rocha, 2019].

3.3.3.2. Anti-cyclic citrullinated peptide (CCPA)

Citrullinated peptide antibodies (CPAs or anti-CPs) comprise a group of highly specific RA antibodies detected during the early stages of the disease. They recognize various peptides and proteins such as filaggrin or fibrinogen, whose arginyl residues are enzymatically transformed into citrullyl residues. The post-translational modification named citrullination reaction (Figure 3.10) consists in the conversion of an arginine into a citrulline. It is catalyzed by peptidylarginine deiminase (PAD) enzyme, which can be released by neutrophils or originate from bacteria [Van Delft, 2019]. CPAs play an important role in inducing inflammation and joint damage, releasing pro-inflammatory cytokines from monocytes/macrophages. It is reported that 70 – 80% of RA patients are CPA positive. Therefore, high levels of CPAs in biological fluids are associated to bad prognosis of the disease [Nielen, 2004] [Watts, 2014].

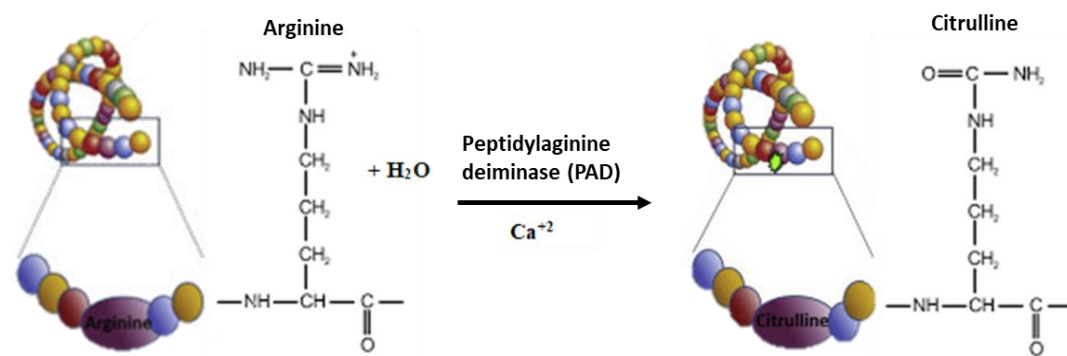


Figure 3.10. Citrullination enzymatic reaction [Van Delft, 2019].

Citrullination reactions cause a charge shift in proteins, which can affect their structure and interactions, and even inducing protein denaturation. This kind of modified proteins are usually present in the cell membrane, cytoplasm, nucleus, and mitochondria appearing frequently during cell death [Jang, 2011]. Accumulation and leakage of PAD enzyme and citrullinated peptides may occur, which produce an abnormal signaling with immune system components and break the self-tolerance. In other words, citrullinated proteins can be recognized as new autoantigen by the immune system and induce the production of anti-citrullinated peptides antibodies [Alghamdi, 2019].

3.3.3.3. Anti-myelin basic protein (anti-MBP)

Autoantibodies play an important role in MS pathogenesis, and their detection can help to evaluate the disease status of patients and predict the response to a specific therapy [Bielekova, 2004]. In particular there is an increasing evidence that antibody-dependent effector mechanisms can play an important role in the immunopathogenesis of demyelination and, therefore, being demyelination a key component of the MS disease progress, autoantibodies against myelin antigens have long been considered as the primary targets of the humoral autoimmune response in MS. In fact, anti-myelin basic protein (anti-MBP) emerging in MS patient's sera and cerebrospinal fluid (CSF) has been regarded as a biomarker of the disease progression [Ponomarenko, 2006]. In addition, understanding the contribution of anti-myelin antibodies to early disease events is increasingly relevant [O'Connor, 2003]. However, despite the importance of anti-MBP biomarker, the autoimmune response in MS is more complex and not only restricted to myelin but much more widespread thorough the brain [Fraussen, 2014].

3.3.4. Enzymes

Some proteins are in charge of the fundamental task of acting as enzymes, biological catalyst that increase the rate of virtually all chemical reaction within cells that would not be carried out or would need years under biocompatible conditions. Related to autoimmune diseases, some antibodies have a special importance expressing enzymatic activity involved in a variety of disorders, such as systemic erythematosus (SLE), scleroderma or autoimmune thyroiditis apart from RA and MS [Wootla, 2011]. Antibodies endowed with enzymatic properties are termed as abzymes (antibody-enzymes) and have shown to be able to specifically hydrolyze thyroglobulin or DNA among other biomolecules. Furthermore, various types of proteases (proteinases or peptidases) are also involved in autoimmune disorders such as RA, playing a direct role in cartilage degradation [Momohara, 1997]. Moreover, as will be seen below, various classes of proteases including matrix metalloproteinases (MMPs) are linked to chemokines participating jointly in the migration of inflammatory cells to the synovial fluid and breakdown of cartilage and bone [Opdenakker, 2002].

3.3.4.1. Matrix metalloproteinase-3 (MMP-3)

As belonging to the metalloproteinase family, MMP3 enzyme (Figure 3.11), also known as stromelysin-1, has an active zinc-dependent site with three bound histidine residues, requiring calcium ions and being functional at neutral pH [Laronha, 2020]. It is secreted by mesenchymal cells, such as chondrocytes and fibroblasts [Lee, 2008], being its expression induced by TNF- α cytokines.

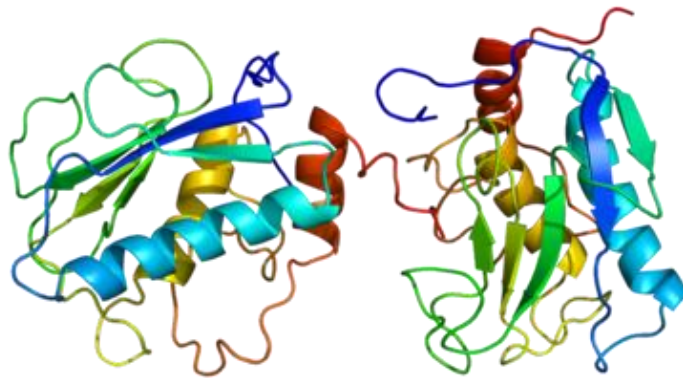


Figure 3.11. Structure of matrix metalloproteinase-3 (MMP3).

Measurement of MMP3 content in serum turns out to be a useful scarcely invasive method to monitor pathological processes underlying inflammation and joint destruction related to autoimmune diseases [Zucker, 1994] [Keyszer, 1999]. Although increased levels of this enzyme are not specific for RA [Akiyama, 1997], monitoring MMP3 in serum of RA patients can be used as a marker for the effectiveness of antirheumatic therapy [Tuncer, 2019], as well as to evaluate the difference in the prognosis, reflecting the disease activity and treatment response with moderate to severe RA patients [Sun, 2020] especially in the early stage of illness.

3.4. ELECTROCHEMICAL PLATFORMS AND IMMUNOSENSORS

The experimental work carried out in this Thesis involved the use of screen-printed electrodes for the implementation of both integrated immunosensors and magnetically assisted bioplatfroms. In the first case, the electrode surface was modified with materials able to immobilize biomolecules also improving the electrochemical responses, and the second type employed functionalized magnetic microbeads as support for immunoreagents immobilization. Furthermore, all the developed designs implied the implementation of immunoassay schemes based on antibody-antigen reactions to quantify the unknown concentration of analytes. The characteristics of the electrodes, materials and strategies used are briefly summarized below.

3.4.1. Electrochemical platforms based on screen-printed electrodes (SPEs)

Screen-printing technology has revolutionized the field of electrochemical (bio)sensing [Yáñez-Sedeño, 2020] [Pingarrón, 2021]. The easy way in which SPEs electrodes can be tailored with different modifiers including nanomaterials, bioreagents or redox mediators, and fabricated onto a variety of substrates such as paper or flexible materials, make them extremely suitable to design sensors for a wide range of analytes and applications. Currently, mass-produced disposable, inexpensive and robust strip solid electrodes suitable to be fabricated with different materials, geometries, miniaturized sizes, and multiplexed formats are available. In fact, one of the more relevant particular advantages exhibited by SPEs is the ability to perform multiplexed analysis in a simple way by exploiting the different SPEs formats. In addition, their flat

shape facilitates the coupling with magnetic micro- and nanomaterials by simple attraction with a magnet positioned under the working electrode [Yáñez-Sedeño, 2016].

In this work, gold SPEs (SPAuEs) modified by oxidative grafting with *p*-aminobenzoic acid (*p*-ABA) for covalent immobilization of anti-TGF or anti-PYY antibodies (Figure 3.12a) were used. Moreover, carbon SPEs (SPCEs) modified with multiwalled carbon nanotubes (MWCNTs) for immobilization of anti-CXCL7 antibodies assisted by click chemistry, and anti-IL-1 β using electro-click methodology (Figure 3.12b) were also used. Furthermore, dual and single SPCEs were also utilized as electrochemical substrates for implementation of magnetically assisted bioplatfroms applied to the simultaneous determination of RF and CCPA, or MMP3 and CXCL7 (Figure 3.12c), and individually for anti-MBP and CCL5.

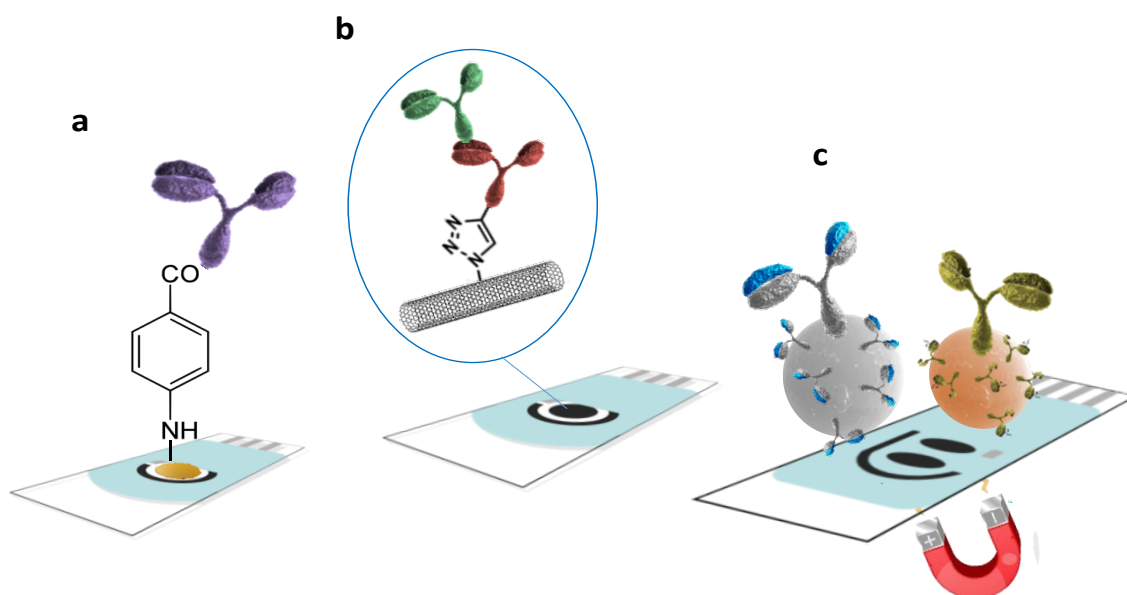


Figure 3.12. Schematic display of bioconjugates prepared with: (a) SPAuE grafted with *p*-ABA; (b) SPCE modified with MWCNTs for click-assisted chemistry immobilization; (c) dual SPCE for implementation of a magnetically assisted bioplatfrom.

3.4.2. Electrode surface functionalization

The good performance of electrochemical biosensors requires the use of electrode surfaces with particular abilities, as well as of sensitive and accurate transduction techniques [Pingarrón, 2021]. Regarding electrodes, the active surfaces must play specific roles to achieve two main goals: to get the best electrochemical responses and, if needed, to allow the stable and suitable immobilization of

biomolecules. These two crucial aspects must be carefully optimized since the adequate performance of the (bio)sensor is closely linked to them. Important features such as high conductivity, electrocatalytic ability and resistance to fouling are largely responsible for the sensitivity, selectivity and reproducibility of the electrochemical measurements.

Currently, the use of nanomaterials as modifiers of the electrode surface is widely extended since they commonly ensure high conductivity and electrocatalytic effects towards the involved electrochemical reactions. In the works carried out in this Thesis, two modes of functionalization of the electrode surface have been applied using CNTs as a modifying material or directly by means of electrochemical grafting with *p*-ABA.

3.4.2.1. Surface functionalization with azide-modified carbon nanotubes

From their discovery in 1991 [Iijima, 1991], CNTs have been consolidated as an extremely useful electrochemical nanomaterial becoming one of the carbon species most studied and applied by both academic and industrial researchers [Rauti, 2018]. Thanks to their unique physical and chemical properties such as electrical conductance, high mechanical strength and the possibilities to create functional groups, this material is widely used in the preparation of electrochemical biosensors [Wang, 2018] [Jin, 2020] [Alagappan, 2020]. However, their employment is not limited to this field, and CNTs-based applications in nanotechnology is extended to reinforcement and modification of material properties in nanocomposites, nanoelectromechanical systems (NEMS), transistors, quantum dots and nanoscale bioprobes [Harik, 2018].

Among the different types of CNTs, in this work, MWCNTs were used as electrode modifier and substrate for implementing click chemistry-assisted preparation of bioconjugates. In this application, the ease modification of the external wall of nanotubes was taken advantage to create an azide group. In addition, their presence in the electrode surface was shown to promote a rapid electron transfer, enhancing the reaction rate thus providing a higher sensitivity [Tîlmăciu, 2015] [Gomes-Filho, 2013]. Figure 3.13 schematizes the steps involved in the preparation of MWCNTs functionalized with azide moieties. As it can be shown, once oxidized with nitric and sulfuric acids, carboxylated nanotubes were treated with 1-azide-3,6,9-trioxaundecan-1-amine (NH₂-PEG-N₃). This polyethyleneglycol (PEG) derivative contains a free

amine which can be covalently conjugated to cMWCNTs after activation by means of carbodiimide-sulfosuccinimide chemistry.

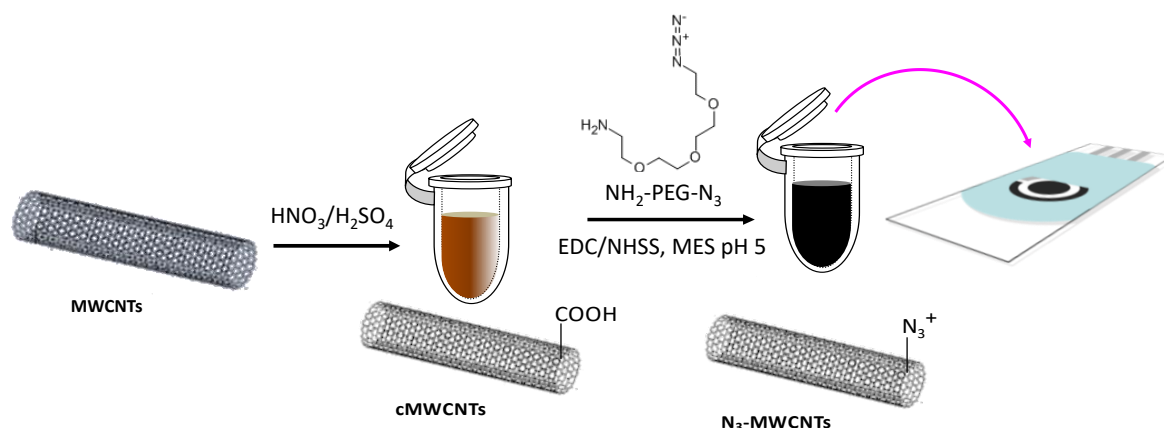


Figure 3.13. Steps involved in the preparation of azide-functionalized MWCNTs.

3.4.2.2. Electrochemical grafting

During the last decades, electrochemical grafting (or electrografting) has been established as a simple, rapid and versatile methodology that sustains the introduction of grafted layers with a large variety of functionalities at conductive substrates by the simple change of aryl ring substituents [Pilan, 2021]. This method for surface modification is very suitable for tuning electrode properties, thus allowing interfacial features to be controlled [Randriamahazaka, 2015]. In addition, implementation of grafting procedures offers many experimental alternatives concerning the process itself, the type of surfaces, and the choice of substituents. One of the most utilized molecules to carry out these processes is *p*-ABA since it not only provides carboxylic groups for covalent immobilization of biomolecules but also keep them oriented because of the flat structure.

As Figure 3.14 shows, two main alternatives can be utilized for application of electrochemical grafting. As it can be seen, one involves the synthesis of diazonium salts from aryl amine derivatives followed by electrochemical reduction, loss of nitrogen and subsequent linking of the carboxyphenyl derivative to the electrode surface (Fig. 3.14.a) [Yáñez-Sedeño, 2018]. Since in 1992, Pinson and co-workers described the mechanism for the reaction, this chemistry has demonstrated to be an efficient way to incorporate functional groups onto carbon surfaces, metals and other materials, with a

reaction time scale of seconds to minutes [Delamar, 1992] [Belanger, 2011]. However, the use of aryl diazonium salt chemistry for the preparation of biosensors may show some drawbacks regarding reactivity of the head group and the presence of side reactions which compromises surface chemistry control.

Other efficient alternative (Fig. 3.14.b) which is used in this work, implies electrochemical oxidation by cyclic voltammetry of arylamines in the presence of lithium perchlorate in ethanol resulting in the formation of amine cation radicals [Liu, 2000]. This procedure, applied in a single stage, is faster than the previous one and provides binding to the substrate via the nitrogen atom. The main problem that can arise with this method is the formation of a multilayer coating that passivates the electrode and hinders the charge transfer. However, this inconvenient can be avoided by a careful optimization of the chemical and electrochemical variables involved, controlling surface coverage [Chira, 2012]. It is worth mentioning that, despite the advantages, oxidative grafting has been much less widely used for the preparation of electrochemical biosensors than the one based on aryl diazonium salts. In our group, this alternative has previously been used to prepare sulfonamide [Conzuelo, 2012], estradiol [Ojeda, 2012] and fetuin [Sánchez-Tirado, 2018] immunosensors using SPCEs.

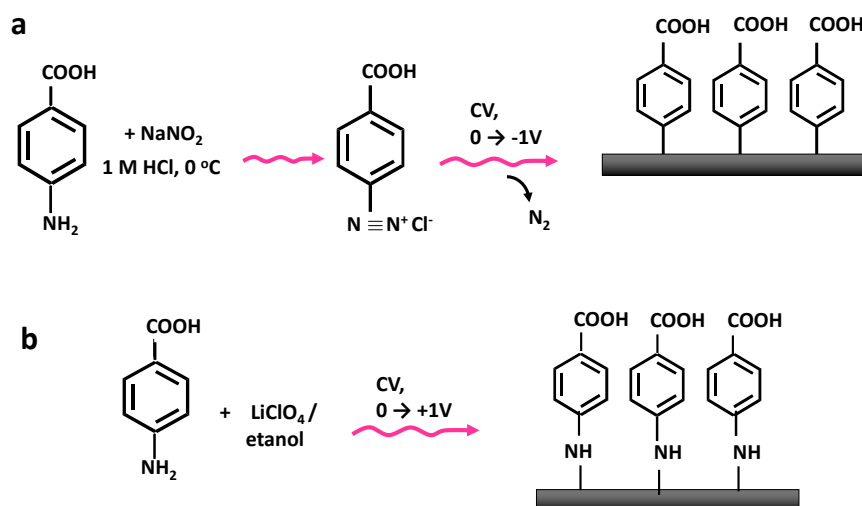


Figure 3.14. Steps involved in electrochemical grafting by: (a) reduction of diazonium salts synthesized from arylamines, and (b) direct oxidation of arylamines.

In this Thesis, *p*-ABA was electrooxidized onto SPAuEs to take advantage of gold surface functionalization though generated NH-Au bond [Fagas, 2007] which competes favorably with fabrication of self-assembled monolayers (SAMs) of thiols. The resulting surface, with a high loading of carboxyl groups, was successfully employed for the development of two different immunosensors designed for the determination of TGF- β 1 cytokine and the hormone PYY.

3.4.3. Electrochemical platforms for magnetically assisted immunodetection

Some configurations developed in this Thesis are based on the use of functionalized magnetic microparticles (MBs) as a substrate for the immobilization of immunoreagents. In these designs, the electrode plays the double role of conductive medium for electronic transfer and support of immunoconjugates magnetically trapped on its surface. The application of MBs, enabling the easy, fast and selective capture of the target molecule from complex samples and further coupling with disposable electrochemical sensors, is nowadays a well-established methodology [Yáñez-Sedeño, 2016] (Figure 3.15). These particles make possible the efficient immobilization of capture bioreceptors allowing fast assay kinetics whilst minimize unspecific adsorptions usually occurring in complex samples. Furthermore, their use also avoids the need for electrode modification that is required for the immobilization of bioreagents at the electrode surface.

As it is known, MBs are micrometric particles provided by a magnetic core mainly consisting of magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$) covered by a protective thin shell made of polymeric stabilizers such as agarose, silica, or polystyrene, which prevent particle aggregation allowing further modifications [Modh, 2018]. MBs provided with a variety of functional groups able to bind several biomolecules for use in different types of bioassays are commercially available [Nikolelis, 2016]. This versatility is one of the interesting features of MBs that joins other important analytical advantages of magnetically assisted biodetection [Kokkinos, 2016]. The large surface area of MBs and the high number of functionalities for binding recognition elements or specific analytes, as well as the magnetic properties of these particles which allow the isolation and preconcentration of target molecules from complex samples due to the easy handling, modification and manipulation of MBs, greatly facilitate the preparation

of bioconjugates and the detection steps leading to strategies that generally exhibit better limits of detection and lower matrix effects than conventional integrated configurations. Finally, as stated above, coupling the use of magnetic microparticles with electrochemical transduction at SPEs provides also the easy and efficient immobilization on the disposable electrode surface without risk for sample components fouling, leading to significant improvements in the performance of electrochemical biosensors for their application to real clinical samples without previous treatment except dilution.

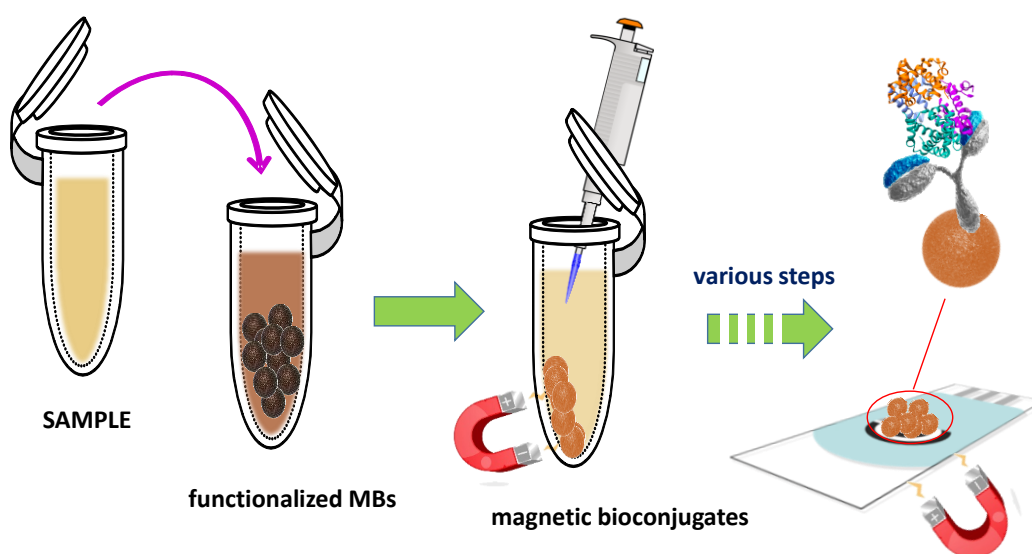


Figure 3.15. Simplified scheme of some steps involved in the preparation of a magnetically assisted electrochemical immunosensor.

In this work, two types of functionalized MBs have been used. Carboxylated MBs (COOH-MBs, cMBs) were used for the development of a magnetically assisted immunosensor for simultaneous determination of CXCL7 and MMP3 and by immobilization of the corresponding specific antibodies. These particles have also been utilized as support for covalent immobilization of MBP peptide and development of an immunosensing platform for the detection of anti-MBP autoantibody, as well as, in combination with neutravidin-MBs (Neutr-MBs), for the construction of a dual immunosensing platform for CCPA and RF autoantibodies. Neutr-MBs have also applied to the individual determination of CCL5 by the immobilization of a biotinylated anti-CCL5 antibody (anti-CCL5-Biotin). Regarding cMBs, these particles provide enough carboxylic groups to assure stable immobilization of a high loading of

immunoreagents with a good reproducibility by covalent amide bindings with amine moieties of biomolecules. Nonetheless, this capability can be seen reduced because of steric hindrance resulted of an excess or improper orientation of immunoreagents. Furthermore, in order to enhance this critical step of immunoconjugates preparation, an activation process involving carbodiimide chemistry is extremely recommended. This strategy is discussed in Section 3.4.4.2. On the other hand, the use of Neutr-MBs allows immobilization of biotinylated molecules taking advantage of the high affinity that exists between neutravidin and biotin. This affinity binding is described in Section 3.4.4.3.

3.4.4. Preparation of immunosensing platforms

All the electrochemical bioplatfroms developed in this work involved immobilization of biomolecules (antibodies or peptides) onto modified SPEs or functionalized MBs, followed by application of immunoassay strategies for detection of analytes on the basis of commercially available immunoreagents for each application. Sandwich-type schemes (Figure 3.16a) were established except in one case, in which a direct competitive assay (Figure 3.16b) was used for the determination of PYY hormone. In previous sections, the methods for modifying the electrode surface and the characteristics of functionalized MBs used in these investigations have been described, so that here the procedures used for the attachment of biomolecules and preparation of immunoconjugates will be considered.

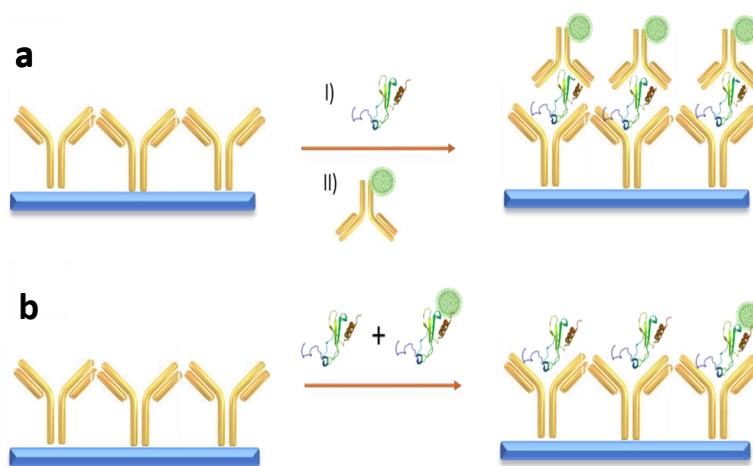


Figure 3.16. Simplified schemes of sandwich-type (a) and direct competitive (b) immunoassays using capture antibodies immobilized onto the electrochemical platform and labelled antibodies (a) or antigens (b) as detection biomolecules.

3.4.4.1. Click chemistry assisted immobilization

The so-called "click chemistry" comprises a set of reactions involving cycloadditions of unsaturated species, nucleophilic substitutions, reactions involving non-aldol carbonyl group or addition reactions to carbon-carbon multiple bonds [Yáñez-Sedeño, 2019]. Among them, probably the most representative is the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) introduced by Sharpless et al. in 2001 [Kolb, 2001]. The high interest in the application of this reaction, for which the term "click chemistry" is usually employed, derives from the high yields achieved, the absence of by-products, and moderate conditions required both in aqueous medium and under physiological conditions. Although it was designed as a tool for organic synthesis, currently, this process is considered as one of the most useful methods for building architectures and to incorporate functionalities onto different materials including electrode surfaces for development of electrochemical sensors [Cermat, 2015]. Click reaction involving azide and alkyne complementary functions is compatible with a wide range of pHs, solvents and temperatures, resulting in an efficient strategy for immobilization of biomolecules to different substrates [Nie, 2015].

It has been shown that CuAAC rate is 10^7 fold higher than the non-catalyzed reaction taking place at room temperature [Bock, 2006]. Therefore, various methods for incorporation of Cu(I) based on the direct addition of cuprous salts or on the *in situ* chemical reduction of Cu(II) salts using hydroquinone or ascorbic acid [Hein, 2010] have been reported. Moreover, Cu(I) catalyst can also be electrochemically generated [Ipek, 2014]. As will be seen, this method, named "electroclick" results in a very interesting alternative for the selective functionalization of electrode platforms [Devaraj, 2006].

As described in Section 3.4.2.1., azide-functionalized MWCNTs were used in this work as modifier for the proper preparation of the electrode surface prior to construction of the electrochemical immunosensors for CXCL7 chemokine and IL-1 β cytokine respectively assisted by click and electroclick chemistry. Thus, the resulting surface allowed a stable immobilization of the biorecognition elements and provided sensitive electrochemical responses. Figure 3.17 shows the steps followed after electrode modification which consists in the Cu(I) catalyzed azide-alkyne reaction between azide-MWCNTs and ethynyl-IgG, the latter used as the alkyne reagent. Then,

as it can be shown, the specific immunoreagents required for the preparation of the immunosensor were also sequentially incorporated.

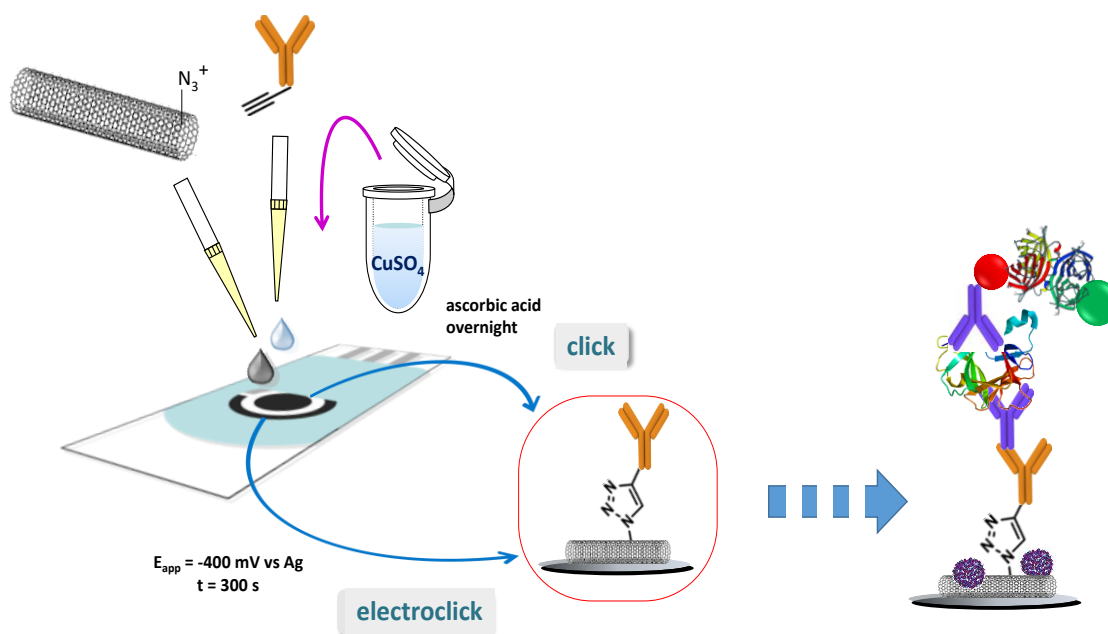


Figure 3.17. Schematic display of steps involved in the preparation of an electrochemical immunosensor by IgG-alkyne-azide-MWCNTs click or electroclick - assisted synthesis followed by sequential incorporation of specific immunoreagents (not shown).

3.4.4.2. Immobilization by covalent binding

Covalent binding is one of the most used methods for biomolecules immobilization. Its implementation takes advantage of a variety of functional groups present in biochemical targets for practical application of bioconjugation techniques: primary amines ($-NH_2$), carbohydrates (sugars), thiols ($-SH$), carbonyls ($-CHO$) and carboxyl groups ($-COOH$) are the most representative [Liébana, 2016]. Amino groups exist at the N-terminus of polypeptides chain and in the side chain of lysine residues, being usually arranged on the outer surface of proteins and positively charged in physiological conditions. Therefore, these groups are normally accessible for conjugation by covalent bond, mostly with carboxyl moieties, without the risk of denaturing the protein structure [Hernández, 2011]. This strategy has been frequently used for the immobilization of bioreagents on electrode surfaces modified with carboxyl groups created either by simple oxidation of carbon materials or by grafting with arylamino carboxylic acids, as well as on carboxyl-functionalized MBs.

Attachment of proteins to carboxyl groups by covalent binding requires the application of a previous activation step which involves the preparation of a succinimidyl ester terminated surface layer for subsequent reaction with an amino linker appended to the lysine groups of protein to form strong covalent peptide bonds. Succinimidyl ester groups are obtained by the reaction of carboxyl moieties with a carbodiimide such as N-ethyl-N'-(3-(dimethylamino) propyl) carbodiimide (EDC) or other similar coupling agents (Figure 3.18) in the presence of N-hydroxysuccinimide (NHS) or its water-soluble analog sulfo-NHS (NHSS). The O-acylisourea intermediate formed by the reaction between EDC and carboxyl groups is unstable in aqueous solution but stabilizes by coupling to NHS, allowing an efficient conjugation to primary amines at physiological pH [Sam, 2010].

This process is a well-established bioconjugation methodology because of simplicity and high efficiency, with the additional advantage that it can be performed in aqueous solution without using organic solvents [Park, 2014]. The activation reaction with EDC and NHSS has shown to be more efficient at pH 4.5-7.2. Although NHSS-esters are stable enough to resist a two-step reaction scheme, however coupling should be done as quickly as possible to avoid hydrolysis of the formed ester which can occur within hours or minutes depending on the pH of the solution.

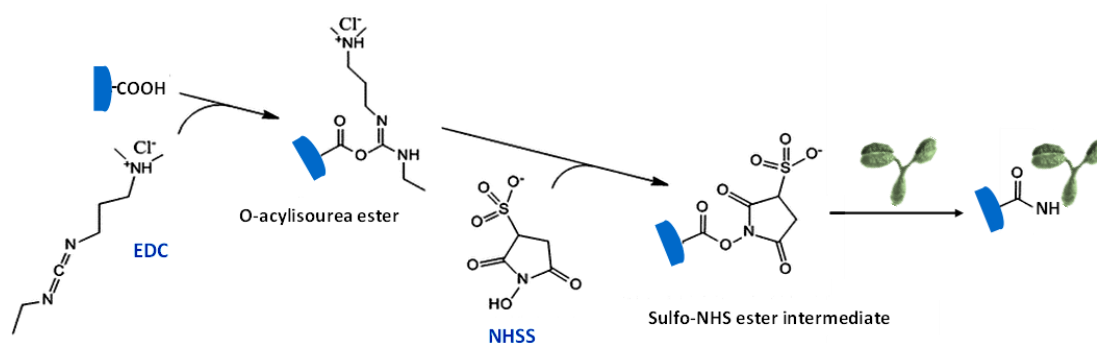


Figure 3.18. Scheme of antibody covalent binding onto a carboxylated surface using EDC/NHSS reactions.

In this work, covalent attachment was implemented onto *p*-ABA grafted SPAuEs for the development of electrochemical immunosensors for TGF- β 1 cytokine and PYY hormone by immobilization of the respective specific antibodies, as well as onto dual SPCEs for the construction of an electrochemical platform magnetically assisted for simultaneous immunosensing of CXCL7 chemokine and MMP3 enzyme. Similar designs were also prepared using cMBs as support for covalent immobilization of a Fc fragment of human IgG (Fc(IgG) or MBP, for the determination of RF or anti-MBP autoantibodies, respectively. For illustrative purposes, Figure 3.19 shows the simplified steps involved in the preparation of immobilized antibody-antigen conjugates using both type of designs.

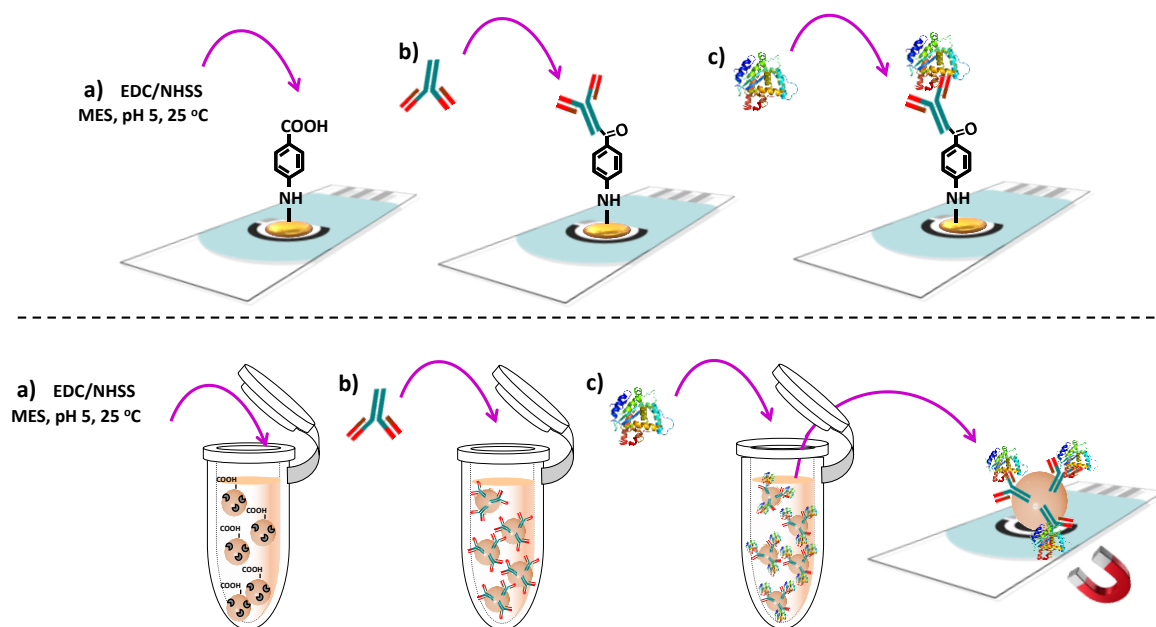


Figure 3.19. Simplified scheme of antibody-antigen conjugates preparation by covalent immobilization onto *p*-ABA-grafted SPAuEs (up) and COOH-MBs (down).

3.4.4.3. Affinity binding with biotinylated immunoreagents

Biotinylation process allows incorporation of biotin groups into biomolecules without losing of chemical and biological properties, making possible a wide range of applications. Biotin affinity to avidin, neutravidin or streptavidin are among the most specific and stable non-covalent protein-ligand interactions. The consequent stability has gained universal recognition standing out its use in diagnostics, treatments, nanoscience techniques and purification and capture purposes [Vetter, 2018]. Avidin is

a tetrameric glycoprotein in which each of the four subunits binds to biotin with the mentioned affinity. Despite its wide applicability, this protein has several limitations including non-specific binding and possible immunogenicity. To face these limitations, some variants of avidin are available, being streptavidin and neutravidin two of the most widely used. Streptavidin is an avidin analogue isolated from the bacterium *Streptomyces avidinii*. Not only it is resistant to denaturing agents, temperature, pH and proteolytic enzymes, but also it protects the biotinyl esters from hydrolysis. In addition, neutravidin (Figure 3.20) is a deglycosylated form of avidin with charge close to neutrality that prevents the nonspecific binding to cell surfaces and proteins [Nguyen, 2012] [Jain, 2017]. Due to the absence of Arg-Tyr-Asp sequence, the possibility the interaction with the membrane receptors of several cells is eliminated.

In this Thesis, streptavidin conjugates with horseradish peroxidase (Strept-HRP) or alkaline phosphatase (Strept-AP) were used as labels of biotinylated detection antibodies for development of various immunosensor configurations. Furthermore, neutravidin-functionalized MBs were utilized for immobilization of biotinylated CCP peptide in the design of immunosensing platform for CCP autoantibody (CCPA).

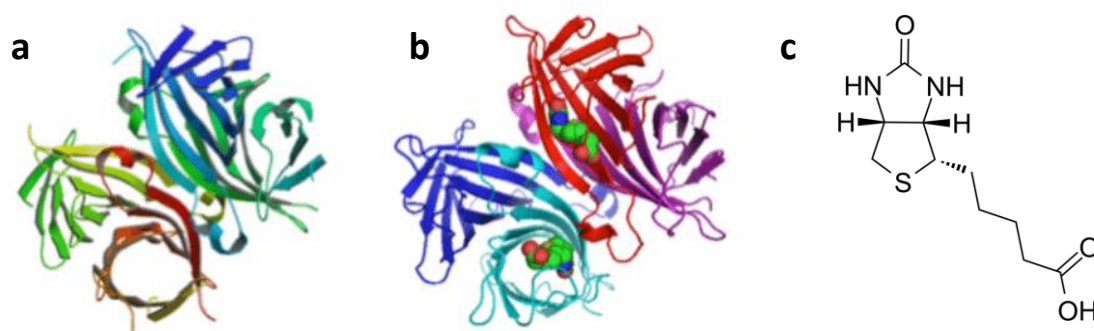


Figure 3.20. (a) Ribbon diagram of the tetrameric structure of neutravidin. (b) Two biotin molecules in space-filling representation bound to the two "pockets" of neutravidin. (c) Chemical structure of biotin (also known as vitamin B 7). Adapted from [Popov, 2015].

3.5. REPORTED METHODS FOR DETERMINATION OF THE TARGET ANALYTES

In this section, a revision of the determination methods described in the literature for the determination of the target analytes involving ELISA methods and affinity biosensors was performed with the results summarized in Tables 3.5.1 - 3.5.9.

3.5.1. Transforming growth factor β 1 (TGF- β 1)

Immunoassay strategies using sandwich-type configurations with peroxidase-labelled or biotinylated anti-TGF- β 1 as detection antibodies are employed in commercial ELISA colorimetric kits. Some are provided by R&D Systems [www.rndsystems.com/products/human-tgf-beta-1-quantikine-elisa-kit_db100b], Abcam [www.abcam.com/human-tgf-beta-1-elisa-kit-ab100647.html], Abnova [www.abnova.com/products/products_detail.asp?catalog_id=KA3106], Thermo-Fisher [www.thermofisher.com/order/genome-database/productdetails/immunoassay/88-8350-88.html?CID=AFLBC-88-8350-88], and LSBio [www.lsbio.com/elisakits/human-tgfb1-tgf-beta-1-sandwich-elisa-elisa-kit-ls-f23380/23380], [www.lsbio.com/elisakits/human-tgfb1-tgf-beta-1-sandwich-elisa-elisa-kit-ls-f2548/2548], and claim to be valid for determining TGF- β 1 in the range from several tens to thousands of pg mL^{-1} with minimum detectable concentrations that can drop to a few units of pg mL^{-1} (Table 3.5.1).

Regarding electrochemical biosensors, few configurations were found in the literature. An aptasensor involving modified Au electrodes integrated with microfluidics and thiolated aptamers labelled with methylene blue self-assembled on gold surfaces was reported [Matharu 2014]. The linear range covered up to 250 ng mL^{-1} with a detection limit of 1 ng mL^{-1} . This sensor was used to monitor TGF- β 1 release from hepatic cells. An impedimetric immunosensor was also developed for the determination of TGF- β 1 in human serum using a polyethyleneglycol SAM prepared onto interdigitated electrodes for the covalent immobilization of antibodies. A linear impedance vs. log TGF- β 1 concentration range between 1 and 1000 ng mL^{-1} was found with a detection limit of 0.570 ng mL^{-1} [Yao, 2016]. Our group prepared various immunosensing configurations for the detection of TGF- β 1 applied to serum samples [Sánchez-Tirado, 2016] and urine [Sánchez-Tirado, 2017]. The latter involved cMBs

for immobilization of specific anti-TGF- β 1 using Mix&Go polymer and amperometric detection with the H_2O_2 /hydro-quinone (HQ) system, providing a calibration range between 15 and 3000 pg mL^{-1} .

Table 3.5.1. ELISA kits and electrochemical biosensors for the determination of TGF- β 1.

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	Sand. HRP-anti-TGF- β 1- TGF- β 1-anti-TGF- β 1	Colorimetry TMB/H ₂ O ₂	31.3 - 2000 pg mL ⁻¹ /4.61 pg mL ⁻¹	4h 30 min	Cell culture, serum, urine, plasma	R&D Systems DB100B
ELISA	Sand. HRP-Strept-Biotin-anti- TGF- β 1-TGF- β 1-anti-TGF- β 1	Colorimetry TMB/H ₂ O ₂	82.3-6x10 ⁴ pg mL ⁻¹ /80 pg mL ⁻¹	4h 45 min	Cell culture, serum, urine, plasma	Abcam ab100647
ELISA	Sand. HRP-Avidin-Biotin-anti- TGF- β 1-TGF- β 1-anti-TGF- β 1	Colorimetry TMB/H ₂ O ₂	15.6 - 1000 pg mL ⁻¹ /8.6 pg mL ⁻¹	4h 30 min	Body fluid, serum, cell culture, lysates	Abnova KA3106
ELISA	Sand. HRP-Avidin-Biotin-anti- TGF- β 1-TGF- β 1-anti-TGF- β 1	Colorimetry TMB/H ₂ O ₂	8 - 1000 pg mL ⁻¹ / 8 pg mL ⁻¹	4h 45 min	Cell culture, serum, plasma	Thermo Fisher 88-8350-88
ELISA	Sand. HRP-Strept-Biotin-anti- TGF- β 1-TGF- β 1-anti-TGF- β 1	Colorimetry TMB/H ₂ O ₂	31.25-2000 pg mL ⁻¹ /18.75 pg mL ⁻¹	3h 15 min	Breast milk, serum, plasma, cell lysates	LSBio F23380
ELISA	Sand. HRP-Avidin-Biotin-anti- TGF- β 1-TGF- β 1-anti-TGF- β 1	Colorimetry TMB/H ₂ O ₂	15.6 - 1000 pg mL ⁻¹ /1 pg mL ⁻¹	2h 30 min	Cell culture, serum, plasma	LSBio F2548
Apta- sensor	MB-Apt-SAM/AuE + anti- TGF- β 1 aptamer	SWV MB	1 - 250 ng mL ⁻¹ / 1 ng mL ⁻¹	2h	Hepatic stellate cells	[Matharau, 2014]
Immuno- sensor	TGF- β 1-anti-TGF- β 1-HOOC-PEG/AuE	EIS	1 - 1000 ng mL ⁻¹ / 0.586 ng mL ⁻¹	1h 15 min	Serum	[Yao, 2016]
Immuno- sensor	Sand. poly-HRP-Strept-Biotin-anti-TGF- β 1- TGF- β 1-anti-TGF- β 1-IgG-MWCNTs/SPCE	Amperometry H ₂ O ₂ /HQ	5 - 200 pg mL ⁻¹ / 1.3 pg mL ⁻¹	1h 20 min	Serum	[Sánchez- Tirado, 2016]
Immuno- sensor	Sand. poly-HRP-Strept-Biotin-anti-TGF- β 1- TGF- β 1-anti-TGF- β 1-HOOC-MBs/SPCE	Amperometry H ₂ O ₂ /HQ	15 - 3000 pg mL ⁻¹ / 10 pg mL ⁻¹	2h 20 min	Urine	[Sánchez- Tirado, 2017]
Immuno- sensor	Sand. V-Phe-SWCNT(-HRP)-anti-TGF- β 1- TGF- β 1-anti-TGF-Biotin-Strept/SPCE	Amperometry H ₂ O ₂ /HQ	2.5 - 1000 pg mL ⁻¹ / 0.95 pg mL ⁻¹	2h 30 min	Saliva	[Sánchez- Tirado,2017a]

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

Abbreviations: Sand., Sandwich-type; HRP, horseradish peroxidase; TMB, 3,3', 5,5'-tetramethylbenzidine; SWV, square-wave voltammetry; MB, methylene blue; EIS, electrochemical impedance spectroscopy; Apt, aptamer; SAM, self-assembled monolayer; PEG, polyethyleneglycol; HQ, hydroquinone; V, viologen.

Our group also synthesized SWCNTs functionalized with viologen redox mediator (V) by aryl-diazonium chemistry in the presence of isoamyl nitrite, and the resulting V-Phe-SWCNT hybrids were used as carrier tags for signal amplification once HRP and detection anti-TGF biotinylated capture anti-TGF antibodies were immobilized onto 4-carboxyphenyl functionalized SPCEs modified with streptavidin and a sandwich-type immunoassay was implemented for TGF- β 1. The linear range exhibited by the as prepared immunosensor extended between 2.5 and 1000 pg mL⁻¹ TGF- β 1 and the LOD value was of 0.95 pg mL⁻¹ [Sanchez-Tirado, 2017].

3.5.2. Interleukin 1 β (IL-1 β)

Various commercial ELISA kits are also available for the determination of this cytokine involving similar sandwich-type configurations to those cited above. As examples, the analytical features of some of them from R&D Systems [www.rndsystems.com/products/human-il-1-beta-il-1f2-quantikine-elisa-kit_dlb50], Abcam [www.abcam.com/human-il-1-beta-elisa-kit-ab214025.html], Biogen Cientifica (Cloud Clone Corp.) [www.biogen.es/es/cloud-clone/584410-elisa-kit-for-interleukin-1-beta-il1b.html], Ray Biotech [www.raybiotech.com/human-il-1-beta-elisa/] and Thermo Fisher (Invitrogen) [www.thermofisher.com/elisa/product/IL-1-beta-Human-ELISA-Kit/KAC1211] are summarized in Table 3.5.2. The most sensitive design provides a limit of detection of 0.3 pg mL⁻¹ requiring an assay time of more than 4 h, and the fastest needs 2 h 15 min showing a higher LOD value of 2 pg mL⁻¹.

Regarding electrochemical biosensors, Krause et al. [Krause, 2015] prepared a microfluidic immunosensing array for various proteins including IL- β 1 where immobilized antibodies and enzyme labels onto MBs were placed in the capture chamber and transported downstream to a detection chamber containing an eight-sensor array of PDDA-glutathion and AuNPs modified SPCEs. Using this design, a LOD value of 0.04 pg mL⁻¹ was obtained in a short time of 30 min. An ultrasensitive method was also described based on the preparation of an immunosensor by covalent immobilization of anti-IL-1 β capture antibodies onto 6-phosphohexanoic acid modified ITO and impedance measurements which were proportional to IL-1 β concentrations in the range of 0.025-3 pg mL⁻¹ with a minimum detectable concentration of 7.5 fg mL⁻¹ [Aydın, 2018]. In addition, a fully integrated bioelectrochemical platform for the determination of IL-1 β

and IL-10 cytokines by impedimetric measurements was proposed using eight gold working microelectrodes functionalized with 4-carboxymethyl aryl diazonium for immobilization of the capture antibodies. A concentration range of 1 to 15 $\text{pg}\cdot\text{mL}^{-1}$ and a LOD value of 0.7 $\text{pg}\cdot\text{mL}^{-1}$ IL-1 β were achieved [Baraket, 2017].

Our group also reported a dual immunosensor involving SPCEs modified with 4-carboxyphenyl-functionalized double-walled carbon nanotubes (HOOC-Phe-DWCNTs/SPCEs) for the determination of IL-1 β simultaneously with TNF- α . A sandwich type immunoassay with signal amplification by means of poly-HRP-streptavidin conjugates and amperometric detection using the H_2O_2 /HRP/HQ system were implemented. The developed method provided a linear range between 0.5 and 100 pg mL^{-1} for IL-1 β and the achieved LOD value was of 0.38 pg mL^{-1} in spiked serum and saliva samples [Sánchez-Tirado, 2017].

3.5.3. Chemokine (C-X-C motif) ligand 7 (CXCL7)

The analytical characteristics of some ELISA methods for the determination of CXCL7 in biological samples are summarized in Table 3.5.3. As it can be shown, protocols of commercial ELISA kits from R&D Systems [www.rndsystems.com/products/human-cxcl7-nap-2-duoset-elisa_dy393], Abcam [www.abcam.com/human-cxcl7-elisa-kit-ab_100613.html], Rockland [www.rockland.com/categories/kits/human-cxcl7-elisa-kit-KO_A0426/] and Aviscera [www.aviscerabioscience.com/Product_Systems/NAP2/SK00434_-01.pdf] involve sandwich type configurations with anti-CXCL7 capture antibodies and a biotinylated secondary antibody which conjugates with HRP-Strept for subsequent detection with H_2O_2 and TMB. This same scheme but using Avidin-HRP conjugates is employed in the kit from Novus Biologicals [www.novusbio.com/products/cxcl7-nap-2-elisa-kit_nbp2-59965], whereas an HRP-labelled instead biotinylated detection antibody is used in that from LS Bio [www.lsbio.com/elisakits/human-cxcl7-ppbp-sandwich-elisa-elisa-kit-ls-f26826/26826]. As it can also be seen, this last method is the one that requires a shorter test time (1h 20 min versus more than 3 or 4 h) but it is also the least sensitive. It should be noted that no electrochemical biosensors have been found in the revised literature for the determination of this chemokine.

Table 3.5.2. ELISA kits and electrochemical biosensors for the determination of IL-1 β .

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	Sand. HRP-Strept-Biotin-anti-IL-1 β -IL-1 β -anti- IL-1 β	Colorimetry TMB/H ₂ O ₂	3.9 - 250 pg mL ⁻¹ / 1 pg mL ⁻¹	4h 30 min	Cell culture, serum, plasma	R&D Systems DLB50B
ELISA	Sand. HRP-Strept-Biotin-anti-IL-1 β -IL-1 β -anti- IL-1 β	Colorimetry TMB/H ₂ O ₂	14.06 - 900 pg mL ⁻¹ / 5.64 pg mL ⁻¹	2h 30 min	Cell culture, serum, plasma	Abcam ab214025
ELISA	Sand. HRP-Strept-Biotin-anti-IL-1 β -IL-1 β -anti- IL-1 β	Colorimetry TMB/H ₂ O ₂	7.8 - 500 pg mL ⁻¹ / 8.6 pg mL ⁻¹	3h	Body fluid, serum, cell culture, lysates	Biogen (CC) 584410
ELISA	Sand. HRP-Strept-Biotin-anti-IL-1 β -IL-1 β -anti- IL-1 β	Colorimetry TMB/H ₂ O ₂	0.3 - 100 pg mL ⁻¹ / 0.3 pg mL ⁻¹	4h 45 min	Cell culture, plasma, serum, cell lysates	Ray Biotech ELH-IL1b-1
ELISA	Sand. HRP-Strept-Biotin-anti-IL-1 β -IL-1 β -anti- IL-1 β	Colorimetry TMB/H ₂ O ₂	33 - 1400 pg mL ⁻¹ / 2 pg mL ⁻¹	2h 15 min	Cell culture supernatant, serum, plasma	Thermo Fisher 88-8350-88
Microfluidic immunosensor array	Sand. HRP-anti-IL-1 β -AuNPs /MBs-IL-1 β -anti-IL-1 β - GSH/AuNPs/ PDDA/SPCE	Amperometry H ₂ O ₂ /HQ	0.04 - 22 pg mL ⁻¹ / 0.04 pg mL ⁻¹	30 min	Serum	[Krause, 2015]
Immuno- sensor	Sand. HRP-Strept-Biotin-anti-IL-1 β -IL-1 β -Phe-DWCNTs/ SPCE	Amperometry H ₂ O ₂ /HQ	0.5 -100 pg mL ⁻¹ 0.38 pg mL ⁻¹	2h	Serum, saliva	[Sánchez- Tirado, 2017]
Immuno- sensor	IL-1 β -BSA-PHA/ITO	EIS	0.025 - 3 pg mL ⁻¹ 0.0075 pg mL ⁻¹	45 min	Serum, saliva	[Aydin, 2018]

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

Abbreviations: Sand., Sandwich type; HRP, horseradish peroxidase; TMB, 3,3',5,5'-tetramethylbenzidine; GSH, glutathion; PDDA, poly-(diallyldimethylammonium); ITO, Indium-tin oxide electrode; PHA, 6-phosphonohehexanoic acid; BSA, bovine serum albumin; EIS, electrochemical impedance spectroscopy.

Table 3.5.3. ELISA kits for the determination of CXCL7.

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	Sand. HRP-Strept-Biotin- anti-CXCL7-CXCL7-anti- CXCL7	Colorimetry TMB/H ₂ O ₂	15.6 - 1000 pg mL ⁻¹ / 15.6 pg mL ⁻¹	4h 40 min	Cell culture, serum, urine	R&D Systems DY393
ELISA	Sand. HRP-Strept-Biotin- anti-CXCL7-CXCL7-anti- CXCL7	Colorimetry TMB/H ₂ O ₂	4.1 - 1000 pg mL ⁻¹ / 8.5 pg mL ⁻¹	4h 45 min	Cell culture supernatants, lysates, serum, plasma	Abcam ab100613
ELISA	Sand. HRP-Strept-Biotin- anti-CXCL7-CXCL7-anti- CXCL7	Colorimetry TMB/H ₂ O ₂	15.6 - 1000 pg mL ⁻¹ / 2 pg mL ⁻¹	3h 30 min	Cell culture supernatants, serum, plasma	Rockland KOA0426
ELISA	Sand. HRP-anti-CXCL7- CXCL7-anti-CXCL7	Colorimetry TMB/H ₂ O ₂	0.5 - 10 ng mL ⁻¹ / 0.1 ng mL ⁻¹	1h 20 min	Cell culture supernatants, lysates, serum, plasma, tissue homogenates	LS BIO LS-F26826
ELISA	Sand. HRP-Avidin-Biotin- anti-CXCL7-CXCL7-anti- CXCL7	Colorimetry TMB/H ₂ O ₂	78 - 5000 pg mL ⁻¹ / 19.5 pg mL ⁻¹	4h 30 min	Serum, plasma, tissue homogenates	Novus Biol. NBP2-59965
ELISA	Sand. HRP-Strept-Biotin- anti-CXCL7-CXCL7-anti- CXCL7	Colorimetry TMB/H ₂ O ₂	125 - 2000 pg mL ⁻¹ / 10 pg mL ⁻¹	4h 45 min	Serum, plasma	Aviscera Bios. SK00434-01

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

3.5.4. Chemokine (C-C motif) ligand 5 (CCL5)

As it occurred for the previous chemokine, in this case no electrochemical biosensor for the determination of CCL5 was found in the revised literature. In addition, all the ELISA methods on which the available commercial kits are based are very similar to each other and to others used for different cytokines. As Table 3.5.4. shows, sandwich-type configurations involving biotinylated detection antibodies and Strept-HRP conjugates are proposed by Thermo Fisher [www.thermofisher.com/elisa/product/RANTES-Human-ELISA-Kit/EHRNTS], Ray Biotech [www.raybiotech.com/human-rantes-elisa/] and Sigma Aldrich (Merck) [www.sigmaaldrich.com/ES/es/product/sigma/rab0076]. These immunoassays are very similar, providing concentration ranges of up to thousands of pg mL^{-1} and detection limits around pg mL^{-1} units, as well as assay times equal to or greater than 3 hours. Furthermore, R&D Systems [www.rndsystems.com/products/human-ccl5-rantes-quantikine-elisa-kit_drn00b] proposes the use of an HRP-labelled detection antibody, directly. This simplification, however, does not shorten the assay time, but slightly increases the detection limit. Finally, Abcam [www.abcam.com/human-rantes-elisa-kit-ccl5-ab174446.html#lb] developed the Simple Step ELISA[®] for CCL5, using an HRP-labelled detector antibody conjugated in solution with the analyte/sample and the capture antibody. This conjugate is in turn immobilized onto an antibody coating the well. This strategy greatly shortens assay time up to 90 min and allows to obtain a higher sensitivity, with a LOD value of 0.091pg mL^{-1} .

3.5.5. Peptide YY (PYY)

Various ELISA methods involving similar immunoassay schemes were developed for PYY (Table 3.5.5.). As examples, sandwich-type configurations involving specific PYY capture antibodies and detection with biotinylated antibodies and HRP-labelled avidin or streptavidin are provided by My Biosource [www.mylbiosource.com/human-elisa-kits/pyy/2512606], Thermo Fisher [www.thermofisher.com/elisa/product/Human-PYY-ELISA-Kit/] or Ray Biotech [https://doc.raybiotech.com/pdf/Manual/ELH-PYY_2021.06.14.pdf]. Linear semilogarithmic calibrations for PYY in the range of units or few tens up to few thousand pg mL^{-1} were achieved except in the kit from Ray Biotech whose calibration covers the range of ng mL^{-1} . The assay times reach nearly five hours. When comparing the tests from Thermo Fisher and My Biosource, note that in the latter, the shorter test, lasting just over three hours, provides a higher detection limit.

Table 3.5.4. ELISA kits and electrochemical biosensors for the determination of CCL5.

Method	Configuration	Detection	Linear range/LOD*	Assay time	Sample	Reference
ELISA	Sand. HRP-Strept-Biotin-anti-CCL5-CCL5-anti-CCL5	Colorimetry TMB/H ₂ O ₂	51.2 - 2000 pg mL ⁻¹ / 2 pg mL ⁻¹	3h	Cell culture supernatant, serum, urine, plasma	Thermo Fisher EHRNTS
ELISA	Sand. HRP-Strept-Biotin-anti-CCL5-CCL5-anti-CCL5	Colorimetry TMB/H ₂ O ₂	3 - 2000 pg mL ⁻¹ / 3 pg mL ⁻¹	4h 45 min	Cell culture supernatants, serum, plasma	Ray Biotech ELH-RANTES-1
ELISA	Sand. HRP-Strept-Biotin-anti-CCL5-CCL5-anti-CCL5	Colorimetry TMB/H ₂ O ₂	2.74-2000 pg/mL ⁻¹ / 3 pg mL ⁻¹	4h 45 min	Serum, plasma, urine, cell culture supernatants	Sigma-Aldrich Human RANTES CCL5 ELISA Kit RAB0076
ELISA	Sand. HRP-anti-CCL5-CCL5-anti-CCL5	Colorimetry TMB/H ₂ O ₂	31.2 - 2000 pg mL ⁻¹ / 6.6 pg mL ⁻¹	3h 30 min	Cell culture supernatants, serum, plasma, urine	R&D Human CCL5/ RANTES Quantikine DR N00B
ELISA	Sand. HRP-anti-CCL5-CCL5-anti-CCL5	Colorimetry TMB/H ₂ O ₂	15.6 - 1000 pg mL ⁻¹ /-	4h 45 min	Cell culture supernatants, serum, plasma, urine	R&D Human CCL5/ RANTES Duo Set Cat. No: DY278-05
ELISA	Sand. anti-tag antibody + (HRP - anti-CCL5-CCL5-anti-CCL5)	Colorimetry TMB/H ₂ O ₂	0.94-960 pg mL ⁻¹ / 0.091 pg mL ⁻¹	90 min	Cell culture supernatants, serum, plasma	ab174446 Human RANTES Simple Step ELISA® Kit

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

As it can also be seen, a competitive configuration from Eagle [\[https://eaglebio.com/product/human-pyy-elisa-assay/\]](https://eaglebio.com/product/human-pyy-elisa-assay/), is also available. However, this assay requires more than 20 h or incubate overnight. Finally, as in the case of CCL5, a SimpleStep ELISA® from Abcam [\[www.abcam.com/human-pyy-elisa-kit-ab255727.html\]](http://www.abcam.com/human-pyy-elisa-kit-ab255727.html) for the detection of PYY using similar experimental conditions is also available.

With respect to electrochemical biosensors, only two configurations developed by our group have been found in the literature. Direct competitive immunoassays were performed in both cases at electrodes modified by grafting of the diazonium salt of 4-aminobenzoic acid onto reduced graphene oxide on which the covalent immobilization of specific anti-PYY antibodies was accomplished. Detection antibodies labelled with alkaline phosphatase (AP) were also used and differential pulse voltammetry upon addition of 1-naphthyl phosphate for monitoring the affinity was applied. Under optimized conditions, the immunosensor prepared at a glassy carbon electrode provided a linear range between 0.1 and $100 \cdot 10^3$ pg/mL and a limit of detection of 0.01 pg/mL PYY [\[Guerrero, 2015\]](#). Based on the previous design, a multiplexed electrochemical immunosensor using modified dual SPCEs was also prepared for the simultaneous determination of PYY and ghrelin (GHRL), this latter hormone playing also important roles in the regulation of hunger and satiety. As Table 3.5.5. shows, the range of concentrations in the calibration plot extended between 10^{-4} and 10 ng mL⁻¹ PYY [\[Martínez-García, 2017\]](#).

3.5.6. Rheumatoid factor (RF)

Some examples of commercial ELISA kit available for RF autoantibody are summarized in Table 3.5.6. In all cases, the determination involved an indirect enzyme linked immunoassay in which Fc fragments of IgG are previously coated onto microwells to specifically bind RF antibodies present in biological samples. Subsequently, an HRP-labelled anti-human IgM conjugate is added to the wells, which binds to the RF conjugate. As it can be shown, quite similar results provide ELISA kits from Demeditec [\[www.demeditec.com/en/products/rheumatoid-factor-igg-elisa-de-7640\]](http://www.demeditec.com/en/products/rheumatoid-factor-igg-elisa-de-7640) and Abcam [\[www.abcam.com/rheumatoid-factor-igm-elisa-kit-ab178653.html\]](http://www.abcam.com/rheumatoid-factor-igm-elisa-kit-ab178653.html), although the latter, with a larger assay time, allows also to obtain a slightly lower LOD value. In the Eagle Biosciences's ELISA kit [\[https://eaglebio.com/product/rheumatoid-factor-igm-elisa-kit/\]](https://eaglebio.com/product/rheumatoid-factor-igm-elisa-kit/), wells are coated with rabbit IgG, and the indirect assay requires a time of 2.5 h.

Table 3.5.5. ELISA kits and electrochemical biosensors for the determination of PYY.

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	Direct Comp. HRP-Strept-Biotin -PYY-PYY-anti-PYY	Colorimetry TMB/ H ₂ O ₂	0.082 - 20 ng mL ⁻¹ 20 pg mL ⁻¹	≈20h	Plasma, serum	Eagle Biosciences YK080
ELISA	Sand. HRP-Avidin-Biotin-anti- PYY-PYY-anti-PYY	Colorimetry TMB/ H ₂ O ₂	31.25-2000 pg mL ⁻¹ 18.75 pg mL ⁻¹	3h 15min	Plasma, serum, cell lysates, tissue homogenates	MyBioSource MBS2512606
ELISA	Sand. HRP-Strept-Biotin-anti- PYY-PYY-anti-PYY	Colorimetry TMB/ H ₂ O ₂	0.156-2000 pg mL ⁻¹ 1.4 pg mL ⁻¹	4h 45min	Plasma, serum, cell culture	Thermo Fisher PYY EH387RB
ELISA	Sand. HRP-Strept-Biotin-anti- PYY-PYY-anti-PYY	Colorimetry TMB/ H ₂ O ₂	15.63 - 10 ng mL ⁻¹ 0.15 ng mL ⁻¹	4h 45min	Plasma, serum, cell culture	Ray Biotech ELH- PYY
ELISA	Sand. anti-tag Ab + (HRP-anti- PYY-PYY-anti-PYY)	Colorimetry TMB/H ₂ O ₂	0.156 - 10 ng mL ⁻¹ 8.4 pg mL ⁻¹	90 min	Plasma, tissue extract	Abcam PYY Simple Step ab255727
Immuno- sensor	Direct Comp. AP-Strept-Biotin -PYY-anti-PYY-Phe-rGO/GCE	DPV 1-NP	10 ⁻⁴ - 10 ² ng mL ⁻¹ / 0.01 pg mL ⁻¹	2h	Serum	[Guerrero, 2015]
Immuno- sensor	Direct Comp. AP-Strept-Biotin -PYY-anti-PYY Phe-rGO/SPCE	DPV 1-NP	10 ⁻⁴ - 10 ng mL ⁻¹ / 0.02 pg mL ⁻¹	2h	Serum, saliva	[Martínez-García, 2016]

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

Abbreviations: AP, alkaline phosphatase; Phe, phenyl; 1-NP, 1-naphtyl phenol.

Table 3.5.6. ELISA kits and electrochemical biosensors for the determination of RF.

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	Indirect HRP-IgM-RF-Fc(IgG)	Colorimetry TMB/ H ₂ O ₂	15 - 500 IU mL ⁻¹ / 1 IU mL ⁻¹	1h 5 min	Plasma, serum	Demeditec Rheumatoid Factor IgG ELISA DE7640
ELISA	Indirect HRP-IgM-RF-Fc(IgG)	Colorimetry TMB/ H ₂ O ₂	3 - 300 IU mL ⁻¹ / <0.5 IU mL ⁻¹	1h 45min	Plasma, serum	Abcam ab178653 Anti- Rheumatoid Factor IgM ELISA Kit
ELISA	Indirect HRP-IgM-RF-IgG	Colorimetry TMB/ H ₂ O ₂	1 - 300 IU mL ⁻¹ / 1 IU mL ⁻¹	2h 30min	Serum	Eagle Biosc. RF IgM ELISA RFM31-K01
EISC based immunosensor	Label free RF-APTES/YbTixOy	Capacitance	1.54-1.54·10 ⁴ IU mL ⁻¹ / 40 IU mL ⁻¹	2h 45min	Serum	[Pan, 2015]
IDW _μ E based biosensor	Label free Fc(IgG)-TA-SAM- IDW _μ E	EIS Fe(CN) ₆ ^{3-/4-}	1 - 200 IU mL ⁻¹ / 0.6 IU mL ⁻¹	10 min	Serum	[Chinnadayya, 2019]

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

Abbreviations: Fc, fragment crystallizable region of antibody; EISC, electrolyte-insulator-semiconductor; APTES, (3-aminopropyl)-triethoxysilane; TA, thioctic acid; IDW_μE, interdigitated wave type microelectrode array.

Regarding biosensors, Pan et al. [Pan, 2015] applied YbTi_xO_y sensing membranes for the construction of a label-free electrolyte-insulator-semiconductor (EIS) sensor to detect the RF in serum samples. EDC/NHS chemistry was used to functionalize the carboxyl groups at the tail of RF antibodies which were immobilized onto an APTES-modified YbTi_xO_y surface. The developed EIS biosensor exhibited a LOD of 40 U/mL and a dynamic range between 1.54 and $1.54 \cdot 10^4$ IU mL⁻¹ (0.1 -1000 μM) requiring an assay time of 2 h 45 min. More recently, an impedimetric-interdigitated wave type microelectrode array (IDW_μE) was also proposed for the label-free and direct detection of RF [Chinnadayya, 2019]. The IDW_μE was functionalized with a self-assembled monolayer (SAM) of thioctic acid for antigen immobilization and the covalent immobilization of IgG-Fc onto the SAM modified electrode. Impedimetric measurements in the presence of redox probe showed a significant change in the impedance spectrum and charge transfer resistance upon IgM-RF binding providing a LOD values of 0.6 IU mL⁻¹. The sensor was applied to the determination of RF autoantibody in human serum in just 10 min.

3.5.7. Anti-cyclic citrullinated peptide (CCPA)

The main features of the reported methods for the determination of CCPA autoantibody are summarized in Table 3.5.7, which includes examples of the most representative ELISA kits commercially available and the few electrochemical designs of biosensors developed so far. As it can be seen, sandwich-type immunoassays were implemented in the ELISA protocols involving well plates coated with the cyclic citrullinated peptide to bond with anti-CCP autoantibodies from the sample. Subsequently, bound antibodies react with HRP-labelled anti-human-IgG and the colorimetric detection is carried out as usually, in the presence of TMB and H₂O₂. The analytical characteristics of the developed methods can be summarized as calibration dynamic ranges up to 2960 IU mL⁻¹ and limits of detection around 1.6 IU mL⁻¹ [ONYVA E-23-0182-11]. The required assay times varies from 1 h 5 min up to 2 h 20 min [www.demeditec.com/en/products/ccp-ab-elisa-de7760?q=c].

Regarding biosensors, cyclic citrullinated peptide (CCP) based on cyclic citrullinated sequences derived from filaggrin [Schellekens, 2000] [Aggarwal, 2009] has commonly been used as the sensitive and specific antigen for the detection of anti-CCP

antibody. Some electrochemical bioplatfroms have been described in the literature. As example, a simple amperometric immunosensor was reported for the detection of serum CCPA using a chimeric fibrin–filaggrin synthetic peptide (CFFCP1) as the recognition element anchored to the surface of an electrode modified with multiwalled carbon nanotube and polystyrene (MWCNT–PS) based electrode. Amperometric detection was performed using HRP-hIgG by addition of H₂O₂ in the presence of TMB as the redox mediator. The immunosensor was validated for application to human serum samples [De Gracia Villa, 2011]. Interdigitated electrodes (IDEs) modified with cyclic antigen (CCP)-coated-AuNPs were also used for determining anti-CCP antibody by impedimetric measurements. The detection method involved signal amplification by conjugation of hIgG with GOD to catalyze glucose producing H₂O₂ that reduce silver ions to Ag⁰ in the form of nanocrystals at the AuNPs. A limit of detection of 0.12 ng/mL was obtained [Vu, 2020]. More recently, Chinnadayala et al. developed a miniaturized, label-free impedimetric immunosensor based on an interdigitated-chain-shaped microelectrode array (ICE) modified with a self-assembled monolayer (SAM) of mercaptohexanoic acid (MHA). The receptor Biotin-CCP was attached onto the surface of SAM modified ICE through a strong avidin-biotin bio-recognition system. The change in the sensor signal upon target analyte (CCPA) binding was probed through the electrochemical impedance spectroscopy (EIS) property of charge-transfer resistance (R_{ct}) of the modified electrodes. Thus, a linear range for the addition of CCPA between 1 - 800 IU/mL and a limit of detection value of 0.82 IU/mL in human serum were achieved [Chinnadayala, 2021]. This team also developed an electrochemical nanobiosensor for detection of CCPA involving SPCEs modified with PANI and MoS₂ and signal amplification by means of PANI-AuNPs. A citrulline-containing cyclic filaggrin peptide (21-mer) was used to recognize the antibody and the determination was made by measuring the decrease in the Fe(CN)₆^{3-/4-} signal by SWV as the antibody concentration increases [Selvam, 2021].

Table 3.5.7. Electrochemical biosensors and ELISA kits for the determination of CCPA.

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	HRP-hIgG-CCPA-CCP	Colorimetry TMB/ H ₂ O ₂	20 - 1,000 IU mL ⁻¹ / 1 IU mL ⁻¹	1h 5 min	Plasma, serum	Demeditec DE7170
ELISA	HRP-hIgG-CCPA-CCP	Colorimetry TMB/ H ₂ O ₂	up to 1,000 IU mL ⁻¹ / 1 IU mL ⁻¹	1h35 min	Plasma, serum	Abnova KA1268
ELISA	HRP-hIgG-CCPA-CCP	Colorimetry TMB/ H ₂ O ₂	20 - 1,000 IU mL ⁻¹ / 1 IU mL ⁻¹	2h15 min	Plasma, serum	IBL International RE75791
ELISA	HRP-hIgG-CCPA-CCP	Colorimetry TMB/ H ₂ O ₂	up to 1,000 IU mL ⁻¹ / 20 IU mL ⁻¹	1h15 min	Plasma, serum	Orgentec ORG601
ELISA	HRP-hIgG-CCPA-CCP	Colorimetry TMB/ H ₂ O ₂	1 - 2,000 IU mL ⁻¹ / 1 IU mL ⁻¹	2h	Plasma, serum	Eagle Biosciences CCP31-K01
ELISA	HRP-hIgG-CCPA-CCP	Colorimetry TMB/ H ₂ O ₂	25 - 2,960 IU mL ⁻¹ / 1.6 IU mL ⁻¹	1h55 min	Serum	ONYVA E-23- 0182-11
Immunosensor	CFFCP1-MWCNT-PS/TFE	Amperometry TMB/ H ₂ O ₂	No data	No data	Serum	[De Gracia Villa, 2011]
Immunosensor	GOD-hIgG-CCPA-CCP- AuNP-IDE	EIS	up to 80 ng mL ⁻¹ / 0.12 ng mL ⁻¹	< 1h	Spiked serum	[Vu, 2020]
Label-free biosensor	CCPA-CCP-Biotin-MHA- ICE	EIS Fe(CN) ₆ ^{3-/4-}	1 - 800 IU mL ⁻¹ / 0.60 IU mL ⁻¹	40 min	Spiked serum	[Chinnandayyala, 2021]
Label-free biosensor	AuNPs-PANI-CCPA-CCP- MoS ₂ /PANI/SPCE	SWV Fe(CN) ₆ ^{3-/4-}	0.25-1,500 IU mL ⁻¹ /0.16 IU mL ⁻¹	1h30 min	Spiked serum	[Selvam, 2021]

***MDD (ELISA):** minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

Abbreviations: CFFCP1, chimeric fibrin-filaggrin synthetic peptide; TFE, thin-film electrode; PS, polystyrene; MHA, mercaptohexanoic acid; ICE, interdigitated-chain-shaped microelectrode array; PANI, polyaniline.

3.5.8. Anti-Myelin Basic Protein (Anti-MBP)

As Table 3.5.8. shows, various ELISA kits for the determination of anti-MBP are commercially available. Sandwich type immunoassays provide calibration plots with concentrations ranging between some units and few hundred ng mL^{-1} with minimum detectable dose (MDD) of around 1 ng mL^{-1} , and assay times of 150 min. Regarding biosensors, only a label-free impedimetric biosensor for anti-MBP has been reported [Derkus, 2013]. It consisted of a platinum electrode with MBP antigen immobilized on either gelatin or gelatin-TiO₂ nanoparticles and achieved a detection limit around 0.15 ng mL^{-1} .

3.5.9. Matrix Metalloproteinase 3 (MMP-3)

Various ELISA kits for MMP3 involving sandwich-type configurations with biotinylated detection antibodies and HRP-Strept conjugates are commercially available (see Table 3.5.9.). Calibrations range from a few tens or hundreds to several thousand pg mL^{-1} and required time-assay are between 1h 30 min [Abcam ab 269371] to 5h [Cusabio CSB-E04677h]. For instance, the ELISA kit from R&D Systems [R&D Systems DMP300] shows a non-linear logarithmic plot with a dynamic range from 0.2 to 10 ng mL^{-1} . The assay time was 4h 30min. A similar method from Abcam (ab269371 Human MMP3 ELISA Kit) exhibits a non-linear logarithmic range between 0.025 and 1.6 ng mL^{-1} , with a minimum detectable concentration of 4.4 pg mL^{-1} . Nevertheless, the most sensitive is that from Invitrogen [Invitrogen BMS2014-3] which provides a LOD value of 0.5 pg mL^{-1} requiring a time of 3h 30min.

With regard to biosensors, very few reports are found in the literature. Among them, it is worth mentioning the electrochemical immunosensor developed by Munge et al. based on vertically aligned single-wall carbon nanotubes (SWCNT) arrays. A sandwich-type configuration was applied by the use of a secondary antibody conjugated with HRP and/or polymer bead loaded with multi-enzyme labels. A detection limit of 400 pg mL^{-1} was attained. Additionally, the implementation of an amplification strategy using a multiply enzyme labelled polymeric bead resulted in a $0.4 - 40 \text{ ng mL}^{-1}$ logarithmic calibration plot and a LOD of 4 pg mL^{-1} [Munge, 2010].

Table 3.5.8. Electrochemical biosensors and ELISA kits for the determination of anti-MBP.

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	HRP-IgG-anti-MBP-MBP	Colorimetry TMB/ H ₂ O ₂	0.25 - 8 ng mL ⁻¹ / 0.1 ng mL ⁻¹	1h 30 min	Plasma, serum, tissue	My Biosource MBS9398507
ELISA	HRP-IgG-anti-MBP-MBP	Colorimetry TMB/ H ₂ O ₂	3.12 - 200 ng mL ⁻¹ / 1.18 ng mL ⁻¹	2h 30 min	Plasma, serum, tissue	Cloud-clone AEA539Hu
ELISA	HRP-IgG-anti-MBP-MBP	Colorimetry TMB/ H ₂ O ₂	15.63-1000 pg/mL ⁻¹ / 9.38 pg mL ⁻¹	3h 30 min	Plasma, serum, tissue	Elabscience E-EL- H0161
ELISA	HRP-IgG-anti-MBP-MBP	Colorimetry TMB/ H ₂ O ₂	0.156 - 10 ng mL ⁻¹ / < 0.07 ng mL ⁻¹	3h	Plasma, serum	Abbexa ELISA 52302
ELISA	HRP-IgG-anti-MBP-MBP	Colorimetry TMB/ H ₂ O ₂	3.12 - 200 ng mL ⁻¹ / 1.24 ng mL ⁻¹	2h 25 min	Plasma, serum	Abclonal RK00896
Label-free immunosensor	anti-MPP-MBP-gelatin- or anti-MBP-MBP-TiO ₂ - gelatin-PtE	EIS Fe(CN) ₆ ^{3-/4-}	0.9750-2,500 ng mL ⁻¹ or 0.4875-2,500 ng mL ⁻¹ / ~0.15 ng mL ⁻¹	-	artificial CSF and serum	[Derkus, 2013]

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.

Table 3.5.9. Electrochemical biosensors and ELISA kits for the determination of MMP3.

Method	Configuration	Detection	Linear range/ LOD*	Assay time	Sample	Reference
ELISA	Sand. HRP-Strep-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.2 -10 ng mL ⁻¹ / 0.045 ng mL ⁻¹	4h 30min	Cell culture, serum, plasma	R&D Systems DMP300
ELISA	Sand. HRP-Strep-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.05 - 3.2 ng mL ⁻¹ / 0.0355 ng mL ⁻¹	1h 30min	Cell culture, serum, plasma	Abcam ab269371
ELISA	Sand. HRP-Strep-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.060-4.0 ng mL ⁻¹ / 5 pg mL ⁻¹	3h 30min	Serum, cell culture, plasma	Invitrogen BMS2014-3
ELISA	Sand. HRP-Avidin-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.078-5.0 ng mL ⁻¹ / 33 pg mL ⁻¹	3h	Serum, plasma, cell culture, tissue homogenates,	ASBOKE H00179
ELISA	Sand. HRP-Avidin-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.156-10 ng mL ⁻¹ / 54 pg mL ⁻¹	5h	Serum, plasma, cell culture supernatants	Cusabio CSBE 04677h
ELISA	Sand. HRP-Strep-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.3-100 ng mL ⁻¹ / 300 pg mL ⁻¹	4h 15min	Serum, plasma, cell culture	RayBiotech ELH-MMP3-1
ELISA	Sand. HRP-Avidin-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.156-10 ng mL ⁻¹ / 10 pg mL ⁻¹	3h 20min	Serum, plasma, cell culture supernatants	LSBio LS-F2514
ELISA	Sand. HRP-Strep-Biotin-anti-MMP3-MMP3-anti-MMP3	Colorimetry TMB/ H ₂ O ₂	0.0625-4 ng mL ⁻¹ / 15 pg mL ⁻¹	3h 45min	Serum, plasma, cell culture	MyBioSource MBS824820
Immunosensor	Sand. HRP/PS bead-anti-MMP3-MMP3-anti-MMP3-SWCNTs/PG	Amperometry H ₂ O ₂ /HQ	0.4 - 40 ng mL ⁻¹ / 0.4 ng mL ⁻¹ or 4 - 300 pg mL ⁻¹ /4 pg mL ⁻¹	1h 15min	Serum	[Munge, 2010]

***MDD** (ELISA): minimal detectable dose (MDD): concentration corresponding to the mean of zero standard replicates + 2 standard deviations.
Abbreviations: PS, polystyrene; PG, pyrolitic graphite.

Parte experimental

Instrumentación
Reactivos y disoluciones
Muestras
Procedimientos experimentales

4.1. INSTRUMENTACIÓN

4.1.1. Aparatos y dispositivos

Las medidas en voltamperometría cíclica (CV) y voltamperometría diferencial de impulsos (DPV) se realizaron utilizando un potenciostato-galvanostato PGSTAT101 provisto del software de análisis electroquímico NOVA 1.8 y un potenciostato μ Autolab tipo III con el software General Purpose Electrochemical System (GPES), ambos instrumentos de la empresa Metrohm AutoLab, siendo estos los que se muestran en la Figura 4.1.

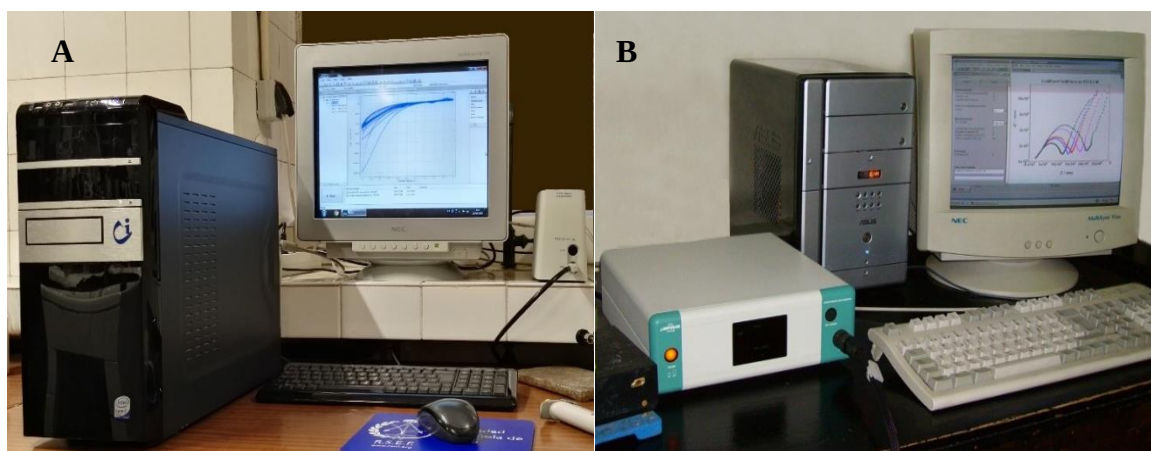


Figura 4.1. A) Potenciostato PGSTAT101 de Metrohm AutoLab y B) Potenciostato μ Autolab tipo III de Metrohm AutoLab.

Para las medidas de espectroscopia de impedancia electroquímica (EIS), utilizadas en el control y caracterización de las etapas de modificación de la superficie electródica, también se hizo uso del potenciostato μ Autolab tipo III utilizando el software FRA2 Frequency Response Analyzer o FRA2, ambos igualmente de Metrohm AutoLab.

Las medidas amperométricas se realizaron con un potenciostato portátil InBea de InBea Biosensores, S.L. mostrado en la Figura 4.2., así como con un potenciostato CHI1030B de Chemical Instruments (Figura 4.3) de uno y dos canales respectivamente, provistos del software correspondiente.



Figura 4.2. Potenciostato portátil InBea (InBea Biosensores, S.L.).



Figura 4.3. Potenciostato CHI1030B de Chemical Instruments de uno o dos canales.

4.1.2. Electrodo

Los procedimientos experimentales puestos a punto en este trabajo se llevaron a cabo empleando distintos tipos de electrodos serigrafiados de Metrohm-DropSens (Oviedo, España) con un electrodo de trabajo propiamente dicho de carbono (SPCE) (DRP-110) o de oro (SPAUE) (DRP-220BT), o con dos electrodos de carbono (dSPCE) (X1110) (configuración dual), así como con un electrodo de pseudo-referencia de plata y un electrodo auxiliar o contraelectrodo de carbono (DRP-110) o de oro (DRP-220BT). Los electrodos indicadores individuales presentan geometría circular con un diámetro de

4 mm, mientras que en el dual las superficies son elípticas con un diámetro respecto al eje mayor de 4 mm y un área de 5.6 mm². En la Figura 4.4. se muestra la fotografía de estos sistemas electródicos, así como una imagen de la superficie de los electrodos de trabajo obtenidas mediante microscopía electrónica de barrido (SEM).

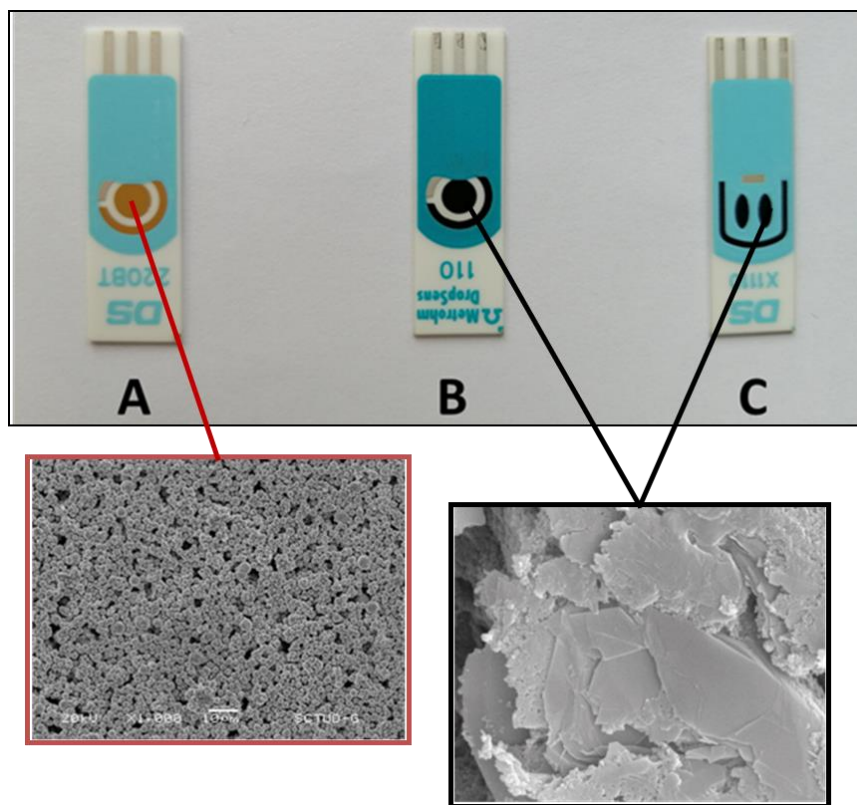


Figura 4.4. SPAuE (A), SPCE (B) y dSPCE (C) e imágenes SEM del electrodo de trabajo del SPCE [Metrohm-DropSens 110] y del SPAuE de baja temperatura de [Metrohm-DropSens 220BT].

4.1.3. Otros dispositivos

La modificación de las micropartículas magnéticas (MBs) y las distintas etapas de bioconjugación se realizaron con ayuda de un concentrador de partículas como el que se muestra en la Figura 4.5. Este dispositivo consiste en un imán de neodimio (AIMAN GZ) ubicado en un soporte fijo y un posicionador de plástico (Dyna MPC-S (nº producto 120.20, Dynal Biotech Dyna MagnsTM-2 (123.21D), Invitrogen Dynal) en el que se colocan los tubos eppendorf que contienen las suspensiones de las MBs. La colocación del posicionador sobre el soporte permite la aproximación y concentración de las micropartículas contenidas en la suspensión de trabajo, lo que posibilita trabajar con ellas,

evitando su pérdida durante las sucesivas etapas de modificación y lavado necesarias en el proceso de formación de los bioconjugados.

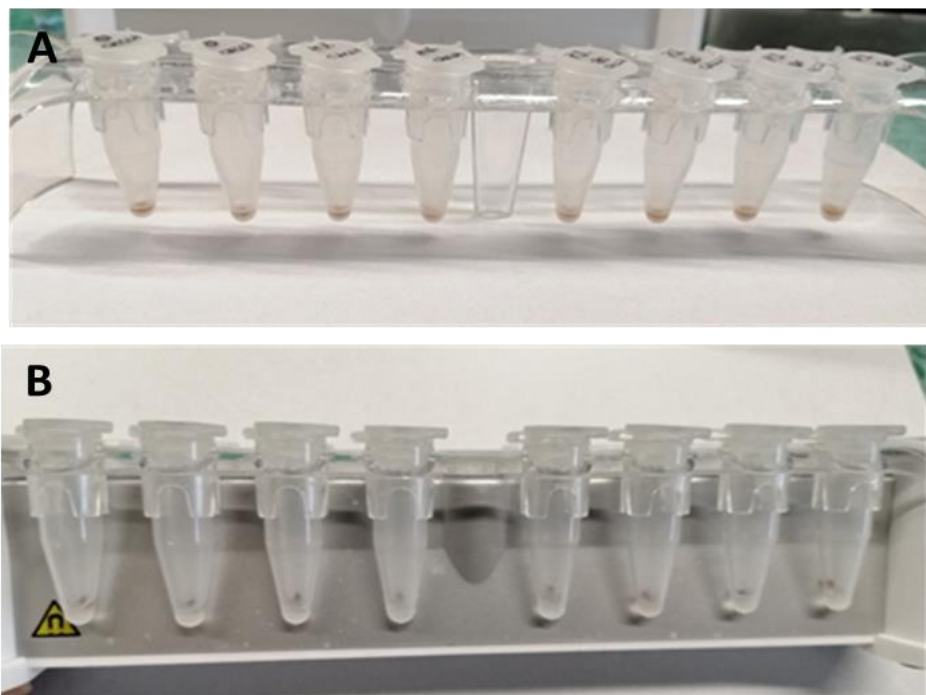


Figura 4.5. Separador magnético Dynal MPC-S. **A)** Eppendorfs con suspensión de MBs sin imantar, **B)** Eppendorfs con suspensión de MBs imantadas.

Los procesos de modificación de las MBs y de incubación de los distintos biorreactivos se efectuaron mediante agitación constante con control de la temperatura para evitar la agregación de dichas partículas, asegurando así una alta eficacia de reacción al tiempo que se garantiza la estabilidad de los biorreactivos. Para estas operaciones se han utilizado los incubadores Optic Yvymen System de Comecta S.A. y Thermo-Shaker Universal de Labortechnik.

Para realizar las medidas amperométricas en disolución con agitación constante, se empleó una célula de medida BAS VC-2, así como imanes entre 2- 5 cm y soportes magnéticos. Estos soportes son bloques de poli(metilmetracrilato) que llevan incorporado un imán cuya ubicación coincide con la del electrodo de trabajo (Figura 4.6) que queda situado en su parte posterior con el fin de asegurar la retención de las MBs durante la medida.



Figura 4.6. Soporte magnético.

La separación y limpieza de anticuerpos modificados (IgG alquilada), se realizó utilizando un filtro Amicon Ultra 0.5 mL Ultracel 10k de Merck Millipore, como el que se muestra en la Figura 4.7.



Figura 4.7. Filtro Amicon Ultra 0.5 mL Ultracel 10k.

Los métodos desarrollados en este trabajo se validaron frente a los procedimientos de inmunoensayo descritos en kits ELISA comerciales para cada uno de los analitos investigados. Para su aplicación, la homogenización de las disoluciones en los pocillos de las placas se realizó empleando un agitador ELISA S-4 Skyline de ELMI, y para la lectura de las mismas se utilizó un lector de absorbancia Sunrise™ de la casa comercial Tecan, provisto del software Magellan.

Por otro lado, las muestras de saliva analizadas se tomaron utilizando un colector de saliva Salivette (Sarstedt) provisto de torunda de algodón y tubo de centrifugación como el que se muestra en la Figura 4.8.



Figura 4.8. Dispositivo colector de saliva Salivette®.

Para la aplicación de los distintos procedimientos experimentales se han empleado otros instrumentos de uso general, como una balanza analítica Sartorius de Thermo Fisher Scientific, un pH-metro de precisión modelo Basic 20+ de Crison calibrado diariamente con distintas disoluciones reguladoras de pH 4.00, 7.00 y 9.21 a $(25 \pm 0.5) ^\circ\text{C}$, un baño de ultrasonidos Elma S 60 de Elmasonic y una estufa P-Selecta. También se ha hecho uso de un homogeneizador de pequeñas muestras de Velp-Scientifica y dos centrifugadoras, Gyrozen 416 de Controltecnica Instruments y P-Selecta Cencom.

4.2. REACTIVOS Y DISOLUCIONES

A continuación, se describen los reactivos y disoluciones utilizadas para la preparación de los inmunosensores y bioplataformas electroquímicas desarrollados en esta Tesis Doctoral. En primer lugar, se consideran los reactivos específicos para cada clase de biosensor o plataforma y después se resumen los de uso general, como es el caso de las disoluciones reguladoras de pH o las especies ensayadas como interferentes potenciales en los estudios de selectividad.

4.2.1. Inmunosensores basados en electrodos modificados por "grafting" electroquímico

La modificación de la superficie de los electrodos de oro se llevó a cabo, con *p*-ABA (Across) en presencia de LiClO₄ (Aldrich, 99.9%) en medio etanol absoluto (Analar Normapur, 99 %). La activación de los grupos carboxilo se realizó con 1-etil-3-[3-dimetilaminopropil] carbodiimida (EDC) (Across) y N-hidroxisulfo-succinimida (NHSS) (Across) 100 mM cada uno de ellos en MES 25 mM de pH 5.0. Para la detección electroquímica se utilizó estreptavidina de *Streptomyces avidinni* [S9210] marcada con fosfatasa alcalina (Ap-Strept) (Thermo Fisher Scientific), reconstituida en disolución reguladora Tris 100 mM de pH 7.2. Finalmente, como sustrato enzimático, se empleó una disolución de 1-naftilfosfato (1-NPP) 5mM (Across) en regulador Trizma de pH 9.6.

Para la construcción del inmunosensor de TGF- β 1 se emplearon los reactivos disponibles en el kit ELISA comercial Human TGF β 1 DuoSet (R&D Systems): un anticuerpo monoclonal de ratón anti-TGF- β 1 humano (anti-TGF) [840116] en PBS 100 mM pH 7.4, un antígeno TGF- β 1 humano (TGF- β 1) [840118] y un anticuerpo monoclonal biotinilado de pollo [840117] anti-TGF- β 1 humano (Biotin-anti-TGF). Estos dos últimos fueron reconstituidos en el reactivo comercial "Reagent Diluent" diluido. Como agente bloqueante se utilizó una disolución comercial de caseína al 1% en medio regulador fosfato (Thermo Scientific).

Para el inmunosensor de PYY, los bioconjugados se prepararon utilizando un anticuerpo monoclonal (IgG) de conejo [G-059-02] anti-péptido YY (3-36) humano (anti-PYY), péptido YY (3-36) humano [059-02] (PYY) y péptido YY marcado con biotina [B-059-02] (Biotin-PYY), todos de la casa comercial Phoenix Pharmaceuticals, Inc. y reconstituídos en disolución reguladora PBS 100 mM de pH 7.4.

Además, se preparó un electrodo modificado con una monocapa de ácido 4-mercaptobenzoico (4-MBA) (Sigma-Aldrich).

4.2.2. Inmunosensores basados en electrodos modificados mediante química "click" y "electroclick"

Se utilizaron nanotubos de carbono de pared múltiple (MWCNTs) (Nanolab) [PD30L5-20] oxidados mediante reflujo en medio ácido HNO₃ (Scharlau, 65 % v/v) y funcionalizados con grupos azida empleando 11-azida-3,6,9-trioxoundecan-1-amino

(Sigma-Aldrich). También se empleó una inmunoglobulina anti-IgG de ratón (IgG) (Sigma) [SAB3700848] alquilada por la región específica Fc utilizando una disolución de NaIO₄ 20 mM de pH 5.5 y etilhidrazida (ChemSpace) en medio regulador PBS 100 mM de pH 7.2. El producto final se reconstituyó en PB 10 mM de pH 7.0.

Para la preparación del inmunosensor de CXCL7, el catalizador Cu(I) de la reacción de cicloadición alquino-azida se obtuvo por reducción de CuSO₄ 5 mM (Sigma-Aldrich) con ácido ascórbico 5 mM (Fluka) y en medio PB 10 mM de pH 7.0.

Los inmunorreactivos utilizados fueron los disponibles en el kit ELISA comercial Human CXCL7/NAP-2 DuoSet (R&D System): un anticuerpo de captura monoclonal de ratón anti-CXCL7 (anti-CXCL7) [840319] en disolución reguladora PBS de pH 7.2-7.4, CXCL7 [840321] como antígeno y un anticuerpo monoclonal biotinilado de cabra (Biotin-anti-CXCL7) [840320] como anticuerpo de detección, ambos preparados en "Reagent Diluent" diluido. Como agente de bloqueo se empleó una disolución comercial de caseína (Thermo Scientific) al 0.6 % (v/v) en PBS 100 mM de pH 7.0.

En el caso de la IL-1 β , se puso a punto un método de síntesis del catalizador Cu(I) por reducción electroquímica. Para la preparación de este inmunosensor se empleó un anticuerpo de captura monoclonal [840168] de ratón anti-IL-1 β (anti-IL1 β) y el antígeno IL-1 β humano [840170], empleándose también un anticuerpo de detección de cabra biotinilado [840169] (Biotin-anti-IL1 β). Estos inmunorreactivos fueron los disponibles en el kit ELISA comercial Human IL-1 beta/IL-1F2 DuoSet de R&D Systems y se prepararon en PBS de pH 7.2-7.4.

Los demás reactivos utilizados fueron los descritos en el Apartado 4.2.1.

4.2.3. Bioplateformas electroquímicas basadas en micropartículas magnéticas funcionalizadas

En primer lugar, se describen los reactivos utilizados para la preparación de la bioplateforma dual para la determinación de los autoanticuerpos factor reumatoide (RF) y anti-péptido citrulinado (CCPA). En el caso del RF se emplearon partículas magnéticas carboxiladas Dynabeads® M-270 de 2.8 μ m (cMBs) (Invitrogen Dynal Biotech ASA; Thermo Fisher Scientific), inmovilizando un fragmento de la fracción constante de inmunoglobulina G humana (Fc IgG) [ab90285], un autoanticuerpo de tipo M dirigido contra la fracción constante de la inmunoglobulina G (RF) [ab178653] y finalmente, un

anticuerpo IgG dirigido contra la cadena pesada de una IgM humana marcada con peroxidasa de rábano [ab178653] (HRP-IgM). Todos estos biorreactivos fueron los disponibles en el kit ELISA comercial [ab178653] (Abcam).

Para la determinación de CCPA se utilizaron partículas magnéticas funcionalizadas con neutravidina (Neutr-MBs, 1 μm -Ø SpeedBeads™) (GE Healthcare) incubando el péptido cíclico citrunilado humano biotinilado (CCP-Biotin) [orb55851] (Biorbyt), así como el anticuerpo específico frente a IgG humana marcada con la enzima peroxidasa (HRP-IgG). Estos dos últimos reactivos fueron los disponibles en el kit ELISA comercial [DE7760] (Demeditec).

La inmunoplateforma dual para la determinación de la enzima metaloproteinasa de matriz 3 (MMP3) y la quimioquina CXCL7 se preparó usando las mismas micropartículas magnéticas carboxiladas Dynabeads® M-270 descritas anteriormente.

Los inmunorreactivos para MMP3 fueron: un anticuerpo de captura de cabra (anti-MMP3) [DNA1018101] en disolución reguladora MES 25 mM de pH 5.0, el antígeno MMP3 [1365804], y un anticuerpo de cabra biotinilado (Biotin-anti-MMP3) como anticuerpo de detección, ambos en PBS de pH 7.2 Estos reactivos fueron los disponibles en el kit comercial ELISA Human total MMP-3 duoset (DY513) (R&D Systems).

Para la determinación de CXCL7 se emplearon los reactivos disponibles en el kit ELISA comercial Human CXCL7/NAP-2 DuoSet (R&D Systems) descritos anteriormente. La proteína CXCL7 humana (CXCL7) [840321] y el anticuerpo monoclonal biotinilado de cabra (Biotin-anti-CXCL7) [840320] se prepararon en PBS de pH 7.4.

La bioplateforma para la determinación de anti-MBP se preparó utilizando las micropartículas magnéticas carboxiladas de Dynabeads® M-270 en las que se incubaba la proteína MBP humana [EPA539Hu61] en medio MES 25 mM de pH 5.0, y un anticuerpo monoclonal humano generado frente a MBP (anti-MBP) [MAA539Hu28] preparado en PBS 100 mM de pH 7.4, ambos de Cloud-Clone Corporation. Como agente de bloqueo se usó etanolamina (Sigma) en PB 100 mM de pH 8.0, y para la detección se empleó un anticuerpo secundario de cabra conjugado con peroxidasa (HRP-IgG) generado frente a la fracción constante de inmunoglobulina IgG de ratón [ab97225] (Abcam), preparado en PB 100 mM de pH 7.4.

La bioplateforma para CCL5 se preparó empleando las Neutr-MBs descritas anteriormente mediante inmovilización de un anticuerpo de captura de cabra biotinilado generado frente a CCL5 humana (anti-CCL5-Biotin) [BAF478] (R&D Systems) en medio PBS 100 mM pH 7.4. La proteína recombinante diana CCL5 humana (CCL5) y el anticuerpo de detección de ratón generado frente a CCL5 humana (anti-CCL5) del kit ELISA DuoSet Human CCL5/RANTES [DY278] (R&D Systems) se acondicionaron en el mismo medio. También se utilizó el conjugado HRP-IgG descrito anteriormente.

A lo largo del trabajo experimental se utilizaron las siguientes disoluciones reguladoras de pH:

- Disoluciones reguladoras fosfato (PB) de diferentes concentraciones y pHs en el intervalo de 7.0 a 8.0 preparadas a partir de Na_2HPO_4 (Panreac) y NaH_2PO_4 (Labkem).
- PB 50 mM de pH 6.0 preparada a partir de K_2HPO_4 (Merck) y de KH_2PO_4 (Labkem).
- PBS pH 7.4: disolución comercial PBS [896036] del DuoSet® Ancillary Reagent Kit 1 (R&D Systems) NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM.
- PBST de pH 7.4: disolución comercial PBS [896036] DuoSet® Ancillary Reagent Kit 1 (R&D Systems) + 0.05% de Tween 20© (Aldrich).
- PBS 100 mM + 150 mM NaCl de pH 7.2: preparada a partir de Na_2HPO_4 (Panreac), NaH_2PO_4 (99%) y NaCl 0.15 M (Labkem).
- MES 25 mM de pH 5.0 y pH 7.0 preparadas a partir de ácido 2-(N-morfolino) etanosulfónico (Gerbu).
- Disolución comercial concentrada de lavado ("Wash Buffer") de pH 7.2-7.4 del kit ELISA de R&D Systems (Ref. WA126) y compuesta por 0.05% Tween®20 en PBS de pH 7.2-7.4.
- Disolución de lavado preparada por dilución de la "Washing Solution" del kit ELISA RF Abcam (ab178653).
- Disolución comercial diluida de lavado "Wash Buffer" diluido de pH 7.2-7.4: preparada a partir de la anterior (Ref. WA126) de R&D Systems en agua Milli-Q, en una relación 1:24.
- Reactivo comercial concentrado "Reagent Diluent" concentrado de pH 7.2-7.4: proporcionada por el kit de R&D Systems (Ref. DY997) y compuesta por el 1% de BSA en PBS de pH 7.2-7.4.

- Reactivo comercial diluido "Reagent Diluent" diluido" de pH 7.2-7.4 preparada adicionando 1.4 mL de Reagent Diluent concentrado (Ref. DY997) de R&D Systems a 98.6 mL de "Wash Buffer" diluido de pH 7.2-7.4.
- Tris 100 mM de pH 7.2 preparada disolviendo Tris-HCl (hidrocloruro de tris(hidroximetil)-aminometano) (Scharlau, 99 %) en agua y ajustando el pH.
- Trizma 50 mM + MgCl₂ 10 mM de pH 9.6 preparada por pesada de las cantidades necesarias de Tris-HCl (Scharlau, 99%) y MgCl₂·6H₂O (Panreac, 99%) y ajuste de pH.

Para los estudios de selectividad se seleccionaron como potenciales interferentes algunas de las siguientes especies:

IgG humana (hIgG) (Sigma), albúmina de suero humano (HSA) (Sigma), hemoglobina (HB) (Sigma), interferon γ (IFN) (BioLegend), ligando de receptor activador para el factor nuclear- κ B (RANKL) (R&D Systems), interleucina 1 β (IL- β) (R&D Systems), interleucina 6 (IL-6) (Abcam), interleucina 8 (IL-8) (Abcam), factor de necrosis tumoral α (TNF- α) (R&D Systems), quimioquina (C-X-C) ligando 4 (CXCL4) (R&D Systems), quimioquina (C-X-C) ligando 7 (CXCL7) (R&D Systems), factor de crecimiento transformante TGF- β 1 (R&D Systems), hormona de crecimiento humana (hGH) (Sigma Aldrich), adiponectina (APN) (Abnova), grelina (GHRL) (Abnova), Leptina (Lep) (BioVendor), E-cadherina (E-cad) (R&D Systems), receptor tirosina quinasa (ERBB2) (Sino Biological Inc.), proteína supresora de tumores humana P53 (P53) (Sigma-Aldrich), cadherina 17 (Cad-17) (R&D Systems), anticuerpo cíclico citrulinado humano (CCPA) (Demeditec), receptor del factor de crecimiento de fibroblastos 4 (FGFR4) (R&D Systems), ácido ascórbico (AA) (Sigma), endoglina (CD105) (R&D Systems), metaloproteinasa de matriz 3 (MMP3) de R&D Systems, factor reumatoide (RF) de Abcam, y ácido úrico (UA) (Sigma).

Los estudios de caracterización mediante EIS y/o CV se realizaron empleando disolución de K₃Fe(CN)₆ (Molekula), K₄Fe(CN)₆·3H₂O (Sigma-Aldrich) 5 mM y KCl (Scharlau) 100 mM.

La detección amperométrica en las configuraciones marcadas con peroxidasa se realizó por amperometría en disoluciones de PB 50 mM de pH 6 que contenían hidroquinona (HQ) 1 mM, por adición de peróxido de hidrógeno (H₂O₂) 50 mM.

La detección colorimétrica en los kits ELISA se llevó a cabo empleando una mezcla de H_2O_2 y 3,3', 5,5' tetrametilbenzidina (TMB) ("Substrate Solution") en relación 1:1 y una disolución de H_2SO_4 2 M ("Stop Solution").

Todos los productos utilizados fueron de calidad analítica. El agua utilizada ($18.2 \text{ m}\Omega\cdot\text{cm}$ a 25°C) se obtuvo utilizando un Sistema de purificación Millipore Milli-Q.

4.3. MUESTRAS

El inmunosensor desarrollado para la determinación de $\text{TGF-}\beta 1$ se aplicó a muestras de plasma (Ref. 8BFP, lote 11/03-8BFP, HyTest Ltd.) enriquecidas con 25 ng mL^{-1} de la proteína. En el caso del inmunosensor de PYY se analizaron muestras de suero liofilizado (Sigma) y saliva de voluntarios contaminadas con 100 y 35 pg mL^{-1} de la hormona, respectivamente.

El inmunosensor de CXCL7 se utilizó para la cuantificación de esta proteína en muestras clínicas reales, suero de pacientes con artritis reumatoide diagnosticado, proporcionadas por el Hospital Clínico San Carlos de Madrid. El inmunosensor para $\text{IL-}1\beta$ se aplicó al análisis de muestras de saliva de un voluntario sano no fumador, sin tratamiento previo ni dilución.

Las muestras de suero proporcionadas por el citado hospital se obtuvieron con el correspondiente consentimiento informado aprobado por los pacientes y el Comité de Ética de las Instituciones que, en el caso de las muestras de epidermólisis bullosa, poseen Código HULP: PI-1602, adherido a las Directrices de Helsinki y a revisiones adicionales, Fortaleza 2013 (<http://www.wma.net>).

En el caso de la plataforma dual para la determinación simultánea de FR y CCPA, se analizaron muestras de suero humano de Abcam (ab178653) y Demeditec (DE7760), respectivamente. La bioplataforma para la determinación simultánea de MMP3 y CXCL7, se aplicó a muestras de suero de individuos sanos y pacientes con artritis reumatoide de Central BioHub® Specimens (Central BioHub GmbH, Hennigsdorf, Alemania). Las bioplataformas para anti-MBP y CCL5 se aplicaron al análisis de muestras de suero de individuos sanos y de pacientes con esclerosis múltiple, de Central BioHub® Specimens (Central BioHub GmbH, Hennigsdorf, Alemania).

Asimismo, las muestras adquiridas a través de Central BioHub® (Hennigsdorf, Alemania) se obtuvieron con el correspondiente consentimiento informado aprobado por los pacientes y cedidas de forma voluntaria, así como fueron aprobados por el Comité de Ética de acuerdo con los protocolos exigidos en la legislación alemana.

4.4. PROCEDIMIENTOS EXPERIMENTALES

4.4.1. Preparación de los inmunosensores basados en electrodos modificados por "grafting" electroquímico

La estrategia de inmovilización sobre electrodos funcionalizados mediante "grafting" electroquímico se aplicó a la construcción de los inmunosensores para la determinación de TGF- β 1 y de PYY empleando electrodos serigrafiados de oro (SPAuEs) siguiendo los procedimientos que se describen a continuación, y que aparecen representados esquemáticamente en las Figuras 4.9 y 4.10.

En primer lugar, la superficie de los electrodos se acondicionó por aplicación de 10 barridos de potencial cíclicos consecutivos entre 0.00 y 1.25 V a 100 mV s⁻¹ en H₂SO₄ 100 mM. Una vez lavado con agua desionizada, cada electrodo se sumergió en una disolución 1.5 mM de ácido *p*-aminobenzoico (p-ABA) y 100 mM LiClO₄ en etanol absoluto y se aplicaron tres barridos de potencial sucesivos mediante CV entre 0.0 y 0.6 V, a 30 mV s⁻¹. Tras el lavado, los grupos carboxilo presentes en la superficie del electrodo se activaron por incubación en la mezcla EDC/NHSS 100 mM en MES de pH 5.0 durante 30 min a temperatura ambiente en humedad y oscuridad.

Para la preparación del inmunosensor de TGF- β 1 (Figura 4.9) se depositaron 5 μ L de disolución de anti-TGF de 10 μ g mL⁻¹ en PBS de pH 7.4 sobre el electrodo, incubando 25 °C durante 30 min. Tras una etapa de lavado, la superficie se bloqueó con 5 μ L de caseína comercial al 1% (v/v) incubando nuevamente 30 min en las mismas condiciones. A continuación, se estableció un diseño de inmunoensayo tipo "sándwich" depositando 5 μ L de disolución de TGF- β 1 patrón o la muestra, dejando incubar durante 60 min en ambiente húmedo y oscuridad a temperatura ambiente. Tras un lavado, se añadieron 5 μ L de Biotin-anti-TGF de 2 μ g mL⁻¹ y, una vez incubado en las condiciones anteriores, el bioelectrodo Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAuE se lavó con agua desionizada y con Tris de pH 7.2, para añadir finalmente 5 μ L de AP-Strept de 5 μ g mL⁻¹

¹ en el mismo regulador incubando durante 30 min. Para la determinación de TGF- β 1 se depositaron 45 μ L de Trizma de pH 9.6 sobre la que se añadieron 5 μ L de 1-NPP 5 mM dejando reaccionar durante 5 min, midiendo a continuación en DPV entre -0.2 y 0.7 V vs Ag, a 50 mV/s.

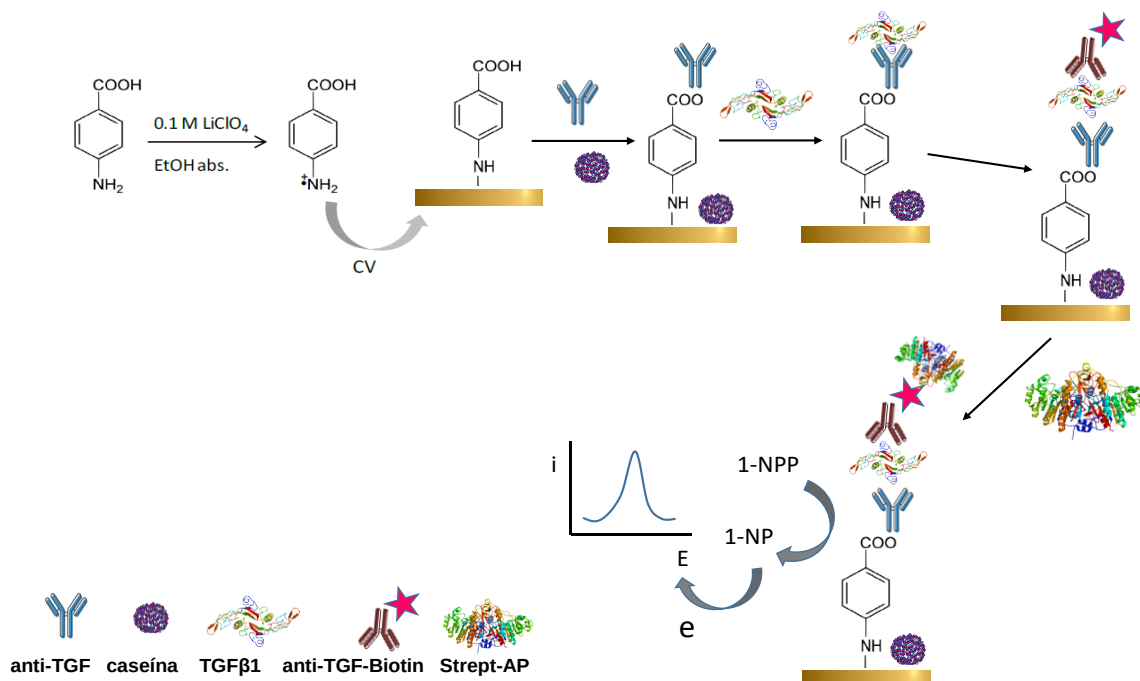


Figura 4.9. Esquema de las etapas de preparación del inmunosensor para la determinación de TGF- β 1 mediante "grafting" electroquímico de *p*-ABA sobre SPAuEs.

Para la preparación del inmunosensor de PYY, una vez funcionalizados los SPAuEs y activados los grupos carboxilo, se inmovilizó el anticuerpo de captura mediante incubación de 10 μ L de disolución de anti-PYY de 20 μ g mL⁻¹ en PBS de pH 7.4 durante 60 min a 25 °C. Después, una vez lavado con el regulador anterior se aplicó una etapa de bloqueo de la superficie electródica con 10 μ L de caseína comercial al 0.2% en PBS de pH 7.4 incubando 30 min a 25 °C. A continuación, se estableció un esquema de inmunoensayo competitivo añadiendo 10 μ L de una mezcla de PYY (o la muestra) y Biotin-PYY de 100 ng mL⁻¹ en el regulador anterior sobre el conjugado anti-PYY/SPAuE, dejando incubarse durante 30 min a 25 °C. Después de lavar con la misma disolución reguladora, se adicionaron 10 μ L de AP-Strept 5 μ g mL⁻¹ y se incubó en las mismas condiciones. Finalmente, la determinación del péptido YY se realizó, como en el caso anterior, depositando 45 μ L de disolución reguladora Trizma de pH 9.6 sobre la que se añadieron 5 μ L de 1-NPP 5 mM dejando reaccionar durante 5 min, y midiendo a continuación en DPV entre -0.2 y 0.7 V vs Ag, a 50 mV/s.

Como se observa en la Figura 4.10, la configuración desarrollada se comparó, utilizando el mismo protocolo, con la que resulta de sustituir la modificación del SPAuE mediante "grafting" electroquímico por la preparación de una monocapa de ácido 4-mercaptobenzoico mediante incubación en medio etanol durante 6h.

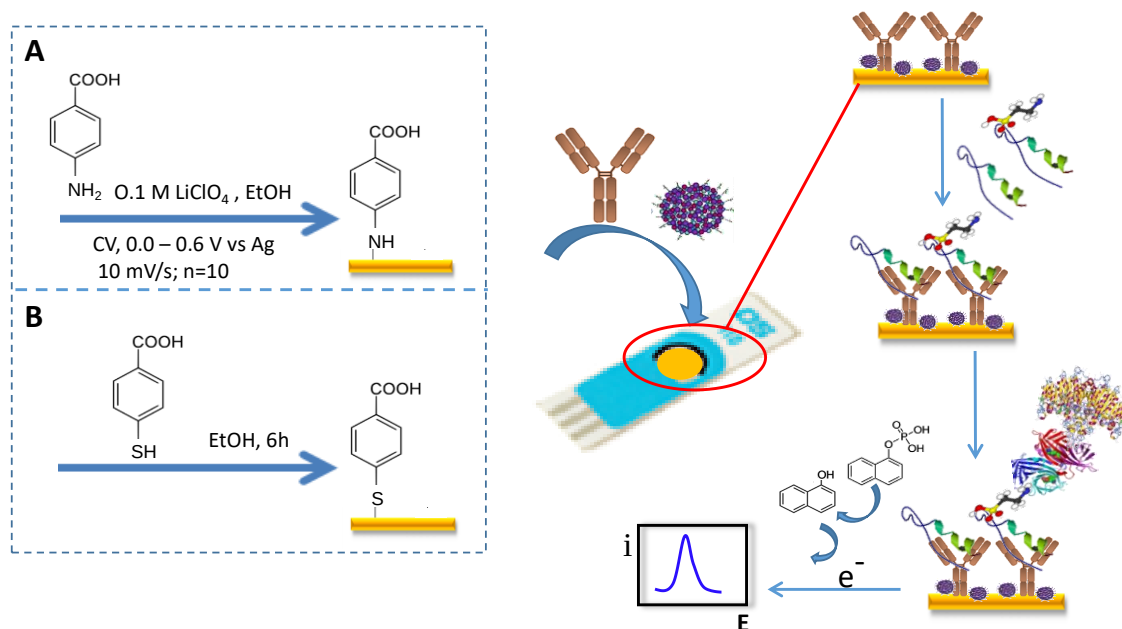


Figura 4.10. Esquema de las etapas de preparación del inmunosensor para la determinación de PYY usando SPAuEs modificados mediante "grafting" electroquímico de *p*-ABA (**A**) o por preparación de una monocapa de 4-MBA (**B**).

4.4.2. Preparación de los inmunosensores basados en electrodos modificados mediante química click y electroclick

La preparación de los inmunosensores para la determinación de CXCL7 e IL-1 β hizo uso de la estrategia "química click" para funcionalizar los electrodos serigrafados de carbono (SPCE) mediante una reacción de cicloadición alquino-azida. Siguiendo un procedimiento puesto a punto anteriormente [Sánchez-Tirado, 2016], los SPCEs modificados con MWCNTs funcionalizados con grupos azida, se hicieron reaccionar con IgG alquilada en presencia del catalizador Cu(I) obtenido por reducción de Cu(II) con ácido ascórbico. Para el inmunosensor de IL-1 β , esta etapa se realizó por reducción electroquímica.

Brevemente, los nanotubos de carbono se oxidaron por tratamiento de 25 mg del producto con HNO₃ al 65% (w/w) calentado a reflujo. Después de centrifugados a temperatura ambiente, los MWCNTs carboxilados (cMWCNTs) se lavaron con agua

desionizada hasta alcanzar pH 7 en las aguas de lavado y se secaron en estufa a 60 °C, conservándolos en la oscuridad a temperatura ambiente. La funcionalización con grupos azida se realizó mezclando en un tubo eppendorf 25 mg de cMWCNTs con 26.8 mg de EDC y 29.8 mg de NHSS en 3.5 mL de MES 25 mM de pH 5.0, manteniendo agitación en la oscuridad a temperatura ambiente durante 2 h. Después se añadieron 4.5 µL de 11-azida-3,6,9-trioxoundecan-1-amino, dejando reaccionar en las mismas condiciones anteriores durante 48 h más. El producto obtenido se centrifugó a 4000 rpm durante 10 min, se aplicaron dos etapas de lavado con PBS 10 mM de pH 7.0 y con agua desionizada, dejando secar el sólido a 37°C.

La alquilación de IgG se llevó a cabo por incubación de 30 µL de una disolución de 2 mg mL⁻¹ de anticuerpo de ratón con 1.88 µL de NaIO₄ 20 mM de pH 5.5 en baño de hielo, con agitación constante y en oscuridad durante 30 min. Una vez centrifugado con filtro Amicon Ultra 10k a 14000 rpm, a 4 °C durante 15 min, se eliminó el exceso de reactivos mediante tres lavados con PB 100 mM y NaCl 150 mM a pH 7.2 en las condiciones anteriores, y el anticuerpo se extrajo del filtro por inversión del mismo y centrifugación sobre un vial limpio y seco a 1000 rpm, a 4 °C durante 2 min. Al producto recuperado se le añadieron 19 µL de una disolución de etinilhidrazina 105 mM en PB 100 mM y NaCl 150 mM de pH 7.2 dejándolo incubar a 950 rpm durante 2 h a 25 °C. Posteriormente, se filtró la mezcla a 14000 rpm a 4 °C durante 15 min y, se aplicaron 5 etapas de lavado con PB 100 mM y NaCl 150 mM de pH 7.2, y con PB 10 mM de pH 7.0, extrayendo de nuevo el anticuerpo funcionalizado en 30 µL de PB 10 mM de pH 7.0.

4.4.2.1. Inmunosensor para la determinación de CXCL7

El esquema de las etapas de preparación del inmunosensor para la determinación de CXCL7 se muestra en la Figura 4.11. La reacción entre los aMWCNTs y el anticuerpo alquilado se realizó mezclando en un tubo de vidrio 34 µL de una disolución 1/100 de IgG-alquino en PB 10 mM de pH 7.0, 22 µL de ácido ascórbico 5 mM, 23 µL de CuSO₄ 5 mM y 79 µL de la suspensión de aMWCNTs. La mezcla se dejó reaccionar con agitación constante en la oscuridad, a 4 °C durante toda la noche. A continuación, una mezcla formada por 5 µL de la suspensión anterior y 5 µL de caseína al 0.6 % en PBS 100 mM de pH 7.0, se depositaron sobre el SPCE dejándolo incubar a 25 °C, en oscuridad y ambiente húmedo durante 30 min. Tras lavar el electrodo por inmersión con agitación

suave en agua desionizada, se depositaron 5 μL de anti-CXCL7 de 2 $\mu\text{g mL}^{-1}$ incubando durante 60 min en las mismas condiciones. Tras otra etapa de lavado con agua desionizada, se incubaron 5 μL de CXCL7 estándar (o la muestra) en las mismas condiciones, durante 60 min. El inmunosensor CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE se lavó con agua desionizada y se le añadieron 5 μL de Biotin-anti-CXCL7 de 100 ng mL^{-1} incubando durante 30 min en las mismas condiciones. Finalmente se lavó con agua desionizada y con regulador Tris de pH 7.2 para depositar 5 μL de Strept-AP 5 $\mu\text{g mL}^{-1}$ sobre el conjugado anterior, incubando a 25 $^{\circ}\text{C}$, en las mismas condiciones 30 min más. La determinación de CXCL7 se realizó tras depositar sobre el inmunosensor 45 μL de una disolución de 1-NPP 5 mM en Trizma 50 mM y MgCl_2 10 mM a pH 9.6 que se dejó reaccionar durante 5 min, obteniendo la respuesta electroquímica mediante DPV entre -0.2 y +0.7 V vs. Ag, a 50 mV s^{-1} .

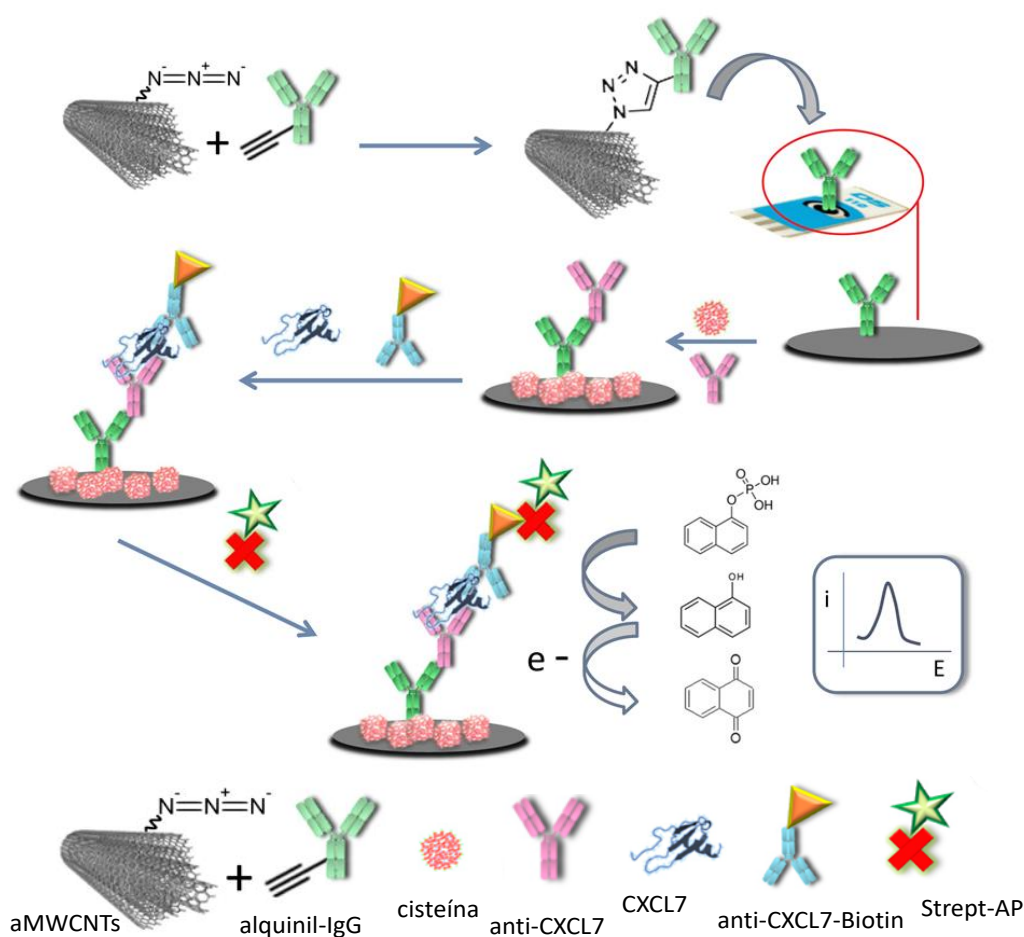


Figura 4.11. Esquema de las etapas de preparación del inmunosensor para la determinación de CXCL7 basado en la modificación de los SPCEs con IgG-alquinoazida-MWCNTs obtenidos por síntesis asistida por "química click".

4.4.2.2. Inmunosensor para la determinación de IL-1 β

El esquema de las etapas de preparación del inmunosensor se ha representado en la Figura 4.12. Esta configuración se construyó sobre un SPCE previamente modificado con 5 μ L de aMWCNTs. De forma paralela se preparó la mezcla de 18.8 μ L de IgG-alquino 1/100 en PB de pH 7.0 con 13.5 μ L de CuSO₄ 5.0 μ M y 13.5 μ L de la disolución reguladora MES 10 mM pH 7.0. Después, 40 μ L de esta disolución se depositaron sobre la superficie del aMWCNTs/SPCE y el catalizador Cu(I) se sintetizó por reducción a -0.4 V vs Ag durante 5 min. A continuación, se lavó el electrodo por inmersión en agua desionizada, conservándolo en humedad.

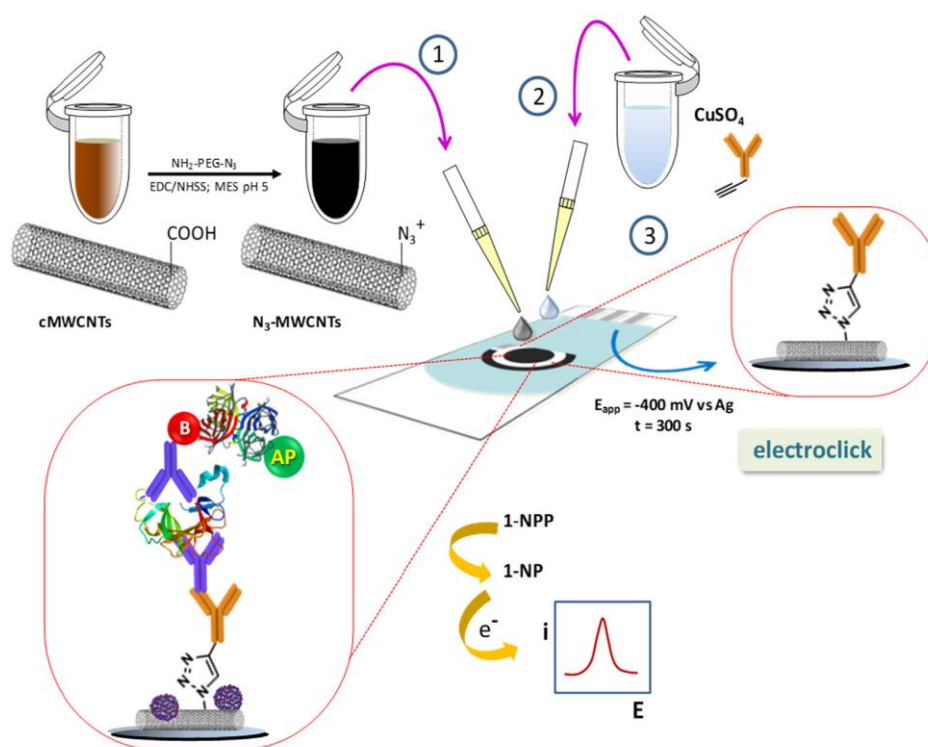


Figura 4.12. Esquema de las etapas de preparación del inmunosensor AP-Strept-Biotin-anti-IL β 1-IL β 1-anti-IL β 1-IgG-MWCNTs/SPCE por química "electro-click".

Para la preparación del inmunosensor, en primer lugar, se bloqueó la superficie del electrodo funcionalizado con 10 μ L de caseína al 1% (v/v) en regulador fosfato incubando durante 30 min. Todas las etapas de incubación se realizaron a temperatura ambiente en ambiente húmedo y en la oscuridad. Tras un lavado con agua desionizada, se inmovilizó el anticuerpo de captura añadiendo 5 μ L de anti-IL1 β de 10 $\mu\text{g mL}^{-1}$ e incubando durante 60 min. Posteriormente se aplicó una etapa de lavado y se añadieron

5 μL de disolución patrón de IL-1 β o la muestra, incubando durante 60 min, seguido de la adición del conjugado Biotin-anti-IL1 β en concentración 0.5 $\mu\text{g mL}^{-1}$ y la incubación en las mismas condiciones. Finalmente, tras lavar con agua desionizada y con Tris 0.1 M pH 7.2, se añadieron 5 μL de Strept-AP de 5 $\mu\text{g mL}^{-1}$ que se incubaron igualmente. La determinación de IL-1 β se realizó del modo descrito en el Apartado anterior.

4.4.3. Preparación de las bioplateformas electroquímicas basadas en micropartículas magnéticas funcionalizadas

4.4.3.1. Bioplateforma para la determinación simultánea de RF y CCPA

La Figura 4.13 muestra un esquema de las etapas de preparación de esta plataforma. Para la determinación del factor reumatoide (RF) se utilizaron 3 μL de una suspensión de cMBs que se lavaron con 50 μL de regulador MES 25 mM de pH 5.0 con agitación constante (950 rpm, 10 min, 25 $^{\circ}\text{C}$) en un agitador-incubador. Tras separar las cMBs del sobrenadante, se añadieron 25 μL de EDC y NHSS 50 mg mL^{-1} en cada una de ellas, dejando incubar durante 60 min en las mismas condiciones. Una vez lavadas con regulador MES 25 mM de pH 5.0, se adicionaron 25 μL de disolución de Fc(IgG) de 25 $\mu\text{g mL}^{-1}$ en MES 25 mM de pH 5, dejándolo incubar 30 min en las condiciones anteriores. Transcurrido este tiempo, se separaron las cMBs y se lavaron como en las etapas anteriores, y se añadieron 25 μL de RF o la muestra incubando durante 60 min a 37 $^{\circ}\text{C}$ con agitación. A continuación, se añadieron 25 μL de HPR-IgM dejando incubar 60 min y finalmente, el bioconjugado formado se lavó dos veces más, resuspendiendo las micropartículas modificadas en 25 μL de PB 50 mM de pH 6.0, dejándolas estar hasta la medida.

Para la determinación de CCPA se transfirieron 3 μL de una suspensión de partículas de neutravidina (Neutr-MBs) a un eppendorf, aplicando el mismo proceso de lavado que el descrito para el inmunosensor de RF. Una vez retirado el sobrenadante se adicionaron 25 μL de CCP-Biotin de 25 $\mu\text{g mL}^{-1}$ en PBS de pH 7.2 y se dejó incubar durante 30 min. Tras dos etapas de lavado en PBS de pH 7.2 se llevó a cabo la incubación de CCPA (25 μL) o de la muestra durante 60 min. Finalizada esta etapa, se realizaron dos lavados con PBS de pH 7.2, y el conjugado se incubó con 25 μL de HRP-IgG durante 60 min. Finalmente se aplicaron dos etapas de lavado con PBS de pH 7.2 y los conjugados se resuspendieron en 50 μL de PB 50 mM de pH 6.0, conservándose hasta la medida.

La determinación simultánea de RF y CCPA se llevó a cabo atrapando cada conjugado magnético sobre una superficie activa del SPCE colocado en el soporte descrito en el Apartado 4.1.3 (Figura 4.5), por inmersión del mismo en 10 mL de PB 50 mM de pH 6.0 que además contenía 100 μ L de HQ 1 mM. Una vez aplicado un potencial constante de -0.2 V vs. Ag, se dejó estabilizar la corriente de fondo y se añadieron 50 μ L de H_2O_2 50 mM registrando el cambio de corriente debido a la reducción de la quinona formada en la reacción enzimática.

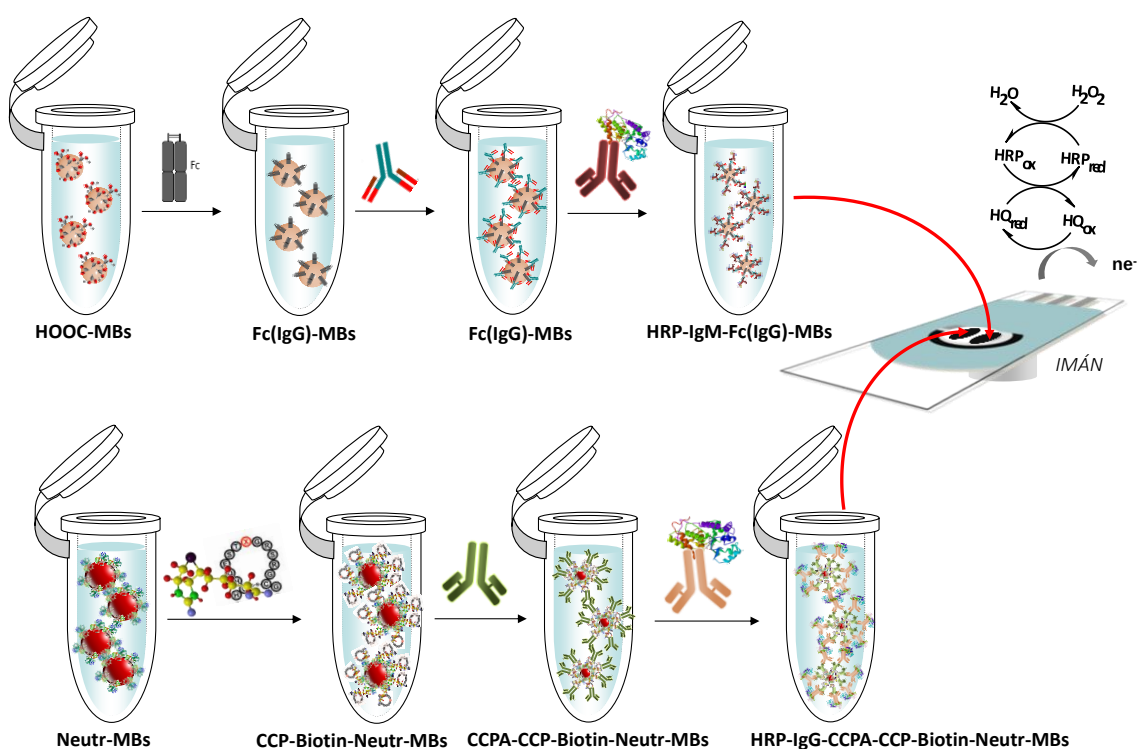


Figura 4.13. Esquema de las etapas de preparación de la bioplataforma basada en micropartículas magnéticas (MBs) funcionalizadas para la determinación simultánea de RF y CCPA.

4.4.3.2. Bioplataforma para la determinación simultánea de CXCL7 y MMP3

Para la preparación de los bioconjugados de CXCL7 y MMP3 (Figura 4.14) se utilizaron cMBs, que se prepararon y activaron con la mezcla EDC/NHSS como se explicó en el Apartado 4.4.3. Después de lavar con regulador MES 25 mM de pH 5.0, se inmovilizaron los anticuerpos de captura respectivos a partir de disoluciones de anti-CXCL7 de 10 μ g mL^{-1} o de anti-MMP3 de 15 μ g mL^{-1} , incubando 45 min. A continuación, tras dos etapas de lavado adicionales, se aplicó una etapa de bloqueo por adición de 25 μ L de etanolamina 1M en PB 0.1 M de pH 8.0. dejando incubar 30 min.

Una vez que los conjugados se lavaron dos veces con 50 μL del mismo regulador y PBS de pH 7.4, se añadieron 25 μL de la disolución correspondiente de CXCL7 o MMP3 o la muestra, incubando durante 60 min. Seguidamente se realizaron dos lavados adicionales con PBS de pH 7.4 y se añadieron 25 μL de Biotin-anti-CXCL7 o Biotin-anti-MMP3 0.25 $\mu\text{g mL}^{-1}$ incubando en las mismas condiciones, y esta secuencia final se repitió añadiendo 25 μL de HRP-Strept en dilución 1/2000 (CXCL7) o 1/500 (MMP3) e incubando durante 20 min. Finalmente, los conjugados HRP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-MBs o HRP-Strept-Biotin-anti-MMP3-MMP3-anti-MMP3-MBs se lavaron dos veces con 50 μL del mismo regulador y se resuspendieron en 25 μL de PB 0.05 M de pH 6.0, dejando estar hasta la realización de las medidas. La determinación simultánea de CXCL7 y MMP3 se llevó a cabo del modo descrito anteriormente, atrapando cada conjugado magnético sobre una superficie activa del SPCE colocado en el soporte descrito en el Apartado 4.1.3 (Figura 4.5), por inmersión del mismo en 10 mL de PB 50 mM de pH 6.0 que además contenía 100 μL de HQ 1 mM. Una vez aplicado un potencial constante de -0.2 V vs. Ag , se dejó estabilizar la corriente de fondo y se añadieron 50 μL de H_2O_2 50 mM registrando el cambio de corriente debido a la reducción de la quinona formada en la reacción enzimática.

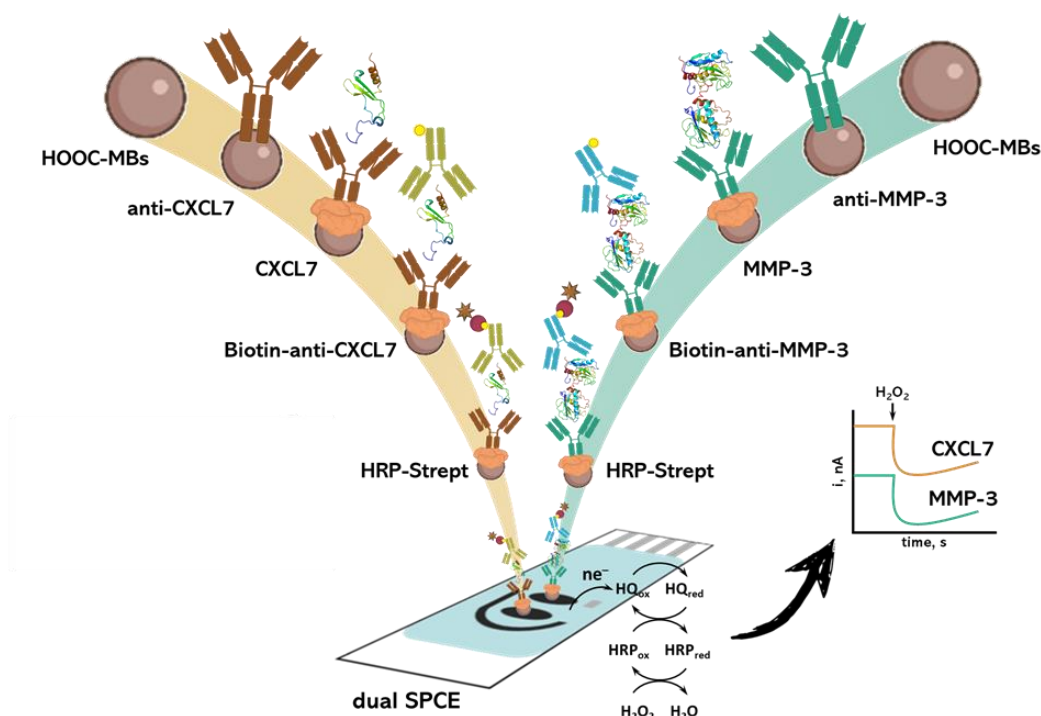


Figura 4.14. Esquema de las etapas de preparación de la bioplataforma basada en micropartículas magnéticas (MBs) funcionalizadas para la determinación simultánea de CXCL7 y MMP3.

4.4.3.3. Bioplataforma para la determinación de anti-MBP

Una vez activados los grupos carboxilo de las cMBs utilizadas para la determinación de este autoanticuerpo, y realizados los lavados correspondientes, se incubó el antígeno específico, MBP, por adición de 25 μL de una disolución de 20 $\mu\text{g mL}^{-1}$ en MES 25 mM de pH 5.0. Transcurridos 45 min, se bloquearon las posiciones reactivas de la superficie de las micropartículas con 50 μL de disolución de etanolamina 1 M en PB 100 mM de pH 8.0 durante 60 min. Después se lavó nuevamente y se añadieron 25 μL de anti-MBP estándar o las muestras en PB 100 mM de pH 7.4, incubando 60 min. Finalmente se adicionaron 25 μL de HRP-IgG 0.5 $\mu\text{g mL}^{-1}$ preparado en disolución comercial de caseína, que se incubó durante 15 min, lavando finalmente con la disolución reguladora de PB 100 mM de pH 7.4. Para realizar las medidas amperométricas, los SPCEs con los conjugados HRP-IgG-anti-MBP-MBP-MBs (50 μL) atrapados en su superficie se dispusieron en el soporte para el electrodo individual representado en la Figura 4.5, y se sumergieron en 10 mL de PB 50 mM de pH 6.0 y 0.1 M de HQ. Una vez aplicando el potencial de -0.2 V vs Ag, se obtuvieron los amperogramas del modo indicado anteriormente.

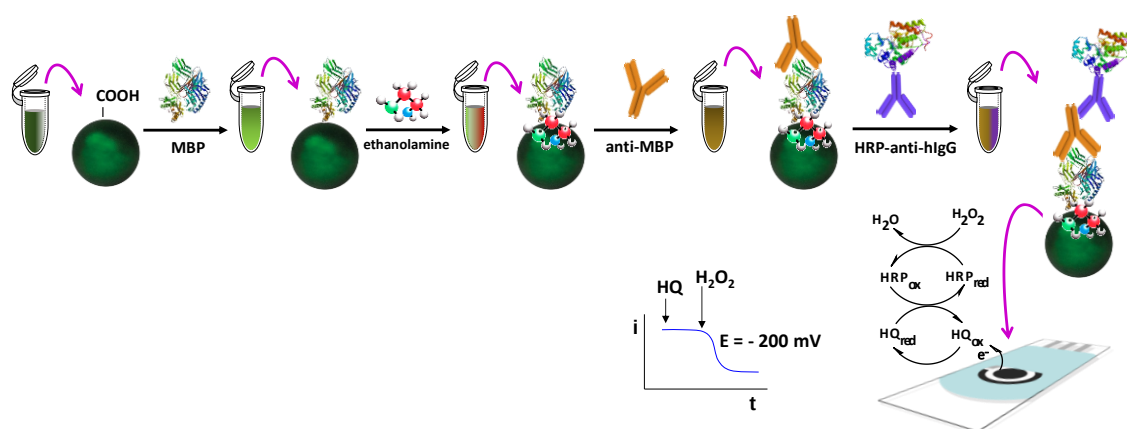


Figura 4.15. Esquema de las etapas de preparación de la bioplataforma basada en micropartículas magnéticas (MBs) funcionalizadas para la determinación de anti-MBP.

4.4.3.4. Bioplataforma para la determinación de CCL5

En primer lugar, se preparó el bioconjugado sobre micropartículas magnéticas funcionalizadas con neutravidina (Neutr-MBs) empleando 3 μL de suspensión de estas partículas sobre las que, una vez lavadas, se procedió a incubar el anticuerpo biotinilado, Biotin-anti-CCL5 añadiendo 25 μL de disolución de 25 $\mu\text{g mL}^{-1}$, dejándolo reaccionar durante 30 min en un agitador-incubador a 600 rpm. Tras dos lavados con 50 μL de disolución reguladora PBST de pH 7.4 se añadieron 25 μL de disolución de CCL5 estándar o la muestra, en PBS 100 mM de pH 7.4, incubando durante 60 min en las mismas condiciones. Después de un nuevo lavado con 50 μL de PBST de pH 7.4, se adicionaron 25 μL de anti-CCL5 de 0.5 $\mu\text{g mL}^{-1}$ en PBS 100 mM de pH 7.4 para su incubación durante 30 min. Finalmente, se lavó y se añadieron 25 μL de HRP-IgG de 0.5 $\mu\text{g mL}^{-1}$ en disolución comercial de caseína, dejándolo incubar durante 30 min. Inmediatamente antes de la medida, se lavó el conjugado con 50 μL de PBST de pH 7.4 resuspendiéndolas en el propio medio para inmovilizarlas posteriormente sobre el SPCE con ayuda de un imán. La medida por amperometría se llevó a cabo introduciendo, como en casos anteriores el dispositivo electrodo-imán (Figura 4.5) en 10 mL de PB 50 mM de pH 6.0 en presencia de HQ 0.1 M. A continuación, se midió la respuesta amperométrica como anteriormente.

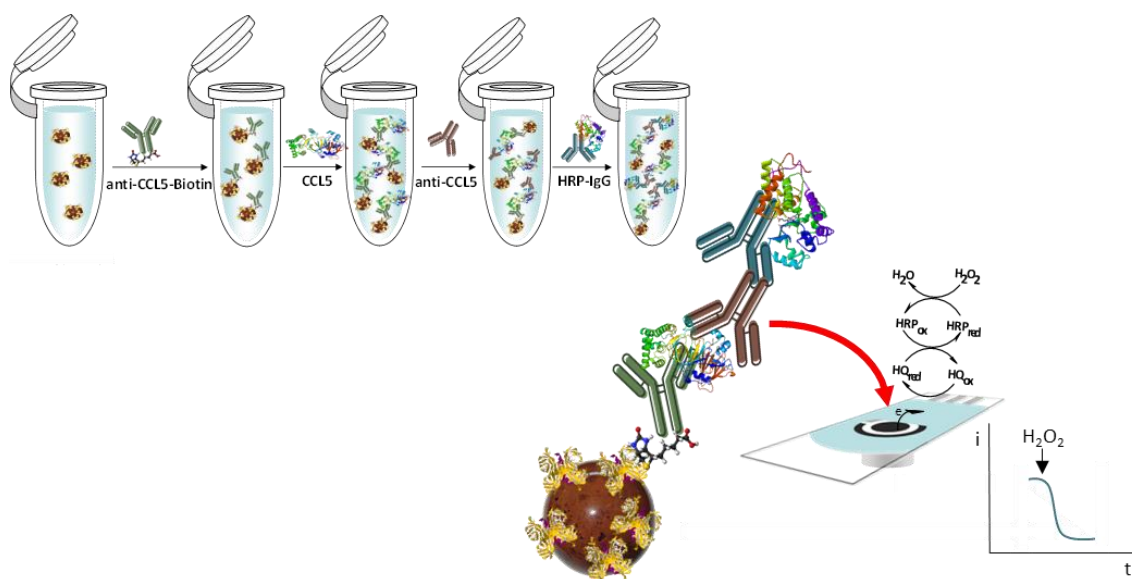


Figura 4.16. Esquema de las etapas de preparación de la bioplataforma basada en micropartículas magnéticas (MBs) funcionalizadas para la determinación de anti-MBP.

4.4.4. Análisis de muestras

4.4.4.1. Determinación de TGF- β 1 en plasma

Se analizaron muestras de plasma comercial (Ref. 8BFP, lote 11/03-8BFP, HyTest Ltd.) enriquecidas con 25 ng mL⁻¹ de TGF- β 1 reconstituidas y diluidas cien veces en disolución reguladora comercial "Reagent Diluent" diluido de pH 7.2-7.4, no siendo necesario ningún tratamiento adicional. Una vez confirmada la ausencia de efecto matriz, la determinación se llevó a cabo por interpolación de la señal proporcionada por las muestras en el calibrado de disoluciones patrón.

4.4.4.2. Determinación de PYY en suero y saliva

En este caso, el método desarrollado se aplicó al análisis de suero liofilizado comercial enriquecido con 100 pg mL⁻¹ de PYY y de saliva humana perteneciente a un individuo sano, contaminada con 35 pg mL⁻¹ del péptido. Cabe destacar que, en ambos casos, el tratamiento de las muestras solo implicó su dilución 1/10 con una disolución del conjugado Biotin-PYY en medio PBS de pH 7.4. Al igual que anteriormente, una vez comprobada la ausencia de efecto matriz, la determinación de PYY se realizó por interpolación de las corrientes en los calibrados de las disoluciones patrón.

4.4.4.3. Determinación de CXCL7 en muestras clínicas de suero

El inmunosensor desarrollado se aplicó a la determinación de CXCL7 en muestras de suero pertenecientes a pacientes con artritis reumatoide diagnosticada, procedentes del Hospital Clínico San Carlos (Madrid, España) obtenidas con el informe de consentimiento aprobado tanto por los pacientes como por el Comité Ético de la Institución. Las muestras se diluyeron en una proporción 1/2500 con "Reagent Diluent" diluido como único tratamiento de las muestras. Tras la confirmación de la ausencia de efecto matriz, la determinación de CXCL7 se realizó por aplicación del método desarrollado e interpolación de la señal de las muestras en el calibrado de disoluciones patrón. Estos resultados se validaron por aplicación de un método de inmunoensayo tipo ELISA empleando el kit comercial ab100613 de Abcam.

4.4.4.4. Determinación de IL-1 β en saliva

Se analizó una muestra de saliva perteneciente a un individuo sano no fumador sin diluir, que no mostraba efecto matriz. La determinación de IL-1 β se llevó a cabo aplicando el procedimiento descrito en el Apartado 4.4.2.2. por interpolación de la señal proporcionada por la muestra en el calibrado de disoluciones patrón y el resultado se

comparó con el proporcionado por el kit DuoSet® ELISA Development Systems DY201-05 de R&D Systems.

4.4.4.5. Determinación simultánea de RF y CCPA en suero

La bioplataforma desarrollada para estos autoanticuerpos se aplicó a muestras de suero humano de Abcam (ab178653) y Demeditec (DE7760) de Demeditec sin necesidad de aplicar ninguna etapa de dilución. Tras comprobar la ausencia de efecto matriz, la determinación de ambas especies se realizó siguiendo el procedimiento descrito en el Apartado 4.4.3.1., por interpolación de la señal proporcionada por las muestras en el calibrado de disoluciones patrón.

4.4.4.6. Determinación simultánea de CXCL7 y MMP3 en suero

Las muestras de suero humano empleadas para la determinación simultánea de CXCL7 y MMP3 pertenecían a individuos sanos y pacientes con artritis reumatoide diagnosticada en distintas fases de la enfermedad, procedentes de Central BioHub® (Hennigsdorf, Alemania). En todos los casos, las muestras fueron diluidas 1/50 en disolución reguladora PBS de pH 7.4. Como en el caso anterior, la ausencia de efecto matriz permitió llevar a cabo la determinación por interpolación de la señal obtenida por las muestras en el calibrado de disoluciones patrón. Los resultados obtenidos por aplicación del método desarrollado, según el procedimiento descrito en el Apartado 4.4.3.2., se validaron frente a kits ELISA comerciales de R&D Systems empleando los mismos inmunorreactivos.

4.4.4.7. Determinación de anti-MBP en suero

Este inmunosensor se aplicó al análisis de muestras de suero humano perteneciente a individuos sanos y a pacientes con esclerosis múltiple proporcionadas por Central BioHub® Specimens a las que se les realizó una dilución 1/100 en disolución reguladora PBS de pH 7.4. Al igual que en otros casos, una vez comprobada la ausencia de efecto matriz, la determinación de este anticuerpo se llevó a cabo aplicando el procedimiento descrito en el Apartado 4.4.3.3., interpolando la señal proporcionada por las muestras en el calibrado de disoluciones patrón. Los resultados se validaron frente a los proporcionados por un kit ELISA comercial (Cloud-Clone Corp.).

4.4.4.8. Determinación de CCL5 en suero

En esta aplicación, las muestras de suero de individuos sanos y de pacientes con esclerosis múltiple (Central BioHub® Specimens) se diluyeron en proporción 1/100 con disolución reguladora PBS de pH 7.4. Una vez comprobada la ausencia de efecto matriz,

la determinación de CCL5 en las muestras se realizó empleando el procedimiento descrito en el Apartado 4.4.3.4. por interpolación de la señal proporcionada por las mismas en el calibrado de disoluciones patrón. La validación se realizó frente al kit ELISA comercial de R&D Systems.

5 Resultados y discusión

Inmunosensores basados en electrodos modificados por "grafting" electroquímico

Inmunosensores basados en electrodos modificados mediante química "click" y "electroclick"

Bioplateformas electroquímicas basadas en micropartículas magnéticas funcionalizadas

5.1. INMUNOSENSORES BASADOS EN ELECTRODOS MODIFICADOS POR "GRAFTING" ELECTROQUÍMICO

Como se comentó en la Introducción, la metodología del llamado "electrografting" o "grafting" (injertado) electroquímico se ha establecido en los últimos años como una de las más simples de las disponibles para la introducción de una amplia variedad de grupos funcionales sobre superficies conductoras, caracterizándose además por su versatilidad, ya que pueden emplearse materiales y reactivos diversos, así como distintos modos de reacción. En estos estudios se emplearon electrodos serigrafados de oro, mucho menos frecuentes que los de carbono, utilizando el ácido *p*-aminobenzoico (*p*-ABA) y la modalidad de "grafting" oxidativo, menos explorada y más rápida que la basada en sales de arildiazonio, para inmovilizar covalentemente los anticuerpos de captura sobre los grupos *p*-carboxifenilamino resultantes, con el fin de desarrollar inmunosensores electroquímicos para la determinación de los antígenos específicos en muestras de interés clínico. Esta metodología se aplicó a la citoquina TGF- β 1 y a la hormona peptídica PYY. Los trabajos experimentales correspondientes a estos dos biomarcadores se realizaron prácticamente a la vez, pero la optimización de variables comunes a ambas configuraciones se investigó primero sobre la plataforma de PYY, por lo que se presentan en primer lugar los resultados obtenidos en este caso.

Además del objetivo de la puesta a punto de inmunosensores electroquímicos rápidos, sensibles y fiables para ambas biomoléculas, los objetivos particulares que se fijaron al comienzo de estas investigaciones fueron:

1. Demostrar la utilidad del método de "grafting" en una sola etapa basado en la oxidación electroquímica de arilaminas para la preparación de superficies biosensoras sobre electrodos de oro.
2. Comparar esta metodología con la utilizada más frecuentemente sobre estos electrodos basada en la fabricación de monocapas de tioles autoensambladas.

5.1.1. Inmunosensor para la determinación de la hormona PYY

5.1.1.1. Configuración del inmunosensor

En la Figura 4.10. del Apartado 4.4.1 se mostró un esquema de las etapas implicadas en la funcionalización y construcción del inmunosensor desarrollado para la determinación de PYY. En primer lugar, la modificación de la superficie de un SPAuE de baja temperatura activado con H_2SO_4 0.1M se llevó a cabo mediante "grafting" electroquímico del radical catiónico de *p*-ABA obtenido por voltamperometría cíclica. Una vez modificado el electrodo, los grupos *p*-carboxifenilamino confinados en su superficie (HOOC-Phe-N-SPAuE) se activaron empleando el método de la carbodiimida (EDC/NHSS 0.1M) y se inmovilizó covalentemente el anticuerpo de captura anti-PYY. Después, se estableció una configuración de tipo competitivo directo entre el antígeno, PYY, y el antígeno biotinilado, Biotin-PYY, seguido de la inmovilización del conjugado de estreptavidina marcada con fosfatasa alcalina (AP-Strept). Finalmente, se llevó a cabo la detección voltamperométrica midiendo en DPV la señal proporcionada por la oxidación electroquímica del 1-naftol (1-NP), producto de la reacción enzimática del sustrato de la fosfatasa alcalina, 1-naftil fosfato (1-NPP). La Figura 5.1 muestra un esquema del inmunosensor y de la reacción implicada.

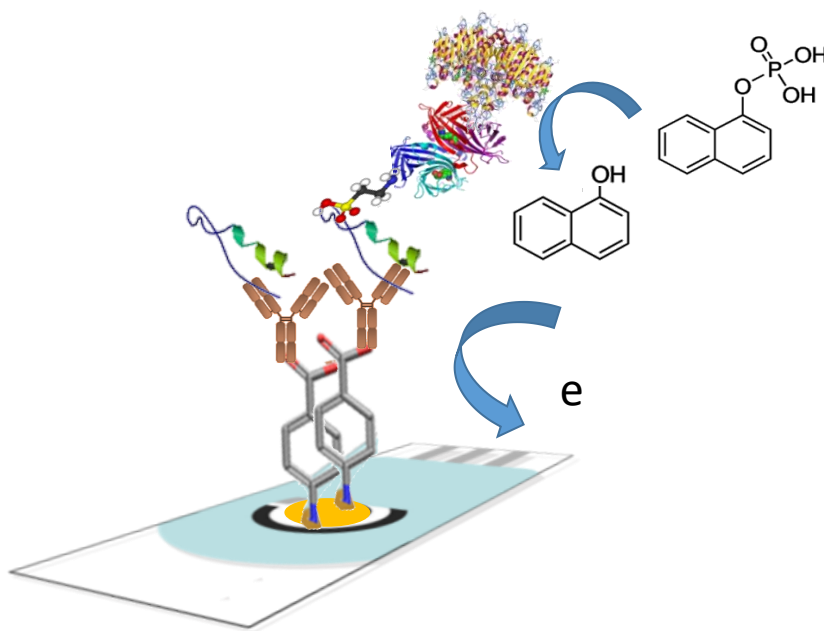


Figura 5.1. Esquema del inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAuE y las reacciones implicadas en la detección.

5.1.1.2. Optimización de las variables experimentales

Con el objetivo de alcanzar la máxima sensibilidad y un amplio intervalo lineal en el calibrado para la determinación del biomarcador, se optimizaron las variables relacionadas con la modificación del electrodo, la construcción del inmunosensor y su funcionamiento. En todos los casos el criterio de optimización fue obtener la mayor relación entre las corrientes medidas con el inmunosensor en presencia (S) y en ausencia (N) de la hormona biotinilada (Biotin-PYY), representando las primeras las respuestas específicas debidas a la interacción antígeno-anticuerpo y las segundas, las respuestas inespecíficas debidas a otras interacciones o corrientes no deseadas sobre la superficie del electrodo. Con respecto a la modificación del SPAuE mediante "grafting" oxidativo, las variables optimizadas fueron: la concentración de *p*-ABA, el intervalo de potencial barrido, el número de ciclos y la velocidad de barrido de potencial en voltamperometría cíclica (CV). El medio de reacción utilizado: LiClO₄ 0.1 M en etanol, fue el mismo empleado en trabajos anteriores [Ojeda, 2012]. Una vez funcionalizada la superficie del electrodo con los grupos *p*-carboxifenilamino, la activación de los grupos carboxilo se realizó en las condiciones optimizadas anteriormente, con EDC/NHSS 0.1 M en disolución reguladora MES de pH 5.0, incubando 30 min a temperatura ambiente, en un recinto húmedo y en la oscuridad [Ojeda, 2014].

Se optimizaron también las concentraciones y los tiempos de incubación de los inmunorreactivos: anti-PYY, agente bloqueante y Biotin-PYY, que son las variables implicadas directamente en la preparación y funcionamiento del inmunosensor. En cuanto a las condiciones de la reacción enzimática en presencia de fosfatasa alcalina (AP), tanto la disolución reguladora (Trizma 50 mM de pH 9.6) como la concentración de sustrato y el tiempo de reacción (1-NPP 0.05 M, 5 min) fueron las utilizadas anteriormente [Martínez-García, 2016]. Por otro lado, con fines comparativos, se construyó otro inmunosensor para PYY basado en la modificación del SPAuE con una monocapa de ácido 4-mercaptobenzoico (4-MBA), incluyéndose al final de los estudios anteriores los resultados obtenidos en la optimización de la preparación del electrodo 4-MBA/SPAuE correspondiendo estos al estudio de la influencia de la concentración de 4-MBA y el tiempo de preparación de la monocapa. El conjunto de datos obtenidos se describe a continuación, resumiéndose estos en la Tabla 5.1.

Influencia de la concentración de *p*-ABA

Para realizar este estudio se modificó la superficie electródica con distintas concentraciones de *p*-ABA en el intervalo comprendido entre 0.35 y 3.6 mM (Figura 5.2). Como se observa, las respuestas inespecíficas se mantienen prácticamente constantes en todo el intervalo estudiado mientras que las corrientes específicas son de baja magnitud hasta alcanzar un valor máximo para una concentración 1.5 mM de *p*-ABA, a partir de la cual disminuyen de nuevo estabilizándose prácticamente después. Probablemente, esta concentración es la que permite inmovilizar una mayor cantidad de anti-PYY, proporcionando la mejor relación S/N. Concentraciones menores darían lugar a un número insuficiente de grupos carboxilo enlazantes mientras que concentraciones muy altas provocarían un efecto de saturación. Teniendo en cuenta estos resultados, la concentración citada se eligió como óptima con el fin de asegurar una funcionalización efectiva que garantice una carga adecuada de anti-PYY sin llegar a saturar el electrodo.

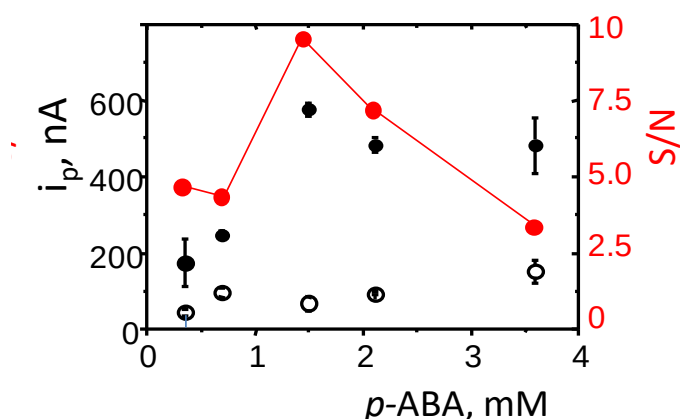


Figura 5.2. Influencia de la concentración de *p*-ABA usada en el proceso de "grafting" sobre la respuesta del inmunosensor en ausencia (○) y en presencia (●) de 100 ng mL⁻¹ de Biotin-PYY, inc. 30 min. 0.0 - +0.6 V; $v = 30 \text{ mV s}^{-1}$; $n = 3$; 20 $\mu\text{g mL}^{-1}$ anti-PYY, inc. 30 min; 0.2 % caseína (BB), inc. 60 min; 5 $\mu\text{g mL}^{-1}$ AP-Strept, inc. 30 min. Detección: 1-NPP 5 mM, 5 min reacción; DPV: -0.2 - +0.7 V vs. Ag; $\Delta E = 50 \text{ mV}$; $v = 10 \text{ mVs}^{-1}$. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Influencia del intervalo de potencial

Para optimizar esta variable se tuvieron en cuenta los datos de trabajos anteriores [Ojeda, 2012] [Sánchez-Tirado, 2018], que recomiendan el intervalo comprendido entre 0.0 y +1.0 V vs Ag para la formación del radical catiónico del *p*-ABA por aplicación de ciclos sucesivos sobre SPCEs. En este caso, tratándose de una superficie de oro, y con el

fin de evitar su oxidación, se estudiaron varios potenciales máximos comprendidos entre +0.6 y +1.0 V vs Ag comenzando el registro del voltamperograma a partir de 0.0 V en todos los casos. En la Figura 5.3. se muestran los resultados obtenidos, indicándose en el eje de abscisas el potencial extremo del intervalo estudiado. Como puede observarse, las respuestas específicas del inmunosensor disminuyen ligeramente a medida que se extiende el intervalo de potencial hacia valores más positivos. Sin embargo, en el caso de las inespecíficas (en ausencia de Biotin-PYY) tiene lugar el efecto contrario, debido probablemente a que se incrementa la posibilidad de oxidaciones no deseadas. Por ello, la mayor relación S/N se obtiene para el intervalo comprendido entre 0.0 y +0.6 V vs Ag, que fue el elegido para estudios posteriores.

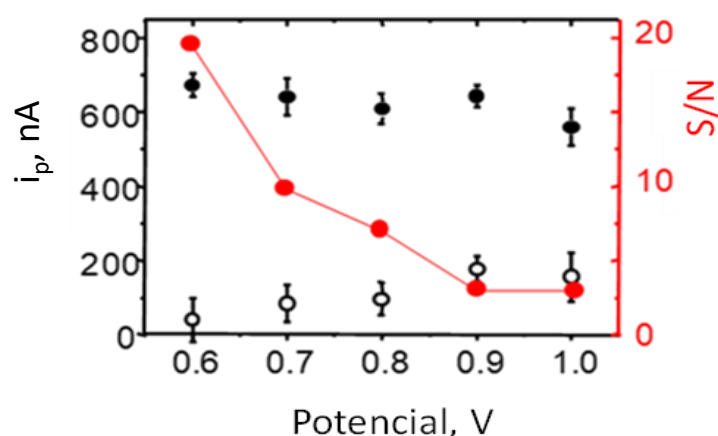


Figura 5.3. Influencia del intervalo de potencial de barrido en el proceso de "grafting" sobre la respuesta del inmunosensor. $v = 30 \text{ mV s}^{-1}$; $n = 3$; $p\text{-ABA } 1.5 \text{ mM}$. Otras condiciones como en la Figura 5.2.

Influencia del número de ciclos

Debido a que la formación del radical de la arilamina mediante "electrografting" oxidativo requiere la aplicación de barridos sucesivos de potencial en CV, el número de ciclos es una variable esencial cuya optimización permitirá asegurar la adecuada funcionalización del electrodo. Para llevar a cabo este estudio se realizaron entre 1 y 10 barridos consecutivos de potencial en el intervalo de 0.0 a +0.6 V vs Ag con los resultados que aparecen en la Figura 5.4a. En ella se observa la disminución de la corriente específica a medida que aumenta el número de ciclos, si bien a partir del tercer ciclo no se observaron diferencias apreciables en los voltamperogramas. Por otro lado, en el estudio de la influencia de esta variable sobre la respuesta del inmunosensor (Figura 5.4b), se obtuvo

la mejor relación S/N al aplicar 3 ciclos consecutivos, resultado que está de acuerdo con los voltamperogramas anteriores. Probablemente, la aplicación de un único ciclo resulta insuficiente para una adecuada inmovilización del anticuerpo de captura, quedando vacantes en la superficie susceptibles de adsorciones inespecíficas. Debido a esto, se obtiene una baja relación S/N en este punto. Después, las señales inespecíficas van disminuyendo con el número de ciclos al tiempo que tiene lugar una pequeña pérdida de señal específica. Esta reducción de señal es mayor cuando se aplican 10 ciclos, lo que se atribuye a la presencia de un exceso de grupos de unión en la superficie del electrodo que causaría impedimentos estéricos debido a la excesiva cantidad de anti-PYY inmovilizado.

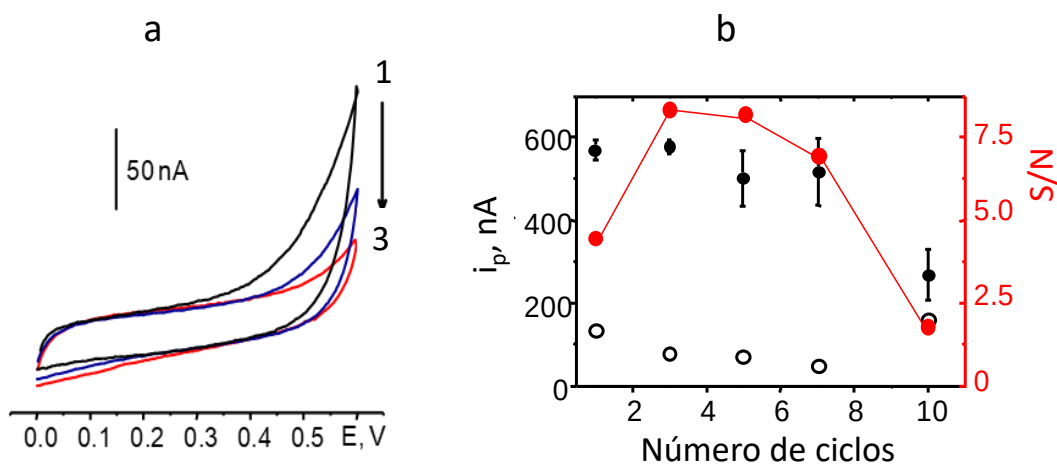


Figura 5.4. (a) Voltamperogramas cíclicos sucesivos en *p*-ABA 1.5 mM; 0.0 - +0.6 V; $v = 30 \text{ mV s}^{-1}$. (b) Influencia del número de ciclos de potencial aplicados en el proceso de "grafting" sobre la respuesta del inmunosensor. Otras condiciones como en la Figura 5.2.

Influencia de la velocidad de barrido de potencial

Esta variable es una de las más importantes a optimizar en la etapa de modificación del electrodo ya que de ella depende la adecuada distribución de los radicales generados en este proceso. Para realizar este estudio se evaluó la influencia sobre la respuesta del inmunosensor del empleo de velocidades de barrido de potencial comprendidas entre 5 y 100 mV s^{-1} . Como puede observarse en la Figura 5.5, las señales específicas del inmunosensor aumentan con la velocidad de barrido hasta 30 mV s^{-1} para llegar a estabilizarse a continuación. Por otro lado, las corrientes inespecíficas permanecen prácticamente constantes en todo el intervalo estudiado. Este comportamiento puede explicarse considerando que altas velocidades de barrido de

potencial pueden generar radicales distribuidos de forma no homogénea y desordenada, haciendo que algunos de los grupos carboxilo no sean adecuados para enlazar anticuerpos mientras que a velocidades de barrido muy lentas el proceso de modificación del electrodo mediante "grafting" no sería eficiente, necesitándose además un tiempo más largo para lograr la funcionalización. Teniendo en cuenta estos resultados, se eligió una velocidad de barrido de potencial de 30 mV s^{-1} para estudios posteriores.

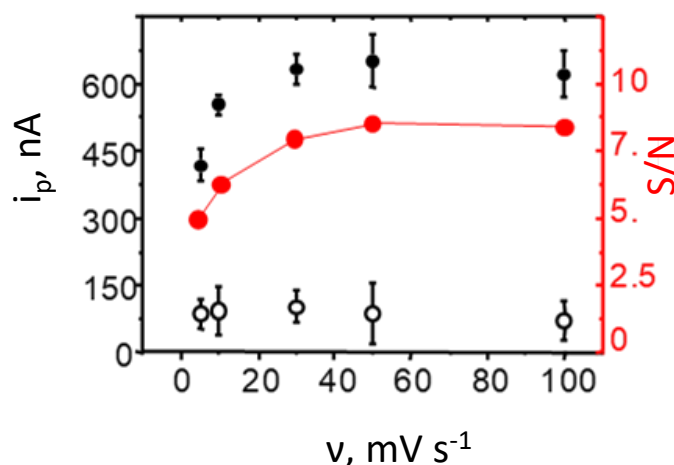


Figura 5.5. Influencia de la velocidad de barrido de potencial aplicada en el proceso de "grafting" sobre la respuesta del inmunosensor. p -ABA 1.5 mM; 0.0 - +0.6 V; $n = 3$. Otras condiciones como en la Figura 5.2.

Influencia de la concentración y el tiempo de incubación de anti-PYY

Tras la optimización de las variables implicadas en la modificación del electrodo, se estudió de la influencia de la concentración y el tiempo de incubación del anticuerpo de captura sobre el electrodo modificado. En primer lugar, se midieron las respuestas del inmunosensor en presencia y ausencia de Biotin-PYY utilizando concentraciones de anti-PYY comprendidas entre 5 y $30 \mu\text{g mL}^{-1}$ e incubando durante 60 min. Como puede observarse en la Figura 5.6a, tanto las respuestas específicas como las inespecíficas aumentan con la concentración de anti-PYY. Estos resultados llevaron a elegir $20 \mu\text{g mL}^{-1}$ como valor óptimo. Aunque este valor no corresponde a la mejor relación S/N debido a la relativamente elevada corriente inespecífica, esta decisión se fundamenta en el hecho de que, como se verá, es posible disminuir en optimizaciones posteriores dicha corriente mediante un bloqueo efectivo, sin que ello implique la pérdida de señal específica. En relación al tiempo de incubación de anti-PYY, se evaluaron las respuestas del

inmunosensor obtenidas tras incubar el anticuerpo de captura durante periodos comprendidos entre 15 y 90 min. Los resultados obtenidos, representados en la Figura 5.6b, permiten apreciar un valor óptimo de la relación S/N para 60 min, siendo este el elegido para la preparación del inmunosensor.

Finalmente se estudiaron tres temperaturas diferentes: 4, 25 y 37 °C, para la incubación del anticuerpo de captura en las condiciones previamente optimizadas, obteniéndose la mejor relación S/N para 25 °C.

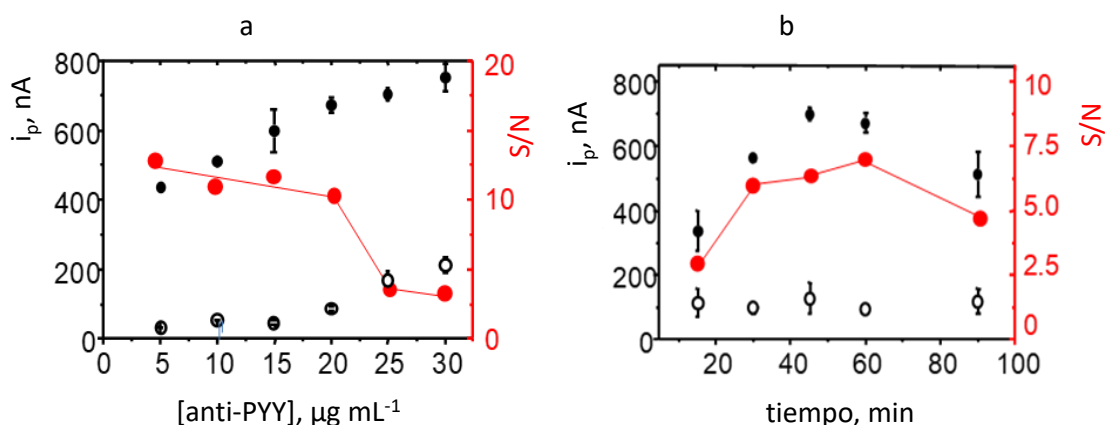


Figura 5.6. Influencia de la concentración (a) y del tiempo de incubación (b) de anti-PYY sobre la respuesta del inmunosensor: p -ABA 1.5 mM; 0.0 - +0.6 V; $v = 30 \text{ mV s}^{-1}$; $n = 3$; $20 \mu\text{g mL}^{-1}$ anti-PYY (b). inc. 60 min (a). Otras condiciones como en la Figura 5.2.

Influencia de la naturaleza del agente bloqueante

Con el objetivo de minimizar las adsorciones inespecíficas sobre la superficie del electrodo modificado, se estudió la eficacia de distintos agentes bloqueantes habituales: etanolamina, albúmina de suero bovino (BSA) y caseína comercial al 1% (Blocking Buffer, BB). Los resultados obtenidos (Figura 5.7) demostraron un comportamiento óptimo para este último agente, que proporcionaba la mejor relación S/N. En el caso del BSA, se observó una fuerte disminución de la respuesta específica, probablemente debido a un bloqueo excesivo, mientras que la etanolamina no mostraba ningún efecto bloqueante, obteniéndose señales inespecíficas incluso superiores a las específicas, fenómeno seguramente causado por la inestabilidad de la superficie del electrodo en presencia de este reactivo.

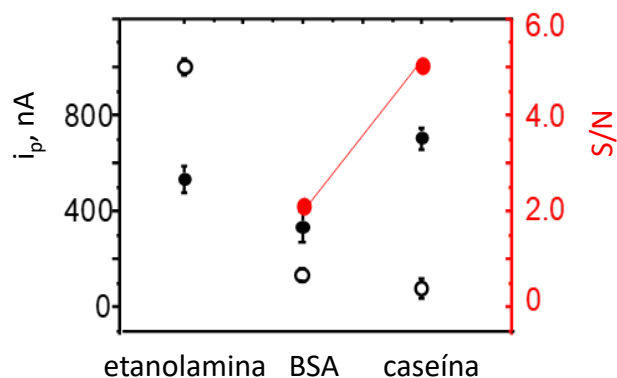


Figura 5.7. Influencia de la naturaleza del agente bloqueante sobre la respuesta del inmunosensor: p -ABA 1.5 mM; 0.0 - +0.6 V; $v = 30 \text{ mV s}^{-1}$; $n = 3$; $20 \mu\text{g mL}^{-1}$ anti-PYY, inc. 60 min; bloqueante inc. 60 min. Otras condiciones como en la Figura 5.2.

Influencia de la concentración y el tiempo de incubación de la caseína

Para optimizar la concentración del agente bloqueante elegido, en primer lugar, se prepararon distintas disoluciones de caseína por dilución del producto "blocking buffer" (BB, caseína comercial al 1%), estudiando la influencia de esta variable sobre la respuesta del inmunosensor. Como se observa en la Figura 5.8a, las corrientes inespecíficas disminuyen, de acuerdo con lo esperado, a medida que aumenta la concentración de bloqueante, mientras que las respuestas específicas más elevadas se obtienen para concentraciones de caseína comprendidas entre el 0.1 y el 0.3 %. Puede verse también que la relación S/N aumenta ligeramente para un porcentaje del 0.6%, pero se obtiene una respuesta específica notablemente inferior debido al exceso de bloqueante además de una peor repetibilidad de las medidas. Estos resultados llevaron a elegir el 0.2 % de caseína, valor que proporciona la mayor relación S/N en el intervalo citado, como concentración óptima para estudios posteriores.

Con respecto al efecto del tiempo de incubación del agente bloqueante (tiempo de bloqueo), esta variable se estudió el intervalo de 15 a 90 min con los resultados representados en la Figura 5.8b. Puede observarse cómo tiempos inferiores a 30 min son insuficientes para asegurar un buen bloqueo, mientras que cuando este tiempo es excesivo, tiene lugar una notable disminución de la respuesta específica.

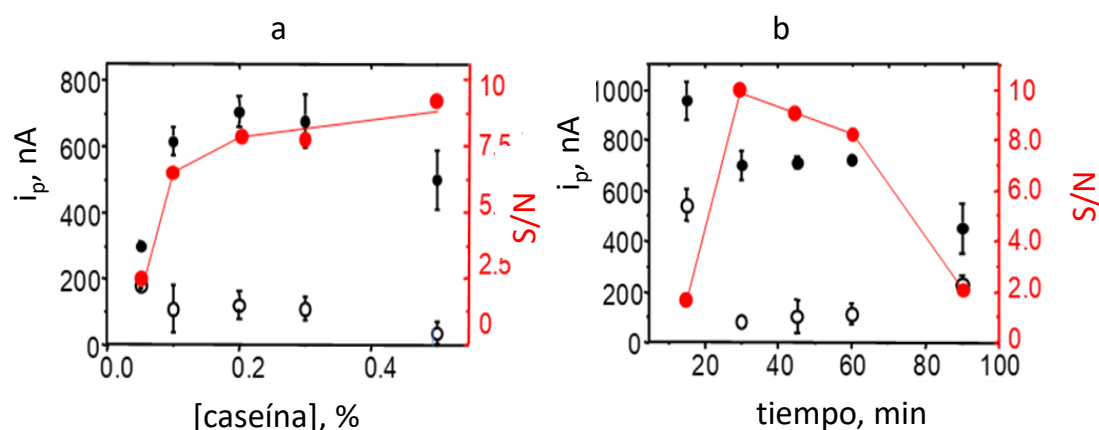


Figura 5.8. Influencia de la concentración (a) y del tiempo de incubación (b) de la caseína sobre la respuesta del inmunosensor: $20 \mu\text{g mL}^{-1}$ anti-PYY, inc. 60 min; 0.2% caseína (b), inc. 60 min. (a). Otras condiciones como en la Figura 5.2.

Influencia de la concentración y el tiempo de incubación de Biotin-PYY

En la Figura 5.9a se han representado las respuestas del inmunosensor obtenidas en ausencia y en presencia de distintas concentraciones de Biotin-PYY en el intervalo de 50 a 400 ng mL^{-1} . Puede verse el aumento rápido de la señal hasta 75 ng mL^{-1} para mantenerse prácticamente constante en el resto del intervalo estudiado. Este resultado llevó a elegir 100 ng mL^{-1} como concentración óptima del antígeno biotinilado. En la Figura 5.9b, se muestran las respuestas obtenidas al incubar durante distintos periodos de tiempo, entre 15 y 60 min, el conjugado Biotin-PYY sobre el bioelectrodo anti-PYY-Phe-N-SPAUE. Al no apreciarse apenas diferencias de corriente a partir de periodos superiores a 30 min, se seleccionó este como tiempo óptimo de incubación.

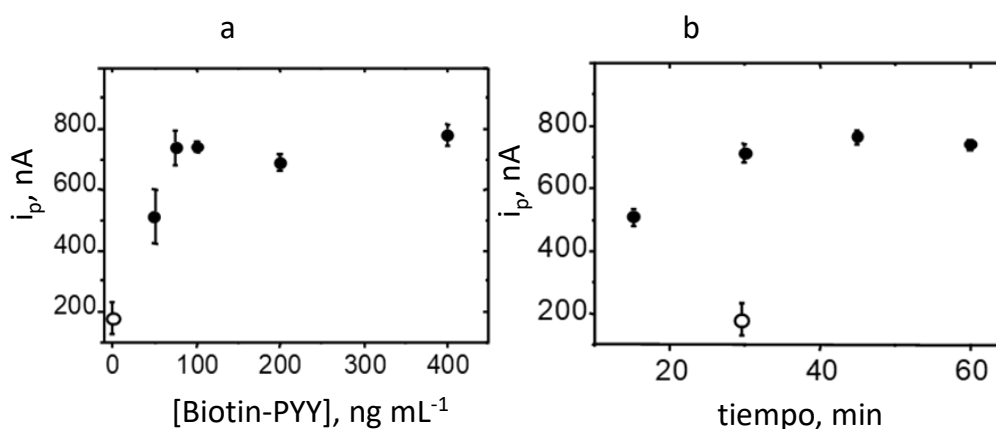


Figura 5.9. Influencia de la concentración (a) y del tiempo de incubación (b) de Biotin-PYY sobre la respuesta del inmunosensor: $20 \mu\text{g mL}^{-1}$ anti-PYY, inc. 60 min; 0.2% caseína, inc. 30 min; 100 ng mL^{-1} Biotin-PYY(b) inc. 30 min (a); Otras condiciones como en la Figura 5.2.

Influencia de la concentración de AP-Strept

Para optimizar la concentración del conjugado empleado para desarrollar la reacción enzimática de la fosfatasa alcalina (AP) se establecieron varios calibrados de PYY en el intervalo de concentraciones comprendido entre 10^{-9} y 10^2 ng mL⁻¹, empleando 2.5 (Δ), 5.0 ($\circ$) y 7.5 ($\bullet$) μ g mL⁻¹ de AP-Strept y un tiempo de incubación de 30 min. Como se muestra en la Figura 5.10, el calibrado obtenido con la concentración intermedia de AP-Strept presentaba un intervalo lineal más amplio y una mayor pendiente, por lo que fue elegida para la preparación del inmunosensor.

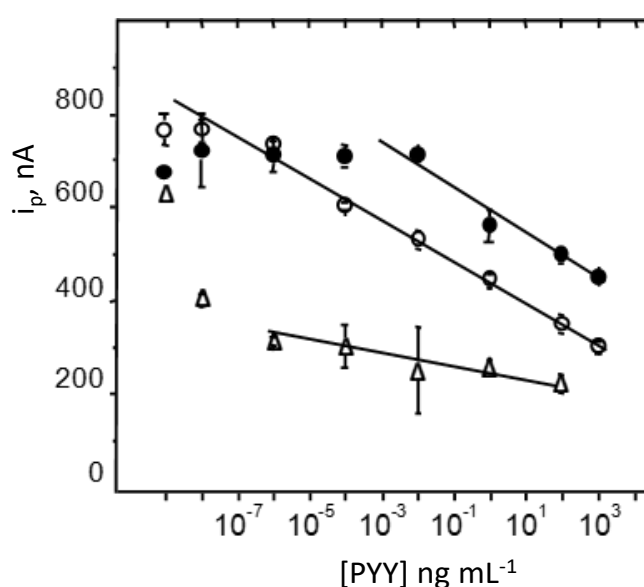


Figura 5.10. Influencia de la concentración de AP-Strept sobre la respuesta del inmunosensor: AP-Strept 2.5(Δ), 5.0($\circ$) y 7.5($\bullet$) μ g mL⁻¹, inc. 30 min; *p*-ABA 1.5 mM; 0.0 - +0.6 V; ν = 30 mV s⁻¹; n=3; 20 μ g mL⁻¹ anti-PYY, inc. 60 min; 0.2% caseína inc. 30 min; 100 ng mL⁻¹ Biotin-PYY, inc. 30 min; Otras condiciones como en la Figura 5.2.

Influencia de la concentración y tiempo de incubación 4-MBA sobre SPAuE

Como se ha indicado al principio de estos estudios, con fines comparativos se preparó otro inmunosensor para PYY basado en la modificación del electrodo serigrafiado de oro con ácido 4-mercaptobenzoico (4-MBA), optimizándose la fabricación de la monocapa con los resultados que se muestran en la Figura 5.11.

Respecto a la concentración de 4-MBA (Figura 5.11a), puede observarse cómo, en las condiciones experimentales elegidas anteriormente, las corrientes específicas aumentan en todo el intervalo estudiado debido al aumento del número de grupos disponibles para enlazar el antígeno biotinilado. Por otro lado, las corrientes inespecíficas son de baja magnitud y permanecen prácticamente constantes a partir de una concentración de 4-MBA 1.0 mM. El resultado de ambas tendencias es un máximo en la relación S/N para una concentración 1.5 mM. En cuanto al tiempo necesario para preparar la monocapa, la Figura 5.11b muestra los resultados obtenidos para periodos de incubación de 2 a 10 horas, encontrándose un valor óptimo de 6h.

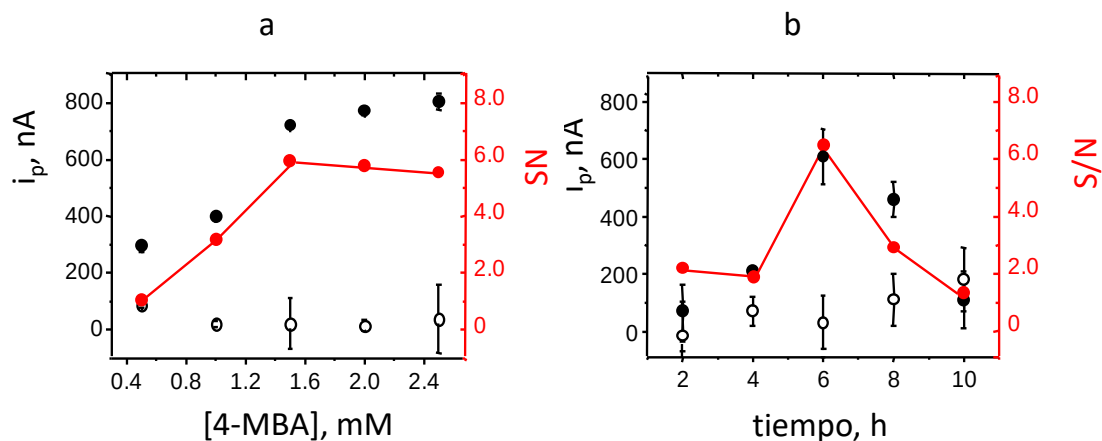


Figura 5.11. Influencia de (a) la concentración y (b) el tiempo de incubación de 4-MBA usados para modificar el SPAuE sobre la respuesta del inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-S-SPAuE. Condiciones: 1.5 mM 4-MBA (b) inc. 6h (a). Otras condiciones como en la Figura 5.2

Finalmente, como resumen de las optimizaciones realizadas, en la Tabla 5.1 se muestran las variables estudiadas y los valores elegidos para la preparación del inmunosensor.

Tabla 5.1. Optimización de las variables experimentales.

Variable	Intervalo estudiado	Valor seleccionado
Concentración de <i>p</i> -ABA, mM	0.35 - 3.6	1.5
Intervalo de potencial, V	0.0-+0.6 - 0.0-+1.0	0.0-+0.6
Número de ciclos	1 - 10	3
Velocidad de barrido, mV s ⁻¹	5 - 100	30
Concentración anti-PYY, µg mL ⁻¹	5 - 30	20
Tiempo de incubación anti-PYY, min	15 - 90	60
Agente bloqueante	etanolamina, BSA, caseína	caseína
Concentración caseína, %	0.1 - 0.5	0.2
Tiempo de incubación caseína, min	15 - 90	30
Concentración Biotin-PYY, ng mL ⁻¹	50 - 400	100
Tiempo de incubación Biotin-PYY, min	15 - 60	30
Concentración AP-Strept, µg mL ⁻¹	2.5, 5.0, 7.5	5.0
Tiempo de incubación AP-Strept	-	30
Concentración 4-MBA, mM	0.5 - 2.5	1.5
Tiempo de incubación, h	2 - 10	6

5.1.1.3. Estudios de caracterización

Para evaluar la efectividad de los procesos de modificación de la superficie electródica mediante "grafting" y los de construcción del inmunosensor, se analizaron todas las etapas implicadas en dichos procesos mediante la técnica de espectroscopia de impedancia electroquímica (EIS). El registro de los espectros de Nyquist y las medidas de la resistencia a la transferencia de carga, R_{CT} , se realizaron empleando una disolución de $\text{Fe}(\text{CN})_6^{3-/4-}$ 5 mM en KCl 0.1 M como sonda redox. Además, los resultados obtenidos en estos estudios se compararon con los del inmunosensor basado en el electrodo MBA/SPAuE.

En la Figura 5.12 aparecen representados los diagramas de impedancias obtenidos sobre el SPAuE desnudo, así como sobre el electrodo funcionalizado, HOOC-Phe-N-SPAuE, y modificado con los inmunorreactivos inmovilizados sucesivamente para la

preparación del inmunosensor. Como puede observarse (Figura 5.12a), el diámetro del semicírculo disminuye notablemente tras la activación del SPAuE con H_2SO_4 0.1M, pasando el valor de R_{CT} de 1950 Ω (curva 1) a 212 Ω (curva 2), lo que pone de manifiesto la utilidad de este proceso. Posteriormente, la incorporación de grupos carboxilo en forma de HOOC-Phe-N-SPAuE provoca un aumento notable de R_{CT} (1075 Ω , curva 3) debido a la repulsión electrostática entre las cargas negativas de la sonda electroquímica, ferro-ferricianuro y las de estos grupos, disociados al pH de trabajo. Sin embargo, tras la activación con EDC/NHSS, las cargas se neutralizan y la resistencia a la transferencia de carga disminuye hasta 12 Ω (curva 4).

Por otro lado, como se observa en la Figura 5.12b, la incorporación secuencial de los inmunorreactivos origina un aumento continuado del diámetro del semicírculo en las curvas de impedancias, debido a la naturaleza aislante de estas biomoléculas. Así, la inmovilización del anticuerpo de captura, anti-PYY, produce un incremento de R_{CT} hasta 170 Ω (curva 5) y, de la misma forma, el bloqueo con caseína seguido de la captura de Biotin-PYY y del conjugado enzimático AP-Strept, da lugar a valores de R_{CT} de 1047 Ω (curva 6), 1355 Ω (curva 7) y 1725 Ω (curva 8), respectivamente. Estos resultados demuestran el éxito en la incorporación de los inmunorreactivos y, por tanto, de la preparación del inmunosensor en las condiciones experimentales elegidas.

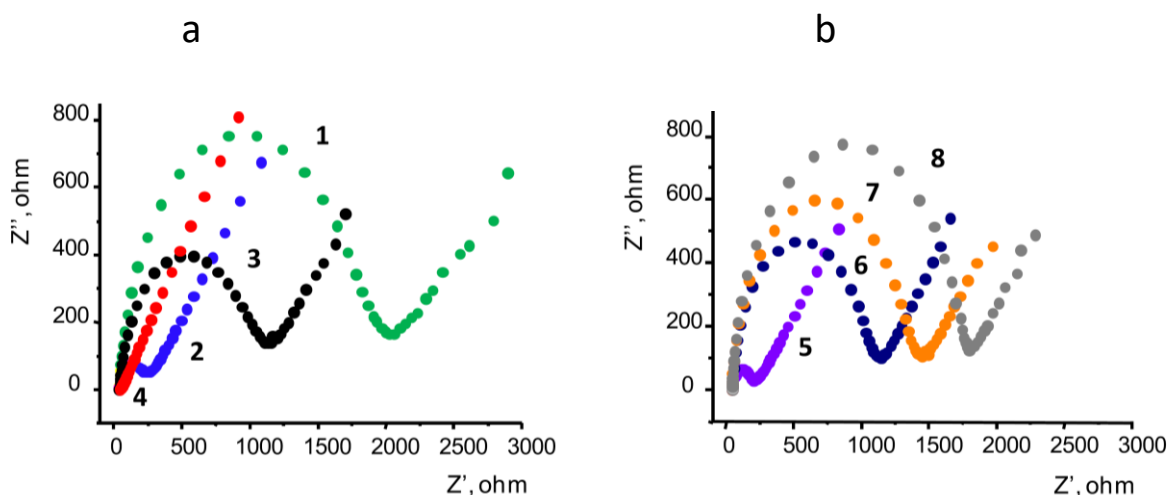


Figura 5.12. Diagramas de Nyquist obtenidos (a) en la etapa de funcionalización del electrodo mediante "grafting" y (b) en las etapas de construcción del inmunosensor: SPAuE sin modificar (1); SPAuE tratado con H_2SO_4 (2); COOH-Phe-N-SPAuE (3); COOH-Phe-N-SPAuE activado con EDC/NHSS 0.1 M (4); anti-PYY-Phe-N-SPAuE (5); caseína/anti-PYY-Phe-N-SPAuE (6); Biotin-PYY-anti-PYY-Phe-N-SPAuE (7); AP-Strept-Biotin-PYY-anti-PYY-Phe-N-SPAuE (8). Condiciones de EIS: número de frecuencia, 50; intervalo de frecuencia, 105 - 0.04 Hz; amplitud 0.01 V. Otras condiciones como en la Tabla 5.1.

Con fines comparativos, en la Figura 5.13 se han representado los espectros de impedancias de los electrodos modificados con 4-MBA (Figura 5.13a) y los correspondientes a las diferentes etapas de preparación del inmunosensor de PYY sobre dichos electrodos (Figura 5.13b). Puede observarse un comportamiento similar al descrito anteriormente cuando se comparan los diagramas del electrodo modificado con 4-MBA antes y después de la activación de los grupos carboxilo con el sistema EDC/NHSS, obteniéndose valores de R_{CT} de 461 Ω (curva 1) y 59 Ω (curva 2), respectivamente. Este comportamiento se debe a los mismos motivos que los ya expuestos. Después, la inmovilización de anti-PYY y el bloqueo posterior con caseína dan lugar a sucesivos incrementos de la resistencia a la transferencia de carga, con R_{CT} = 384 Ω (curva 3) y R_{CT} = 1074 Ω (curva 4), respectivamente. Sin embargo, a diferencia de lo visto anteriormente, la inmovilización de Biotin-PYY provoca solo un débil aumento de R_{CT} (1155 Ω , curva 5) y la del conjugado AP-Strept no origina ningún cambio apreciable, con R_{CT} = 1160 Ω (curva 6). Estos resultados sugieren que la cantidad de Biotin-PYY inmovilizada sobre la plataforma anti-PYY-Phe-S-SPAUE es menor que cuando se utiliza el electrodo modificado por "grafting", lo que hace que posteriormente, la incorporación de AP-Strept no sea significativa.

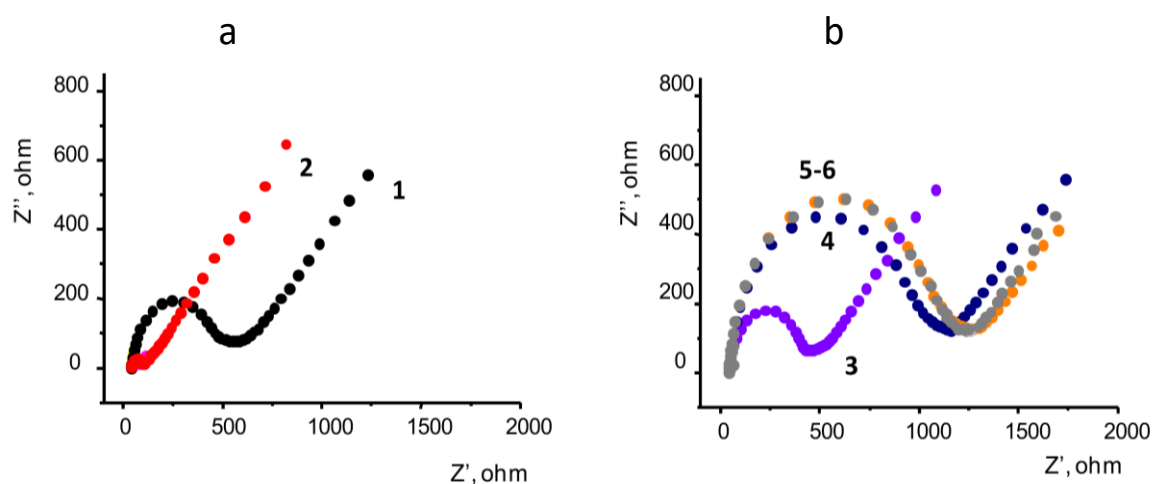


Figura 5.13. Diagramas de Nyquist obtenidos (a) en la etapa de funcionalización del electrodo con 4-MBA y (b) en las etapas de construcción del inmunosensor: 4-MBA-SPAUE (1); 4-MBA-SPAUE activado con EDC/NHSS 0.1 M (2); anti-PYY-Phe-S-SPAUE (3); caseína/anti-PYY-Phe-S-SPAUE (4); Biotin-PYY-anti-PYY-Phe-S-SPAUE (5); AP-Strept-Biotin-PYY-anti-PYY-Phe-S-SPAUE (6). Condiciones de EIS: número de frecuencia, 50; intervalo de frecuencia, 105 - 0.04 Hz; amplitud 0.01 V. Otras condiciones como en la Tabla 5.1

5.1.1.4. Calibrado y características analíticas

El calibrado para la determinación de PYY con el inmunosensor AP-Stept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAuE obtenido en las condiciones experimentales previamente optimizadas se ha representado en la Figura 5.14a. Como era de esperar teniendo en cuenta la configuración de inmunoensayo competitivo desarrollada, la forma de la curva es de tipo sigmoideal decreciente. El ajuste de la relación entre la corriente de pico en DPV y el logaritmo de la concentración de PYY se realizó de acuerdo con un modelo de regresión no lineal en el intervalo de 10^{-9} a 10^6 ng mL⁻¹ ($R^2=0.991$) definido por la siguiente ecuación:

$$i_p = i_{\min} + \frac{i_{\max} - i_{\min}}{1 + \left(\frac{x}{EC_{50}}\right)^{-h}}$$

en la que $i_{\max} = 760 \pm 22$ nA e $i_{\min} = 288 \pm 13$ nA. El valor de EC_{50} , que es la concentración de antígeno que corresponde al cincuenta por ciento de competición, es igual a 0.007 ng mL⁻¹ PYY. Finalmente, la pendiente de Hill, que determina la curvatura del sigmoide [Lu, 2007] es $h = -0.20 \pm 0.04$. Por otro lado, además del ajuste descrito, también se aplicó un tratamiento de ajuste de la curva por regresión logística (logit), representando $\ln(p/1 - p)$ vs $\ln x$, siendo $p = (y - i_{\min})/(i_{\max} - i_{\min})$, lo que permitió obtener la ecuación de una recta, con $R^2 = 0.997$, de pendiente $m = -0.2$, valor que coincide con la pendiente de Hill calculada anteriormente. Por otro lado, la corriente de pico puede relacionarse linealmente con el logaritmo de la concentración de PYY en el intervalo comprendido entre 10^{-6} y 10^3 ng mL⁻¹. En este intervalo, el ajuste por mínimos cuadrados, con $R^2 = 0.990$, proporcionó una pendiente de -49 ± 8 nA por década de concentración.

Como puede verse, el calibrado anterior se compara en la Figura 5.14b con otro calibrado de PYY obtenido con el inmunosensor basado en 4-MBA. Se aprecia que los valores de la corriente de pico son menores que los del calibrado anterior y que el intervalo lineal, con $R^2 = 0.999$, es más corto, extendiéndose desde 10^{-2} hasta 10^2 ng mL⁻¹ PYY. Sin embargo, la pendiente en este intervalo es algo superior a la obtenida anteriormente, con un valor de -89 ± 4 nA por década de concentración.

Las diferencias encontradas entre ambos calibrados se deben probablemente a la menor cantidad de anticuerpo de captura, anti-PYY, inmovilizado sobre el electrodo preparado con 4-MBA ya que, como es sabido, en las configuraciones de ensayo

competitivo, una menor concentración de este anticuerpo origina un aumento de la sensibilidad acompañado de un acortamiento del intervalo lineal ya que se requiere una menor concentración de antígeno para alcanzar la respuesta máxima [Ruth, 2001] [Eguílaz, 2010]. Cabe destacar, además, la baja reproducibilidad de las respuestas de corriente obtenidas con este inmunosensor. En efecto, las medidas repetidas de disoluciones de 0.01 ng mL^{-1} PYY realizadas con cinco inmunosensores preparados en diferentes días proporcionaron un valor de RSD del 17.5 %, resultado muy superior al obtenido con la configuración preparada por "grafting", RSD = 4.6%. Esta escasa precisión se atribuye a la baja estabilidad de la monocapa de 4-MBA sobre el electrodo en el ambiente húmedo en que se realizan las incubaciones para preparar el inmunosensor. Como se señaló en la Introducción, el enlace -N-Au generado en el proceso de "electrografting" oxidativo compite favorablemente con el -S-Au del grupo tiol [Fagas, 2007].

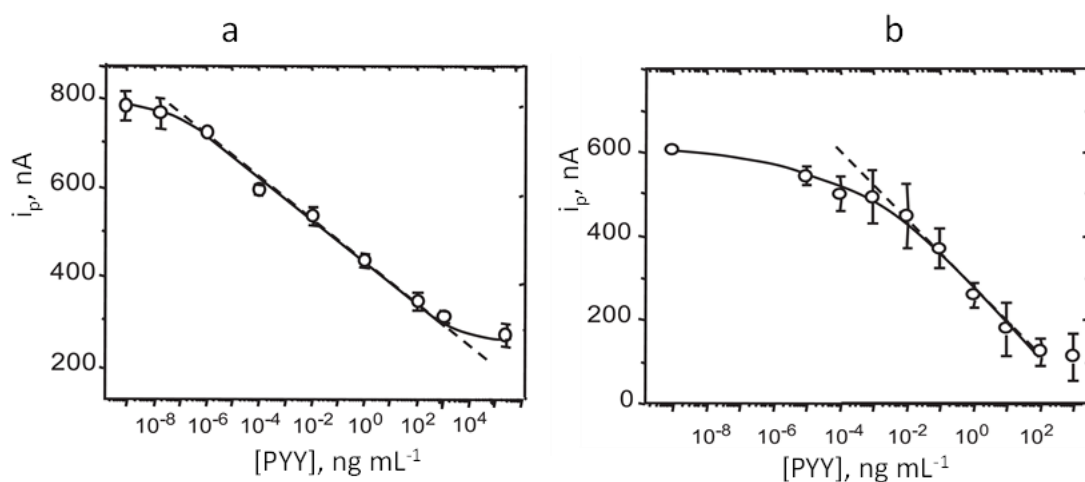


Figura 5.14. Calibrados para la determinación de PYY con los inmunosensores AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE (a) y AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-S-SPAUE (b). Las barras de error se han estimado como el triple de la desviación estándar ($n=3$).

El límite de detección del método desarrollado con el inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE se calculó como la concentración de analito para el que la señal voltamperométrica se reducía en un 10%. Con este criterio, el valor estimado fue: $LOD = 3 \times 10^{-7} \text{ ng mL}^{-1}$. Además, este resultado se comparó con el calculado a partir de la ecuación [Lu, 2007]:

$$LOD = EC_{50} \{ [i_{m\acute{a}x} - i_{m\acute{i}n}) / (i_{m\acute{a}x} - i_{m\acute{i}n} - 3s)] - 1 \}^{-1/h}$$

en la que s es la desviación estándar de las medidas repetidas de diez disoluciones preparadas en ausencia de PYY, $s = \pm 20$ nA, obteniéndose: $\text{LOD} = 5 \times 10^{-7} \text{ ng mL}^{-1}$, resultado que concuerda con el anterior. Señalar también que aplicando el primer criterio al calibrado obtenido con el inmunosensor basado en el electrodo 4-MBA-SPAUE, el límite de detección resultó ser aproximadamente igual a $10^{-5} \text{ ng mL}^{-1}$ PYY, concentración doscientas veces mayor que el LOD obtenido anteriormente.

El intervalo lineal del calibrado establecido con el inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE y el límite de detección alcanzado se compararon con los que presentan varias configuraciones de inmunoensayo tipo ELISA y con los inmunosensores descritos hasta el momento para esta hormona haciendo uso de los datos presentados en la Tabla 3.5.4. de la Introducción. En relación a los kits ELISA, la mayoría de los calibrados semilogarítmicos obtenidos abarcan intervalos comprendidos entre algunas decenas y miles de pg mL^{-1} con límites de detección de décimas o unidades de pg mL^{-1} , por lo que estas características son mejores en el inmunosensor desarrollado en este trabajo.

Por otro lado, en el caso de los inmunosensores, solo existen dos configuraciones, ambas puestas a punto en el Grupo de Investigación [Guerrero, 2015] [Martínez-García, 2016], basados igualmente en un esquema de inmunoensayo competitivo. Es de destacar la mayor amplitud del intervalo lineal que se obtuvo con el inmunosensor descrito en esta Memoria, 10^{-6} - 10^3 ng mL^{-1} frente a 10^{-4} - 10^2 ng mL^{-1} [Guerrero, 2015] o 10^{-4} - 10 [Martínez-García, 2016], así como el límite de detección, que es más de un orden de magnitud inferior. Otra característica a destacar es el menor tiempo de ensayo requerido cuando se utiliza el inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE. En efecto, desde la incubación de anticuerpo de captura, los tiempos necesarios suman un total de 90 min, que es muy inferior a las más de tres o casi cinco horas que se necesitan en la mayor parte de los kits ELISA, y media hora más breve que el tiempo de ensayo de los inmunosensores descritos anteriormente.

5.1.1.5. Estabilidad y selectividad

El estudio de la estabilidad se realizó sobre una batería de conjugados anti-PYY-Phe-N-SPAUE preparados en un mismo día y almacenados a 4°C en la oscuridad y con humedad controlada. Después, cada día de medida se preparaban los inmunosensores en las condiciones óptimas establecidas anteriormente, con 100 ng mL^{-1} Biotin-PYY,

registrando los voltamperogramas en diferencial de impulsos. La Figura 5.15 muestra un gráfico de control en el que se fijaron los límites a $\pm 3s$, donde s es la desviación estándar de las medidas ($n=10$) realizadas el primer día de trabajo (día 0). Como puede apreciarse, fue posible obtener respuestas de corriente dentro de los límites de control establecidos durante 10 días. Este resultado representa una aceptable estabilidad de almacenamiento.

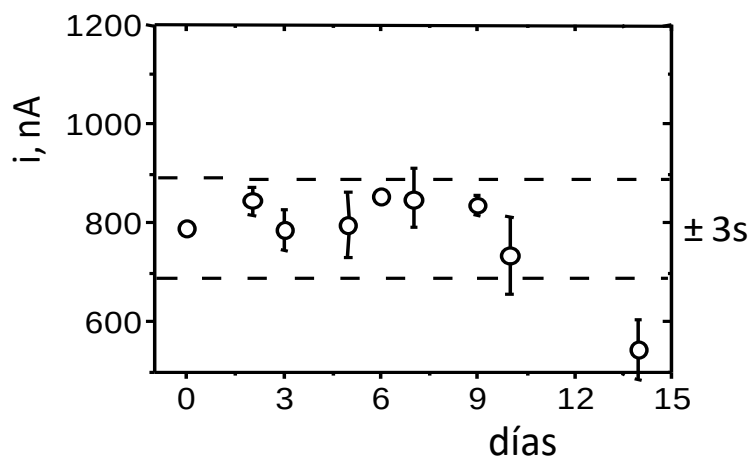


Figura 5.15. Gráfico de control para evaluar la estabilidad de almacenamiento de los conjugados anti-PYY-Phe-N-SPAUE a 4 °C. Ver el texto para más información.

Para estudiar la selectividad del inmunosensor desarrollado se investigó el efecto sobre la respuesta voltamperométrica de la presencia de otras proteínas que pueden acompañar al PYY en sus muestras clínicas. Las especies ensayadas fueron: hormona del crecimiento humana (hGH), leptina (Lep), grelina (GHRL), adiponectina (APN), hemoglobina (HB) y factor de crecimiento transformante $\beta 1$ (TGF- $\beta 1$), todas ellas a un nivel de concentración de 100 ng mL⁻¹. En la Figura 5.16 se han representado las corrientes de pico obtenidas con el inmunosensor al medir distintas disoluciones de 100 ng mL⁻¹ de Biotin-PYY en presencia de distintos potenciales interferentes, que se comparan con la respuesta de la disolución que no contenía ninguna de estas proteínas, en ambos casos en ausencia de PYY. Como puede verse, todas las respuestas aparecen dentro de los límites del gráfico de control establecidos como $\pm 3s$, donde s es la desviación estándar de tres medidas realizadas con el inmunosensor, no apreciándose, además, diferencias significativas entre las señales obtenidas, lo que pone de manifiesto la elevada selectividad del diseño propuesto para la determinación de PYY.

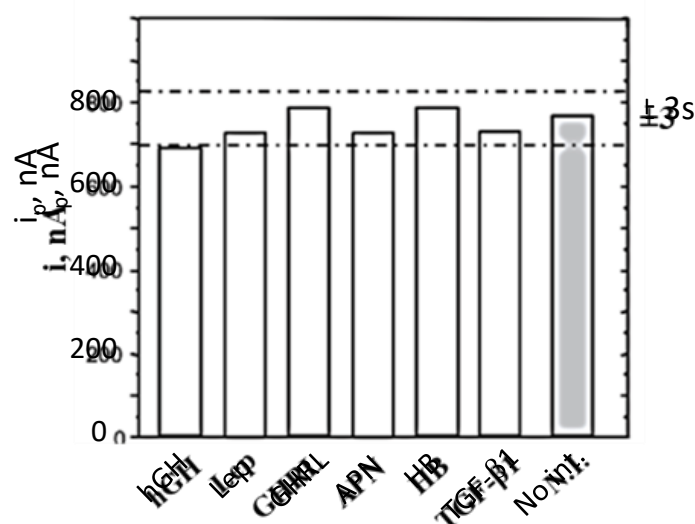


Figura 5.16. Efecto de la presencia de 100 ng mL^{-1} de hGH, Lep, GHRL, APN, HB y TGF- β 1 en la respuesta del inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE en ausencia de PYY.

5.1.1.6. Determinación de PYY en suero y saliva

Para demostrar la utilidad analítica del inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N/SPAUE, el método desarrollado se aplicó al análisis de dos muestras de interés clínico: suero y saliva humanas. Es importante señalar que no se encontraron muestras que contuvieran PYY, por lo que no fue posible determinar el contenido endógeno en muestras reales y hubo que realizar estudios de recuperación sobre muestras contaminadas según el procedimiento descrito en la Parte Experimental (Apartado 4.4.4.2). Con el fin de ajustar las concentraciones de hormona en las muestras a las que cabe esperar en suero y saliva reales, se realizó una búsqueda bibliográfica amplia, encontrándose una gran variabilidad en los datos recogidos en los diferentes estudios acerca de los niveles de PYY esperables en muestras biológicas, ya que dichos niveles dependen de varios factores, principalmente el sexo y BMI del individuo, así como del momento de extracción de la muestra en relación al periodo de ingesta. Sin embargo, algunos estudios relativamente recientes sitúan los niveles de referencia en torno a 100 pg mL^{-1} PYY en suero [Cahill, 2014] y $15 - 75 \text{ pg mL}^{-1}$ PYY en saliva [Acosta, 2011] de individuos sanos. Como puede apreciarse, el intervalo lineal i_p vs $\log [\text{PYY}]$ de concentraciones que abarca el calibrado, comprendido entre 10^{-6} y 10^3 ng mL^{-1} , es adecuado para llevar a cabo la determinación de PYY en ambos tipos de muestras.

Antes de proceder a la determinación, se investigó la posible existencia de efecto matriz comparando las pendientes de los calibrados en suero y saliva contaminadas con PYY y diluidas 10 veces con la del calibrado de patrones. Las pendientes de los calibrados en las muestras, en el intervalo comprendido entre 10^{-4} y 10^2 PYY, fueron -48 ± 7 (suero) y -43 ± 6 (saliva) nA por década de concentración, valores similares al de calibrado de patrones -49 ± 8 nA por década de concentración. La comparación estadística de dichas pendientes se realizó mediante el ensayo de t-Student, obteniendo un valor de $t_{\text{exp}} = 0.215$ en suero y $t_{\text{exp}} = 1.227$ en saliva, ambos inferiores al valor teórico $t_{\text{tab}} = 2.228$ ($n=6$; $\alpha=0.05$), por lo que no existen diferencias significativas entre ellas, descartándose la existencia de efecto matriz en las muestras diluidas diez veces. Seguidamente, se realizaron los estudios de recuperación de PYY en suero y en saliva contaminados con 100 pg mL^{-1} o 35 pg mL^{-1} , respectivamente, por interpolación de las respuestas obtenidas en DPV en el calibrado de disoluciones estándar. Los resultados obtenidos se recogen en la Tabla 5.2, en la que se observa que los valores medios de los porcentajes de recuperación fueron del 96% en suero y 99% en saliva, lo que pone de manifiesto la utilidad analítica de la plataforma desarrollada para el análisis de PYY en este tipo de muestras.

Tabla 5.2. Determinación de PYY en suero y saliva contaminados empleando el inmunosensor AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE.

Muestra	PYY, pg mL^{-1}	Recup. %	Muestra	PYY, pg mL^{-1}	Recup. %
Suero 1	99.6; 102	101 ± 3	Saliva 1	36; 33	100 ± 3
Suero 2	102; 95,0	99 ± 5	Saliva 2	36; 33	99 ± 4
Suero 3	105; 101	103 ± 3	Saliva 3	32; 35	96 ± 4
Suero 4	102; 99.4	101 ± 3	Saliva 4	35; 36	102 ± 1
Suero 5	99.6; 101	100 ± 2	Saliva 5	35; 36	97 ± 2
Suero 6	102; 102	101 ± 2	Saliva 6	35; 37	102 ± 3
Suero 7	100; 98.8	99 ± 2	Saliva 7	34; 35	99 ± 2

5.1.1.7. Conclusiones

En este trabajo se ha desarrollado un inmunosensor electroquímico para la determinación de PYY basado en la técnica de "grafting" oxidativo de *p*-ABA sobre electrodos serigrafados de oro (SPAuEs) que implica la inmovilización covalente del anticuerpo de captura, anti-PYY, y el establecimiento de un esquema de inmunoensayo competitivo directo entre el antígeno PYY y el antígeno marcado con biotina, Biotin-PYY. Esta configuración se ha comparado con el inmunosensor equivalente basado en la preparación de una monocapa de 4-MBA sobre SPAuEs, demostrándose que las características analíticas del primer diseño son mejores en términos de amplitud del intervalo de linealidad, límite de detección y reproducibilidad, requiriéndose además mucho menos tiempo para la preparación de la plataforma electroquímica.

Otras características ventajosas del inmunosensor preparado por "grafting", como la elevada sensibilidad, la estabilidad del conjugado anti-PYY-Phe-N-SPAuE y la alta selectividad han permitido aplicar el método desarrollado al análisis de suero y saliva contaminados con PYY a los niveles normales presentes en estas muestras, obteniéndose buenos resultados sin necesidad de tratamiento previo excepto dilución 1/10.

5.1.2. Inmunosensor para la determinación de la citoquina TGF- β 1

5.1.2.1. Configuración del inmunosensor

Las etapas implicadas en la preparación de este inmunosensor se describieron en el Apartado 4.4.1. de la Parte Experimental, representándose esquemáticamente en la Figura 4.9. Algunas de estas etapas son similares a las aplicadas en la configuración anterior, principalmente en lo que se refiere a la modificación del SPAuE mediante "grafting" oxidativo en presencia de *p*-ABA, la inmovilización del anticuerpo de captura anti-TGF y la detección. Sin embargo, en este caso se estableció una configuración de inmunoensayo tipo sándwich empleando TGF- β 1 y Biotin-anti-TGF.

La Figura 5.17 muestra un esquema del inmunosensor y del modo de detección que, igualmente, implica la medida en DPV de la señal de oxidación del 1-naftol (NP), producto de la reacción enzimática del sustrato de la fosfatasa alcalina, 1-naftilfosfato (NPP).

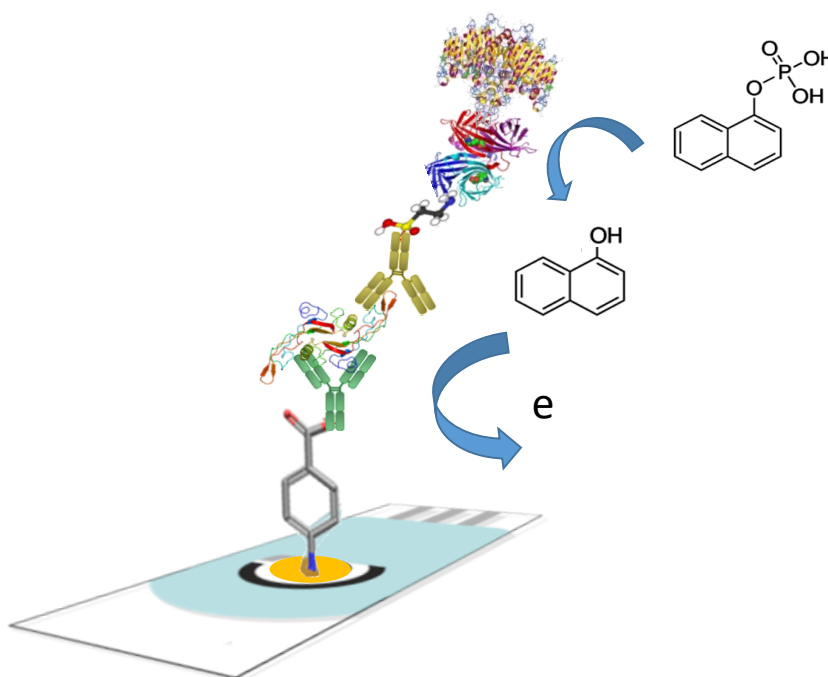


Figura 5.17. Esquema del inmunosensor AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-Phe-N-SPAuE y de las reacciones implicadas en la detección

5.1.2.2. Optimización de las variables experimentales

En el capítulo anterior se optimizaron las variables implicadas en la funcionalización del electrodo mediante "grafting" por voltamperometría cíclica (concentración de *p*-ABA, intervalo de potencial, número de ciclos y velocidad de barrido de potencial) para la preparación del inmunosensor competitivo para PYY. Aunque el electrodo y la técnica utilizados son los mismos, sin embargo, la diferente configuración del inmunoensayo, de tipo sándwich frente a la competitiva anterior, hace que algunas de estas variables puedan afectar en mayor o menor medida a la respuesta electroquímica. Por ello, se fijó el intervalo de potencial barrido (0.0 - +0.6 V vs Ag) elegido anteriormente y se optimizaron nuevamente las demás. Por otro lado, también se estudió la influencia de las variables implicadas en la construcción y funcionamiento del inmunosensor AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-Phe-N-SPAuE sobre la respuesta voltamperométrica en DPV: concentración y tiempo de incubación del anticuerpo de captura, anti-TGF; naturaleza, concentración y tiempo de incubación del agente bloqueante; concentración y tiempo de incubación del anticuerpo de detección biotinilado, Biotin-anti-TGF, y concentración y tiempo de incubación del conjugado enzimático AP-Strept. Las demás variables relacionadas con la activación de los grupos carboxilo, la reacción enzimática y la detección fueron las mismas usadas anteriormente. Los resultados obtenidos en estos estudios se describen a continuación, resumiéndose finalmente en la Tabla 5.3.

Influencia de la concentración de p-ABA

Este estudio se realizó midiendo la respuesta del inmunosensor para TGF- β 1 preparado con electrodos modificados en presencia de diferentes concentraciones de *p*-ABA comprendidas entre 0.73 y 3.6 mM (Figura 5.18). Los resultados obtenidos revelan un comportamiento parecido al encontrado en el capítulo anterior, destacando la mayor relación S/N para *p*-ABA 1.5 mM, que coincide con la concentración elegida en el caso del PYY. En estas condiciones, la respuesta específica del inmunosensor es la mayor de todo el intervalo estudiado, probablemente porque la cantidad de anti-TGF inmovilizada es la más alta. Concentraciones menores son insuficientes para lograr una adecuada modificación del electrodo mientras que concentraciones superiores a esta provocan, aparentemente, un efecto de saturación. Estos resultados llevaron a elegir la concentración de *p*-ABA citada como óptima para estudios posteriores.

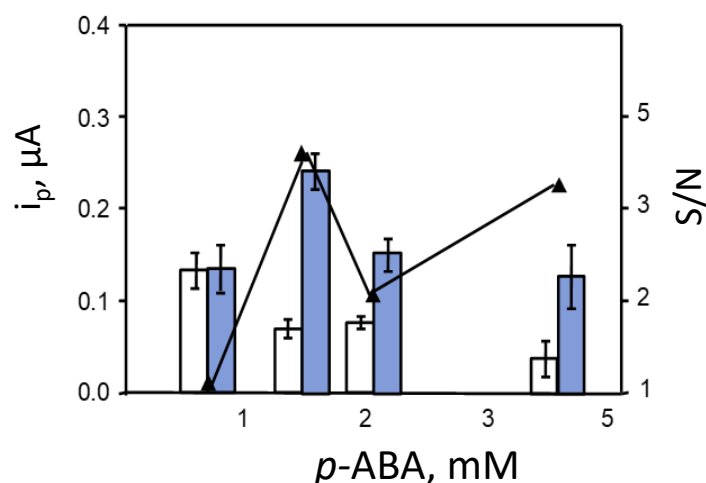


Figura 5.18. Influencia de la concentración de *p*-ABA usada en el proceso de "grafting" sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL⁻¹ de TGF- β 1 (●) inc. 30 min. 0.0 - +0.6 V; ν = 30 mV s⁻¹; n = 3; 10 μ g mL⁻¹ anti-TGF, inc. 30 min; 1 % caseína (BB), inc. 30 min; 2 μ g mL⁻¹ Biotin-anti-TGF, inc. 60 min; 5 μ g mL⁻¹ AP-Strept, inc. 30 min. Detección: 1-NPP 5 mM, 5 min reacción; DPV: -0.2 - +0.7 V vs. Ag; ΔE = 50 mV; ν = 10 mVs⁻¹. Barras de error estimadas como el triple de la desviación estándar (n=3).

Influencia del número de ciclos

En las condiciones experimentales anteriores se estudió la respuesta del inmunosensor al aplicar entre 1 y 7 ciclos de potencial sucesivos mediante CV, con los resultados representados en la Figura 5.19. De un modo similar a lo observado en el caso del inmunosensor de PYY, la señal específica disminuye al aumentar el número de barridos a partir de tres ciclos consecutivos. Como se sugirió, probablemente la aplicación de un único ciclo proporciona pocos grupos carboxilo disponibles para la inmovilización del anticuerpo de captura, mientras que un número elevado origina muchas posiciones de unión que pueden dar lugar a impedimentos estéricos y, por tanto, a una pérdida de efectividad del proceso de inmovilización del anticuerpo. Por otro lado, respecto a las respuestas inespecíficas, la que se obtiene al aplicar un único barrido de potencial es la de mayor magnitud, debido probablemente a que quedan vacantes en el electrodo sobre las que pueden darse interacciones no deseadas. Después se observa una importante disminución de la corriente al aplicar tres barridos de potencial, pero a partir de este punto las señales inespecíficas tienden a aumentar ligeramente con el número de ciclos, siendo este comportamiento contrario al observado en la configuración anterior. Esta tendencia se atribuye a la diferente composición del inmunosensor: en la configuración de tipo

sándwich, en ausencia de antígeno, la deficiente inmovilización del anticuerpo de captura puede dar lugar a un incremento de la cantidad de anticuerpo biotinilado sobre el electrodo que interaccionaría con el conjugado AP-Strept. Esto no ocurre en la configuración competitiva del inmunosensor de PYY, ya que en este caso las señales inespecíficas se miden en ausencia de péptido biotinilado. Como consecuencia, en este caso, la mayor relación S/N se obtiene al aplicar tres ciclos de potencial, que será lo que se utilice para la preparación del inmunosensor.

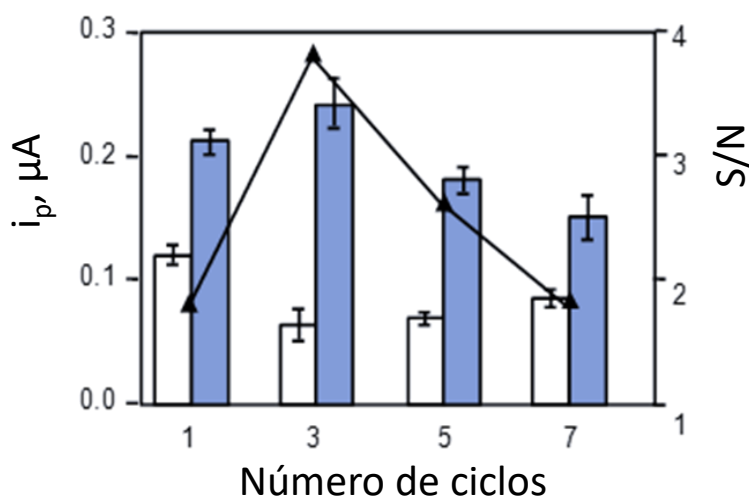


Figure 5.19. Influencia del número de ciclos aplicados en el proceso de "grafting" sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL^{-1} de TGF- $\beta 1$. Condiciones: *p*-ABA 1.5 mM , $v = 30 \text{ mV/s}$; $0.0 \text{ V} - (+) 0.6 \text{ V}$. Otras condiciones como en la Figura 5.18.

Influencia de la velocidad de barrido de potencial

La Figura 5.20 muestra los resultados obtenidos en el estudio del efecto de la velocidad de barrido de potencial en la etapa de "electrografting" sobre la respuesta del inmunosensor. En el margen evaluado, entre 5 y 100 mV s^{-1} , se observa un máximo de la relación S/N para 30 mV s^{-1} , que coincide con el elegido en el caso anterior. Aquí también se observa un aumento de las respuestas específicas hasta esta velocidad, aunque a continuación estas no se estabilizan, como ocurría en el inmunosensor de PYY, sino que disminuyen progresivamente. Por otro lado, y también de un modo diferente al caso anterior, las corrientes inespecíficas tienden a aumentar ligeramente en todo el intervalo estudiado. Teniendo en cuenta la influencia de esta variable en la generación y distribución de los radicales del *p*-ABA sobre el electrodo, este comportamiento puede

explicarse, como ya se explicó anteriormente, considerando que velocidades de barrido lentas darían lugar a un proceso de "grafting" ineficaz, mientras que altas velocidades pueden originar una distribución no homogénea y desordenada de los radicales generados sobre el electrodo. Sin embargo, el hecho de que en esta configuración la tendencia de las señales específicas e inespecíficas a partir de 30 mV s^{-1} sea distinta la observada en el inmunosensor anterior se atribuye nuevamente a la diferente composición de este diseño: en el de tipo sándwich, la funcionalización imperfecta de la superficie del electrodo da lugar a una menor carga de anticuerpos de captura, lo que explica la disminución de las respuestas en presencia de TGF- β 1, mientras que las inespecíficas aumentan al emplear altas velocidades de barrido de potencial debido probablemente a la presencia de anticuerpo biotinilado sobre el electrodo. Al igual que se indicó, este efecto no se aprecia en el inmunosensor competitivo de PYY debido a la ausencia de Biotin-PYY.

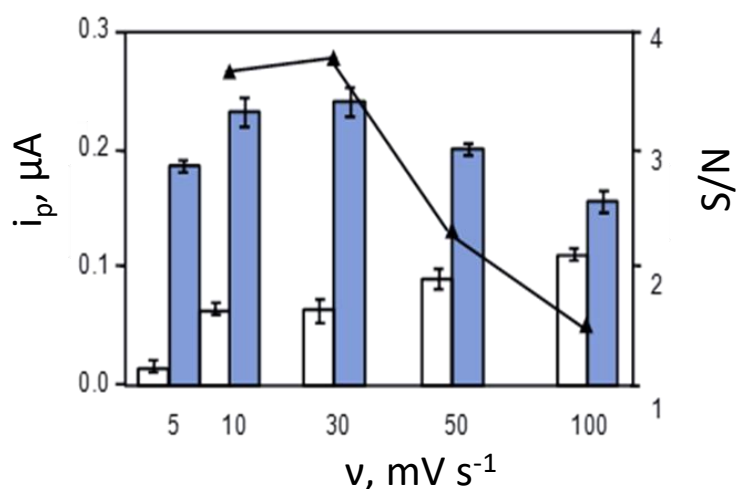


Figure 5.20. Influencia de la velocidad de barrido de potencial aplicada en el proceso de "grafting" sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL^{-1} de TGF- β 1. Condiciones: p -ABA 1.5 mM , $n = 3$; $0.0 \text{ V} - + 0.6 \text{ V}$. Otras condiciones como en la Figura 5.18.

Influencia de la concentración y el tiempo de incubación de anti-TGF

La concentración del anticuerpo de captura influye decisivamente en la respuesta del inmunosensor y para su optimización se mantuvieron las condiciones seleccionadas previamente, investigando la variación de la corriente en DPV en el intervalo de 2.5 a $15 \mu\text{g mL}^{-1}$ anti-TGF. Tal como se observa en la Figura 5.21a, las respuestas específicas aumentan ligeramente con la concentración de anticuerpo como consecuencia del aumento de posiciones de unión para el antígeno. Sin embargo, esta tendencia cambia a partir de una concentración de $10 \mu\text{g mL}^{-1}$ de anti-TGF debido probablemente a un efecto

de saturación. Por otro lado, las señales inespecíficas disminuyen de forma continua en todo el intervalo estudiado, probablemente debido a la menor conductividad del electrodo en presencia de una alta concentración de biomoléculas. Estos resultados llevaron a elegir la concentración citada, que proporciona la mayor relación S/N, como la más adecuada para la preparación del inmunosensor. En cuanto al tiempo de incubación de anti-TGF, los resultados representados en la Figura 21b permiten apreciar un ligero aumento de las respuestas específicas en el intervalo de 15 a 45 min, disminuyendo después para el periodo de 60 min. En este caso, las señales inespecíficas no varían apreciablemente en todo el margen de tiempos estudiado. Teniendo en cuenta que las corrientes específicas a 30 y 45 min son muy parecidas, y considerando el corto tiempo de análisis como una ventaja importante, se eligió un periodo de incubación óptimo de 30 min.

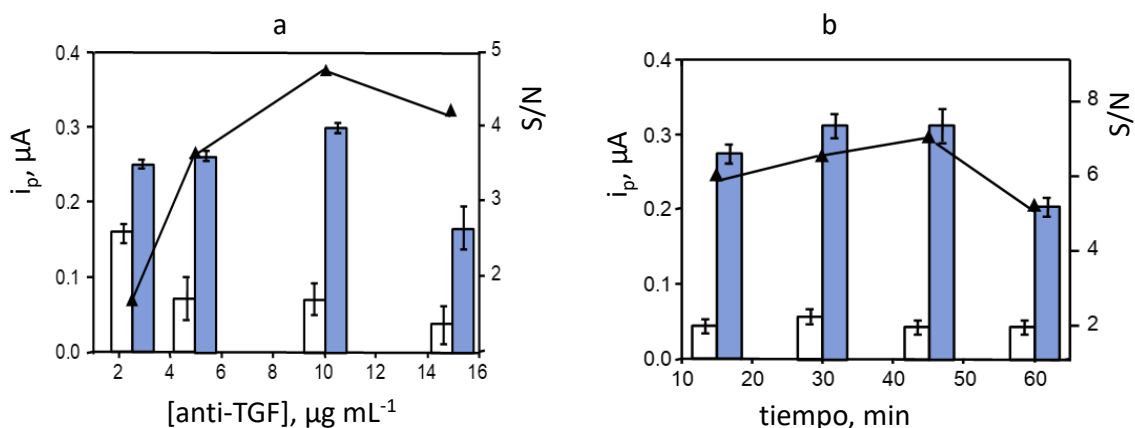


Figura 5.21. Influencia de la concentración (a) y del tiempo de incubación (b) de anti-TGF sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL^{-1} de TGF- $\beta 1$. Condiciones: $p\text{-ABA } 1.5 \text{ mM}$, $n = 3$; $0.0 \text{ V} - +0.6 \text{ V}$; $v = 30 \text{ mV s}^{-1}$; $15 \mu\text{g mL}^{-1}$ anti-TGF (b) inc. 30 min (a). Otras condiciones como en la Figura 5.18.

Influencia de la naturaleza del agente bloqueante

Con el objetivo de minimizar las adsorciones inespecíficas sobre el electrodo, se investigó el funcionamiento del inmunosensor de TGF- 1β utilizando como agentes bloqueantes los reactivos estudiados en el capítulo anterior: etanolamina, BSA y caseína (BB), empleando una concentración del 1% en todos los casos (Figura 5.22). Al igual que anteriormente, también ahora se observó un comportamiento anómalo en presencia de etanolamina, obteniéndose corrientes muy elevadas tanto en las respuestas específicas como en las inespecíficas. Como se señaló, este efecto puede atribuirse a la inestabilidad del electrodo recubierto con este bloqueante. Puede verse que la mejor relación S/N se obtuvo también en esta configuración cuando se empleaba caseína, por lo que fue elegida para la preparación del inmunosensor.

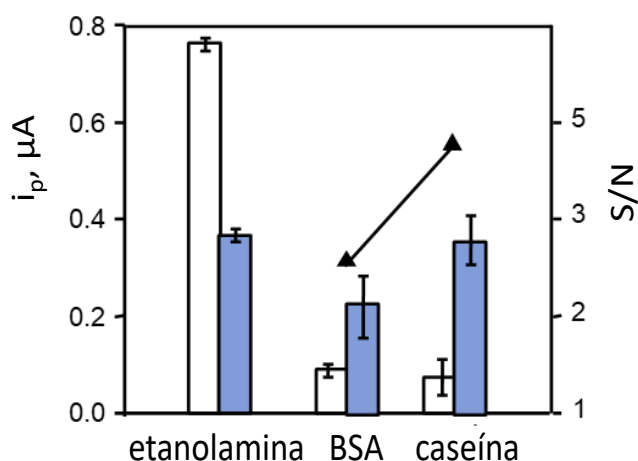


Figura 5.22. Influencia de la naturaleza del agente bloqueante sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL^{-1} de TGF- $\beta 1$. Condiciones: p -ABA 1.5 mM , $n = 3$; $0.0 \text{ V} - +0.6 \text{ V}$; $v = 30 \text{ mV s}^{-1}$. $15 \text{ } \mu\text{g mL}^{-1}$ anti-TGF, inc. 30 min. Otras condiciones como en la Figura 5.18.

Influencia de la concentración y el tiempo de incubación de la caseína

Como se muestra en la Figura 5.23a, a partir de un 0.2%, las señales específicas tienden a aumentar al hacerlo la concentración de caseína, mientras que se van reduciendo las respuestas inespecíficas como consecuencia de un mejor bloqueo del electrodo. Teniendo en cuenta este efecto, se eligió el 1% de caseína para preparar el inmunosensor. Con respecto al tiempo de incubación del agente bloqueante (Figura 5.23b), el efecto de esta variable se estudió en el intervalo de 15 a 90 min. Se observa que periodos inferiores a 30 min no son suficientes para asegurar un buen bloqueo, mientras que cuando este tiempo es excesivo, tiene lugar una notable disminución de la respuesta específica debido probablemente a la presencia de una gran cantidad de material aislante en la superficie del electrodo. Por ello, se eligió el tiempo de incubación indicado, que es el que proporciona la mayor relación S/N. Cabe destacar que, en las condiciones elegidas, la respuesta específica del inmunosensor es cuatro veces la señal inespecífica, por lo que la reacción de formación del inmunocomplejo puede detectarse con una alta sensibilidad.

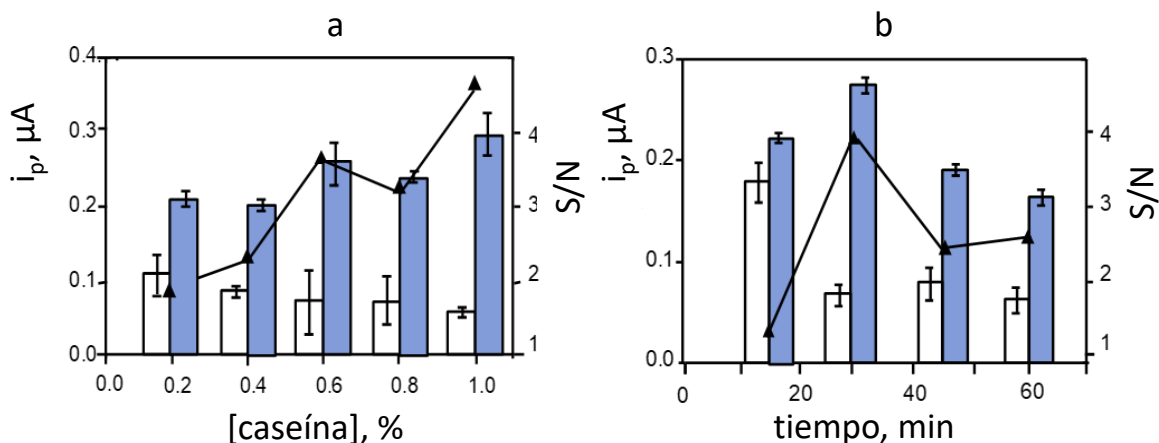


Figura 5.23. Influencia de la concentración (a) y del tiempo de incubación (b) de la caseína sobre la respuesta del inmutosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL⁻¹ de TGF- β 1. Condiciones: *p*-ABA 1.5 mM, *n* = 3; 0.0 - + 0.6 V; *v* = 30 mV s⁻¹. 15 μ g mL⁻¹ anti-TGF, inc. 30 min; 1% caseína (b), 30 min (a). Otras condiciones como en la Figura 5.17.

Influencia de la concentración y el tiempo de incubación de Biotin-anti-TGF

En las condiciones optimizadas previamente, se estudió el efecto sobre la respuesta del inmutosensor de la concentración del anticuerpo secundario, Biotin-anti-TGF, en el intervalo de 0.5 a 5 μ g mL⁻¹, obteniendo los resultados que aparecen representados en la Figura 5.24a. Como puede observarse, para lograr una buena respuesta específica se requiere al menos una concentración de 2 μ g mL⁻¹ de anticuerpo biotinilado. Concentraciones menores proporcionan corrientes más bajas, mientras que, a partir de este valor, las señales específicas prácticamente se estabilizan, pero las respuestas inespecíficas tienden a aumentar ligeramente, lo que provoca la disminución de la relación S/N. Estos resultados llevaron a elegir la concentración citada como óptima para estudios posteriores. Por otro lado, según se observa en la Figura 5.24b, las respuestas específicas del inmutosensor aumentan con el tiempo de incubación entre 15 y 60 min, del mismo modo que lo hacen, aunque más ligeramente, las respuestas inespecíficas en todo el intervalo estudiado. Sin embargo, cuando se mantiene un periodo de incubación de 90 min, la señal decae, disminuyendo la relación S/N. Debido a esta tendencia, se escogió un tiempo de 60 min para la incubación de Biotin-anti-TGF para estudios posteriores.

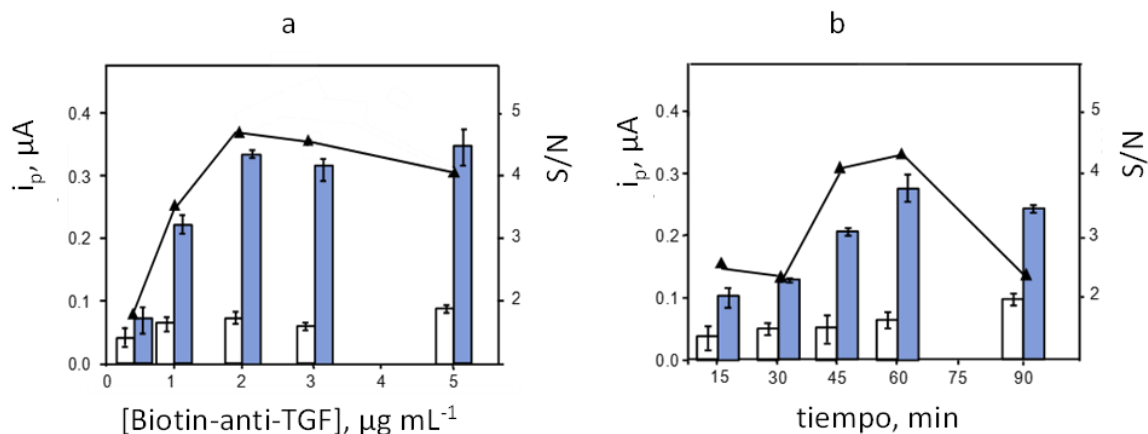


Figura 5.24. Influencia de la concentración (a) y del tiempo de incubación (b) de Biotin-anti-TGF sobre la respuesta del inmutosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL^{-1} de TGF- $\beta 1$. Condiciones: p -ABA 1.5 mM , $n = 3$; $0.0 \text{ V} - + 0.6 \text{ V}$; $v = 30 \text{ mV s}^{-1}$; $15 \mu\text{g mL}^{-1}$ anti-TGF, inc. 30 min ; 1% caseína, inc. 30 min ; $2 \mu\text{g mL}^{-1}$ Biotin-anti-TGF (b), inc. 60 min (a). Otras condiciones como en la Figura 5.18.

Influencia de la concentración y el tiempo de incubación de AP-Strept

Se evaluó la influencia sobre la respuesta del inmutosensor de la concentración del conjugado AP-Strept en el intervalo de concentraciones comprendido entre 1 y $10 \mu\text{g mL}^{-1}$ con los resultados que se muestran en la Figura 5.25a, en la que se observa el aumento de la señal específica hasta $5 \mu\text{g mL}^{-1}$, disminuyendo a concentraciones superiores. Esta disminución, debida probablemente a la saturación de los puntos de unión en el electrodo, se produce a la vez que aumentan las señales inespecíficas, lo que origina la rápida disminución de la relación S/N. En cuanto al tiempo de incubación, el estudio de la influencia de esta variable (Figura 5.25b), hizo posible apreciar el incremento de la señal específica del inmutosensor hasta un periodo de 30 min , permaneciendo a continuación prácticamente constante. Este resultado indica que es suficiente con mantener el periodo de incubación citado para obtener la máxima cantidad de conjugado en el electrodo.

En la Tabla 5.3 se resumen los estudios de optimización realizados, indicando la variable y el intervalo estudiado, así como el valor elegido en cada caso.

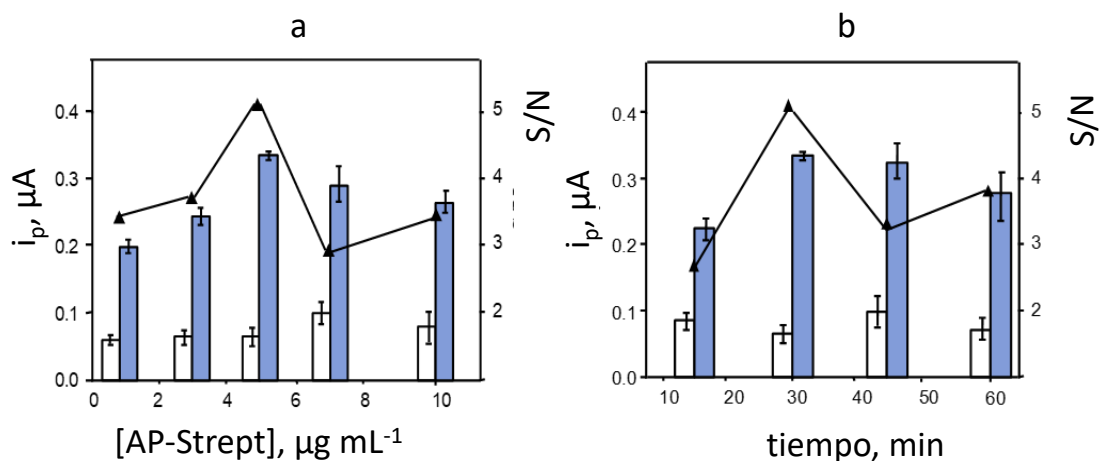


Figura 5.25. Influencia de la concentración (a) y del tiempo de incubación (b) de AP-Strept sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (azul) de 1 ng mL^{-1} de TGF- $\beta 1$. Condiciones: p -ABA 1.5 mM , $n = 3$; $0.0 \text{ V} - +0.6 \text{ V}$; $v = 30 \text{ mV s}^{-1}$; $15 \text{ } \mu\text{g mL}^{-1}$ anti-TGF, inc. 30 min; 1% caseína, inc. 30 min; $2 \text{ } \mu\text{g mL}^{-1}$ Biotin-anti-TGF, inc. 60 min; AP-Strept $5 \text{ } \mu\text{g mL}^{-1}$ (b), 30 min (a). Otras condiciones como en la Figura 5.18.

Tabla 5.3. Optimización de las variables experimentales.

Variable	Intervalo estudiado	Valor seleccionado
Concentración de p -ABA, mM	0.73 - 3.6	1.5
Intervalo de potencial, V	-	0.0-+0.6
Número de ciclos	1 - 7	3
Velocidad de barrido, mV s^{-1}	5 - 100	30
Concentración anti-TGF, $\mu\text{g mL}^{-1}$	2.5 - 15	10
Tiempo incubación anti-TGF, min	15 - 60	30
Agente bloqueante	etanolamina, BSA, caseína	caseína
Concentración caseína, %	0.2 - 1	1
Tiempo incubación caseína, min	15 - 60	30
Concentración Biotin-anti-TGF, $\mu\text{g mL}^{-1}$	0.5 - 5	2
Tiempo incubación Biotin-anti-TGF, min	15 - 90	60
Concentración AP-Strept, $\mu\text{g mL}^{-1}$	1 - 10	5
Tiempo incubación AP-Strept	15 - 60	30

5.1.2.3. Estudios de caracterización

Las etapas de funcionalización del electrodo de oro y las de preparación del inmunosensor se caracterizaron por aplicación de las técnicas de voltamperometría cíclica (CV) y espectroscopia de impedancia electroquímica (EIS) empleando una disolución 5 mM de $\text{Fe}(\text{CN})_6^{3-/4-}$ en KCl 0.1 M como sonda redox. En la Figura 5.26a se muestran los voltamperogramas cíclicos del electrodo desnudo, tratado con H_2SO_4 , y funcionalizado por "grafting" oxidativo. Como era de esperar, el electrodo sin modificar al que no se le aplicó pretratamiento (1) exhibe un peor comportamiento electroquímico con respecto al electrodo tratado con H_2SO_4 : las corrientes de los picos de oxidación y de reducción son menores y la separación pico anódico-pico catódico (ΔE) es mayor. Como referencia, se midieron los siguientes valores: $\Delta E = 286 \text{ mV}$; $i_{\text{pa}} = 50 \mu\text{A}$. Sin embargo, el tratamiento de limpieza y activación con H_2SO_4 (2) da lugar a un comportamiento más reversible con un valor de ΔE de 124 mV y una corriente de oxidación más alta ($i_{\text{pa}} = 92 \mu\text{A}$). Finalmente, una vez modificada la superficie del SPAuE por grafting electroquímico con *p*-ABA (3), el electrodo muestra un comportamiento similar al anterior, aunque con corrientes algo menores ($i_{\text{pa}} = 62.5 \mu\text{A}$) debido a la repulsión electrostática ya mencionada entre las cargas negativas de los grupos carboxilo disociados y las de la sonda electroquímica utilizada.

Por otro lado, los diagramas de Nyquist representados en la Figura 5.26b ponen de manifiesto una menor resistencia a la transferencia de carga (R_{CT}) sobre el electrodo tras el tratamiento electroquímico con H_2SO_4 con respecto a la superficie electródica sin activar, con valores de 37 Ω (curva 2) y 796 Ω (curva 1), respectivamente. La introducción de grupos carboxilo mediante "grafting" con *p*-ABA (curva 3) produce un significativo aumento de la resistencia a la transferencia de carga, como era de esperar, debido principalmente al efecto de repulsión electrostática ya descrito, obteniéndose un valor de $R_{\text{CT}} = 266 \Omega$. Como se observa, estos resultados concuerdan totalmente con los obtenidos mediante CV. A partir de esta, las siguientes etapas aplicadas para la preparación del inmunosensor se caracterizaron únicamente mediante EIS, ya que los resultados obtenidos por CV sobre las superficies electródicas poco conductoras no permitían apreciar claramente las diferencias.

La activación de los grupos carboxilo con la mezcla EDC/NHSS y la consiguiente neutralización de la carga de los grupos funcionales confinados en la superficie del electrodo (curva 4) da lugar a una notable disminución de R_{CT} , obteniéndose un valor de 39 Ω , produciéndose a continuación incrementos sucesivos de esta resistencia a medida

que se incorporaban los inmunoreactivos, anti-TGF (curva 5), con $R_{CT}=119\ \Omega$, y Biotin-anti-TGF (curva 6), con $R_{CT}=502\ \Omega$. Para representar el circuito equivalente de Randles al que mejor se ajustan los resultados experimentales obtenidos mediante EIS se han tenido en cuenta las consideraciones de Lisdat [Lisdat, 2008] acerca del uso de esta técnica en el diseño de biosensores, y de Ramanavicius [Ramanavicius, 2010] sobre su empleo en la caracterización de inmunosensores. Así, se ha propuesto como más adecuado el circuito representado en la parte superior de la Figura 5.26b, en el que R1 es la resistencia de la disolución, CPE es el elemento de fase constante atribuido a la capacidad de la doble capa eléctrica, R2 se refiere al flujo de corriente producida por las reacciones redox en la interfase, y W1 es el elemento Warburg, impedancia semi-infinita debida a la difusión desde el seno de la disolución a la interfase.

El conjunto de resultados confirma la utilidad del procedimiento de inmovilización de los inmunorreactivos sobre el SPAuE funcionalizado por "electrografting" de *p*-ABA.

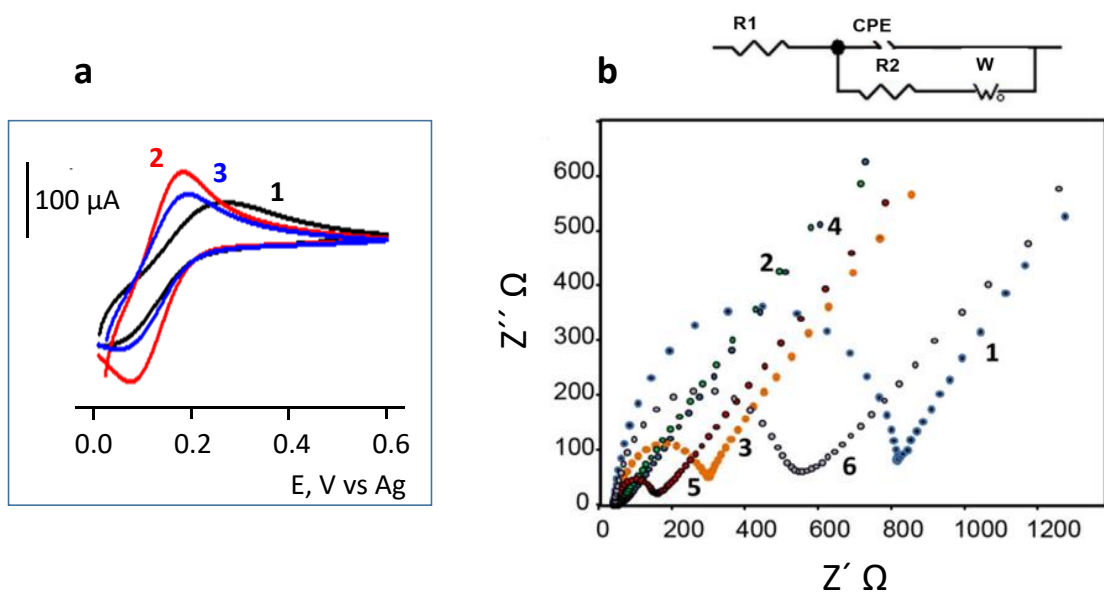


Figura 5.26. Voltamperogramas cíclicos (a) y diagramas de Nyquist (b) de $Fe(CN)_6^{3-/4-}$ 5.0 mM en KCl 0.1 M sobre: SPAuE sin modificar (1); SPAuE tratado con H_2SO_4 (2); COOH-Phe-N-SPAuE (3); COOH-Phe-N-SPAuE activado con EDC/NHSS 0.1M (4); anti-TGF-Phe-N-SPAuE (5); Biotin-anti-TGF-TGF- β 1-anti-TGF-Phe-N-SPAuE (6). Sobre (b) aparece el circuito equivalente de Randles. Condiciones: CV: $v=50\text{ mV s}^{-1}$; EIS: número de frecuencia, 50; intervalo de frecuencia, 105 - 0.04 Hz; amplitud 0.01 V. Otras condiciones como en la Tabla 5.2.

5.1.2.4. Calibrado y características analíticas

En las condiciones optimizadas previamente se obtuvo un calibrado para la determinación de la citoquina TGF- β 1 con el inmunosensor desarrollado. Los resultados obtenidos se han representado en la Figura 5.27. Como era de esperar teniendo en cuenta el inmunoensayo de tipo sándwich empleado, las respuestas de corriente aumentan con la concentración del antígeno en todo el intervalo ensayado, observándose una región de saturación para concentraciones elevadas de analito. El ajuste por mínimos cuadrados del calibrado obtenido proporcionó la siguiente ecuación: $i_p, \mu A = (0.184 \pm 0.004) [TGF-\beta 1, ng mL^{-1}] + (0.047 \pm 0.005)$, con $R^2 = 0.992$. El intervalo lineal representado abarca desde 0.05 hasta 3 ng mL $^{-1}$ de TGF- β 1. Con el criterio de $3s_b/m$, donde s_b es la desviación estándar obtenida a partir de diez medidas de corriente con el inmunosensor en ausencia de TGF- β 1, y m la pendiente del calibrado, se calculó el límite de detección, obteniéndose un valor de LOD = 10 pg mL $^{-1}$. El límite de cuantificación resultó ser 33 pg mL $^{-1}$.

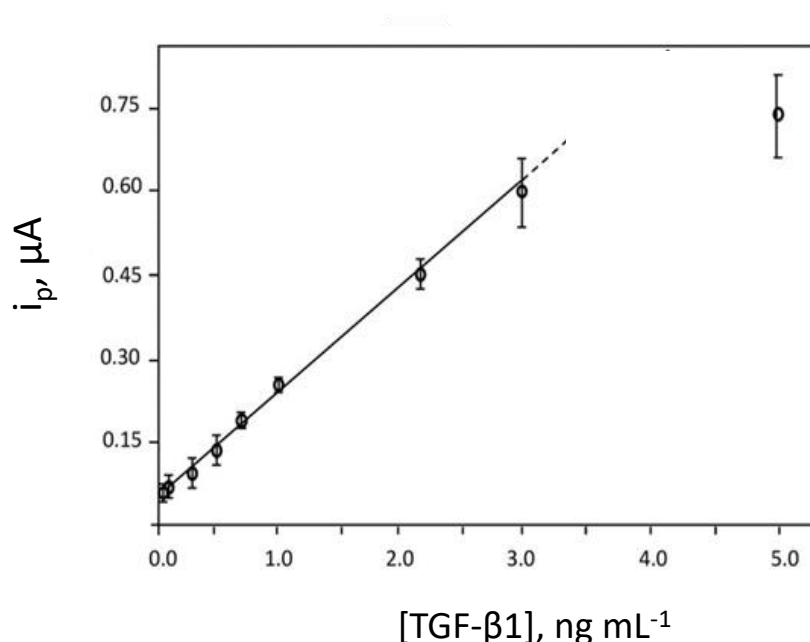


Figura 5.27. Calibrado para la determinación de TGF- β 1 con el inmunosensor AP-Strept-Biotin-anti-TGF-TGF-anti-TGF-Phe-N-SPAUE. Las barras de error se han estimado como el triple de la desviación estándar ($n=3$).

Las características analíticas del método establecido con el inmunosensor AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-Phe-N-SPAUE se compararon con las que proporcionan algunos kits ELISA comerciales, así como con las que presentan los biosensores electroquímicos descritos hasta el momento para esta citoquina. Los datos

que se comentan a continuación se han tomado de los resúmenes en el Apartado 3.5.1. de la Introducción. En relación a los kits ELISA, la mayoría de los métodos disponibles proporcionan calibrados semilogarítmicos que se extienden en intervalos comprendidos entre algunas decenas y miles de pg mL^{-1} , siendo la concentración mínima detectable de pocas unidades de pg mL^{-1} (Tabla 3.5.1.). Como puede apreciarse, los resultados obtenidos con el inmunosensor son similares a los reseñados. Respecto a los biosensores electroquímicos, el intervalo lineal obtenido con el inmunosensor es mucho más amplio que el que presenta el aptasensor descrito por Matharu para TGF- β [Matharu, 2014], y el límite de detección es cien veces inferior. Resultados similares se encuentran al comparar estas características con las de una configuración de inmunoensayo impedimétrico [Yao, 2016], que proporciona un $\text{LOD} = 570 \text{ pg mL}^{-1}$, más de cincuenta veces mayor. Finalmente, con respecto a otros diseños puestos a punto anteriormente por nuestro Grupo, el inmunosensor desarrollado presenta características similares, con un intervalo lineal más amplio en algún caso [Sánchez-Tirado, 2016], pero el límite de detección alcanzado es igual [Sánchez-Tirado, 2017] o algo mayor [Sánchez-Tirado, 2017a] que el encontrado en las configuraciones anteriores.

En relación al tiempo de ensayo requerido, aunque esta característica varía entre los diseños existentes, puede decirse que este periodo es más breve cuando se utiliza el inmunosensor AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-Phe-N-SPAuE. En efecto, desde la incubación de anticuerpo de captura, los tiempos necesarios suman un total de 120 min, que es muy inferior a las más de cuatro horas que se necesitan en la mayor parte de los kits ELISA, y algo más breve que el tiempo de ensayo de algunos inmunosensores descritos anteriormente.

Asimismo, se evaluó la reproducibilidad de las respuestas voltamperométricas empleando diferentes inmunosensores preparados el mismo día o en días diferentes. Los resultados obtenidos, expresados como el valor de la desviación estándar relativa (RSD) fueron del 3.2 % ($n=6$) para medidas de disoluciones de 1 ng mL^{-1} de TGF- β 1 realizadas en el mismo día, y del 3.7 % y 2.2 % ($n=8$), respectivamente, en ausencia y en presencia de 1 ng mL^{-1} de TGF- β 1, para las realizadas en días diferentes. Estos resultados revelan la buena precisión del método de preparación y de medida con el inmunosensor.

5.1.2.5. Estabilidad y selectividad

La estabilidad de almacenamiento fue investigada preparando el mismo día varios inmunosensores anti-TGF-Phe-N-SPAUEs que se mantuvieron a 4 °C y en ambiente húmedo hasta el momento de su empleo. A partir de ellos, en días diferentes, se prepararon los inmunosensores utilizados para medir la respuesta de disoluciones de 1 ng mL⁻¹ de TGF-β1. Los resultados obtenidos se han representado en un gráfico de control (Figura 5.28) en el que se han situado los límites en el intervalo $\pm 3s$, en el que s es la desviación estándar de diez medidas realizadas el primer día de trabajo (día 0). Como se puede observar, las señales obtenidas se mantienen entre los límites de gráfico de control durante al menos 20 días (no se estudiaron periodos más largos), lo que demuestra la buena estabilidad de los conjugados estudiados cuando se almacenan en las condiciones indicadas.

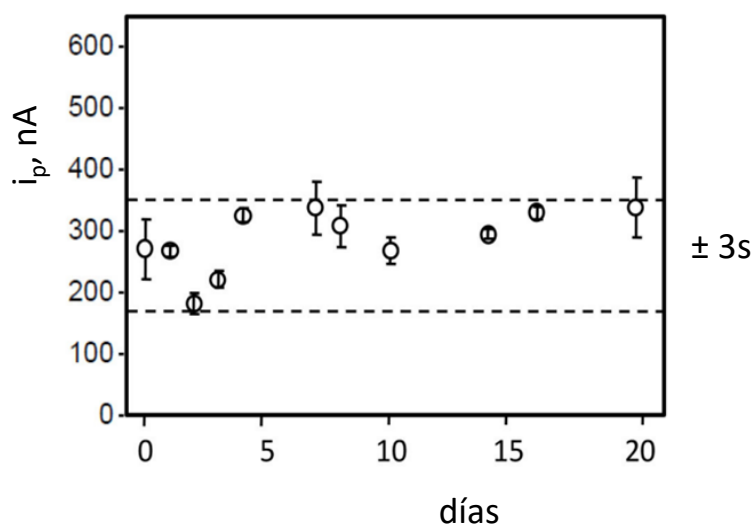


Figura 5.28. Gráfico de control para evaluar la estabilidad de almacenamiento de los conjugados anti-TGF-Phe-N-SPAUE a 4 °C. Ver el texto para más información.

Se estudió la selectividad del inmunosensor AP-Strept-Biotin-anti-TGF-TGF-β1-anti-TGF-SPAUE frente a otras especies que pueden estar presentes en las muestras de interés midiendo su respuesta en disoluciones preparadas en ausencia de TGF-β1 que contenían péptido YY (PYY), interleucina 6 (IL-6), grelina (GHRL), bilirrubina (BR), adiponectina (ADP), factor de necrosis tumoral α (TNF- α), hemoglobina (HB), colesterol (CHRL) y proteína C reactiva (CRP), todos ellos a una concentración de 1 ng mL⁻¹.

Como muestra la Figura 5.29, no se observan interferencias significativas por parte de ninguna de las especies ensayadas, apareciendo sus respuestas entre los límites situados a $\pm 3s$, donde s es la desviación estándar de diez medidas realizadas con el inmunosensor en ausencia de TGF- $\beta 1$. Esta elevada selectividad se atribuye a la práctica especificidad de los anticuerpos utilizados para la preparación del inmunosensor.

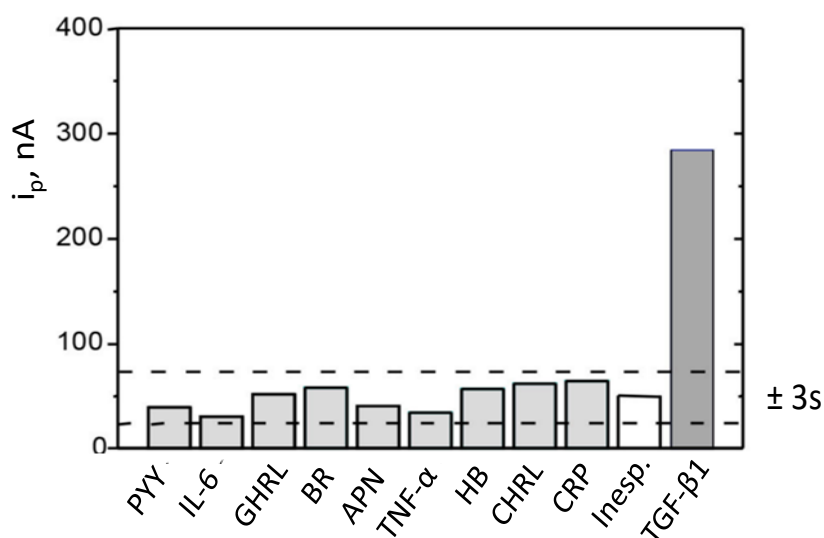


Figura 5.29. Efecto de la presencia de 1 ng mL^{-1} de PYY, IL-6, GHRL, BR, APN, TNF- α , HB, CHRL y CRP en la respuesta del inmunosensor AP-Strept-Biotin-anti-TGF- $\beta 1$ -TGF-anti-TGF-Phe-N-SPAUE en ausencia de TGF- $\beta 1$.

5.1.2.6. Determinación de TGF- $\beta 1$ en plasma humano

La utilidad analítica del inmunosensor desarrollado se demostró aplicándolo a la determinación de TGF- $\beta 1$ en plasma humano, en el que se sabe que la concentración esperable en individuos sanos se encuentra comprendida entre 0.1 y 25 ng mL^{-1} [Grainger, 1995]. Como se ha visto, el intervalo lineal obtenido ($0.05 - 3.0 \text{ ng mL}^{-1}$ de TGF- $\beta 1$) resulta adecuado para la determinación de esta citoquina en este tipo de muestras. No obstante, debido a la falta de disponibilidad de materiales clínicos en los que se pudiera determinar el contenido endógeno de TGF- $\beta 1$, se llevaron a cabo estudios de recuperación en muestras de plasma contaminadas al nivel de 0.250 ng mL^{-1} . El procedimiento seguido y las características de la muestra analizada se han descrito en el Apartado 4.4.4.1 de la Parte Experimental.

Se investigó, en primer lugar, la existencia de efecto matriz en el plasma empleando muestras contaminadas con diferentes concentraciones de TGF- β 1 a las que se aplicó una dilución 1/100 y con las que se construyó un calibrado en el intervalo lineal del obtenido con disoluciones patrón. En la Figura 5.30 se compara este calibrado con el de las muestras, cuya ecuación es: $i_p, \mu A = (0.192 \pm 0.003) [TGF-\beta 1, ng mL^{-1}] + (0.030 \pm 0.007)$, observándose que ambos coinciden apreciablemente. La comparación estadística de las pendientes de los calibrados se realizó mediante el test de t-Student, que proporcionó un valor de $t_{exp} = 1.76$ inferior al tabulado: $t_{tab} = 2.46$. Este resultado demuestra que no existen diferencias significativas entre dichas pendientes, por lo que no existe efecto matriz, lo que hace posible llevar a cabo la determinación de TGF- β 1 en muestras de plasma por interpolación de las corrientes de pico en el calibrado de disoluciones estándar.

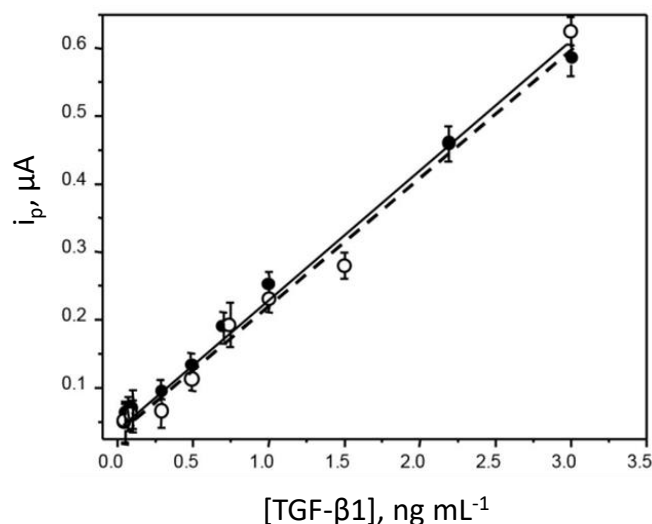


Figura 5.30. Calibrados de disoluciones patrón de TGF- β 1 (puntos negros) y de plasma contaminado con TGF- β 1 y diluido 100 veces (puntos blancos) obtenidos con el inmunosensor AP-Strept-Biotin-anti-TGF- β 1-anti-TGF-Phe-N-SPAuE en DPV. Ver el texto y la Tabla 5.3 para otras condiciones.

En la Tabla 5.4 se han resumido los resultados obtenidos para el análisis por triplicado de ocho muestras de plasma humano contaminadas con TGF- β 1 al nivel de 0.250 ng mL⁻¹. Como puede verse, la recuperación media alcanzada fue del $95 \pm 4 \%$. Estos resultados indican la fiabilidad del método basado en el empleo del inmunosensor desarrollado para la determinación de TGF- β 1 a bajas concentraciones en muestras clínicas.

Tabla 5.4. Determinación de TGF- β 1 en plasma humano contaminado con el inmunosensor AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-Phe-N-SPAUE.

Muestra	TGF- β 1, ng mL ⁻¹	Media $\pm$ s, ng mL ⁻¹	Recup. %
Plasma 1	0.230; 0.220	0.225 $\pm$ 0.007	90 $\pm$ 3
Plasma 2	0.255; 0.246	0.250 $\pm$ 0.006	100 $\pm$ 2
Plasma 3	0.243; 0.234	0.239 $\pm$ 0.006	95 $\pm$ 3
Plasma 4	0.253; 0.252	0.252 $\pm$ 0.001	101 $\pm$ 1
Plasma 5	0.253; 0.252	0.252 $\pm$ 0.001	101 $\pm$ 1
Plasma 6	0.236; 0.244	0.240 $\pm$ 0.006	96 $\pm$ 2
Plasma 7	0.230; 0.220	0.225 $\pm$ 0.007	90 $\pm$ 3
Plasma 8	0.238; 0.229	0.234 $\pm$ 0.006	93 $\pm$ 2

5.1.2.7. Conclusiones

En este trabajo se ha desarrollado un inmunosensor electroquímico para la determinación de la citoquina TGF- β 1 basado en el empleo de electrodos serigrafados de oro de baja temperatura funcionalizados con grupos carboxilo mediante "electrografting" utilizando ácido *p*-ABA y LiClO₄ en medio etanol. Tras establecerse un esquema de inmunoensayo de tipo sándwich empleando un conjugado enzimático de AP-Strept como marcador enzimático, la determinación se llevó a cabo por voltamperometría diferencial de impulsos (DPV) mediante la detección de 1-naftol como producto de la reacción del 1-naftilfosfato usado como sustrato de la fosfatasa alcalina. La metodología empleada permitió obtener mejoras en las características analíticas, principalmente con respecto a las proporcionadas por los kits ELISA colorimétricos para esta proteína. Todo ello ha hecho posible alcanzar un bajo límite de detección, un tiempo de ensayo reducido, así como buenos resultados en su aplicación a la determinación de TGF- β 1 en muestras de plasma humano.

5.2. INMUNOSENSORES BASADOS EN ELECTRODOS MODIFICADOS MEDIANTE QUÍMICA "CLICK" Y "ELECTROCLICK"

Como se ha descrito en la Introducción, en esta Tesis se han puesto a punto dos configuraciones de inmunoensayo electroquímico basadas en el uso de las herramientas de la química "click" y "electroclick" para modificar la superficie electródica, que han permitido desarrollar estructuras funcionalizadas estables y reproducibles útiles para su aplicación a la determinación de biomarcadores de interés. Los inmunosensores desarrollados haciendo uso de estas metodologías implican el empleo de la reacción de cicloadición azida-alquino catalizada por Cu(I) (CuAAC), en la que el catalizador se ha obtenido mediante reacción química, en presencia de un reactivo adecuado, o electroquímica, aplicando un potencial en las condiciones apropiadas, y utilizando en ambos casos dos reactivos complementarios para la reacción de cicloadición: una inmunoglobulina (IgG) etinilada y nanotubos de carbono de pared múltiple (MWCNTs) funcionalizados con un grupo azida. Esta estrategia permitió construir un inmunosensor electroquímico para la quimioquina CXCL7, biomarcador de enfermedades autoinmunes como la artritis reumatoide, y otro para la citoquina interleucina IL-1 β , relacionada con los procesos inflamatorios que acompañan a esta y otras enfermedades.

Los inmunosensores que se describen a continuación suponen un avance pionero en la aplicación de estas metodologías a la construcción de plataformas electroquímicas útiles para la determinación de biomarcadores de interés en muestras reales.

Además de los objetivos generales de puesta a punto y aplicación de dispositivos de inmunodetección sensibles y fiables, los objetivos particulares que se establecieron al dar comienzo a estas investigaciones fueron:

1. Hacer uso por primera vez de la reacción CuAAC, fundamental dentro de la llamada química "click", como medio de funcionalización de superficies electródicas serigrafiadas para la construcción de inmunosensores electroquímicos.
2. Poner a punto la técnica de "electroclick" para demostrar las ventajas que comporta la síntesis electroquímica del ion cuproso, que actúa como catalizador de dicha reacción.

5.2.1. Inmunosensor para la determinación de CXCL7

5.2.1.1. Configuración del inmunosensor

Como se ha señalado anteriormente, la preparación de plataformas electroquímicas para la inmovilización de biomoléculas empleando la metodología "click" requiere la síntesis de los reactivos complementarios azida-etinil. Los procedimientos seguidos para la incorporación de un grupo azida sobre MWCNTs, para la síntesis de IgG etinilada, y para la formación del conjugado IgG-alquino-azida-MWCNTs en presencia de Cu(I) se describieron en el Apartado 4.4.2 de la Parte Experimental. Además, en la Figura 4.11. del Apartado 4.4.2.1. se resumió el método de construcción del inmunosensor cuyo esquema se muestra en la Figura 5.31. Brevemente, una vez modificado un SPCE con el conjugado preparado anteriormente (ver inserto en la Fig. 5.30), se inmovilizó el anticuerpo de captura anti-CXCL7, estableciéndose una configuración de tipo sándwich con el antígeno CXCL7 y un anticuerpo biotinilado, Biotin-anti-CXCL7, seguido del conjugado AP-Strept. La detección se llevó a cabo mediante DPV, midiendo la corriente de oxidación del 1-naftol, 1-NP, producto de la reacción enzimática del 1-naftil fosfato (1-NPP).

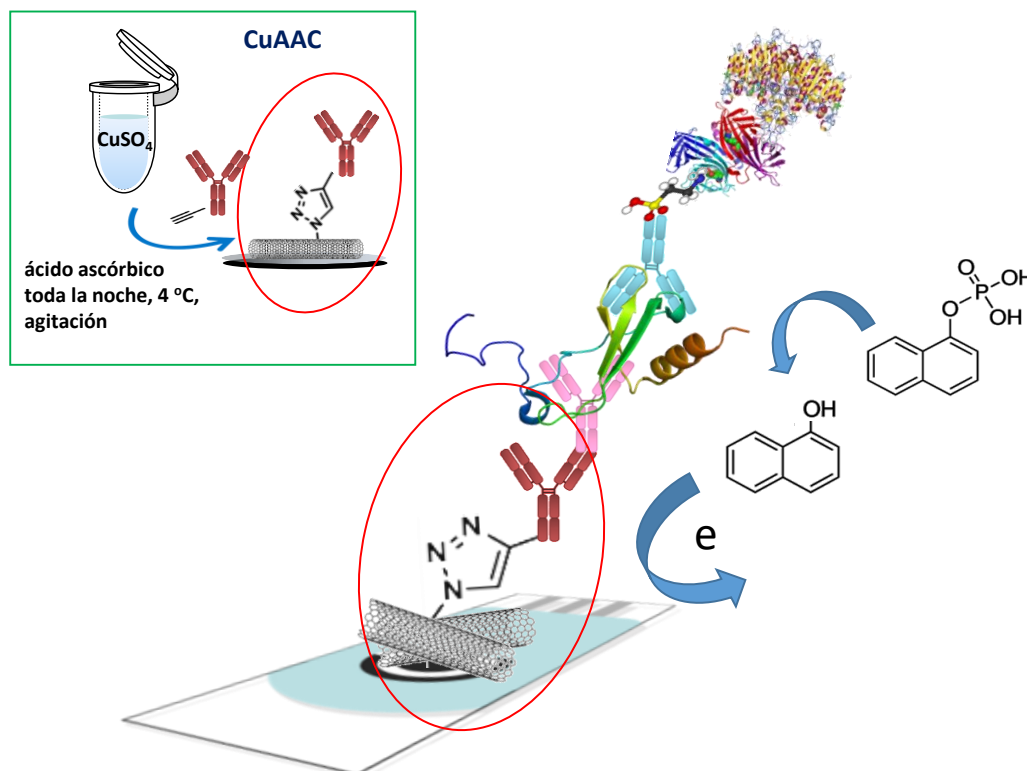


Figura 5.31. Esquema del inmunosensor AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE y las reacciones implicadas en la detección. Inserto: preparación del conjugado IgG-etinil-azida-MWCNTs.

5.2.1.2. Optimización de las variables experimentales

Los procedimientos seguidos para la síntesis de los reactivos complementarios azida-MWCNTs y etinil-IgG en las condiciones optimizadas en trabajos anteriores [Sánchez-Tirado, 2016] se han descrito en la Parte Experimental. Por ello, en esta parte, los estudios de optimización se centraron en evaluar la influencia de: la concentración y el tiempo de incubación de la caseína como agente bloqueante; la concentración y el tiempo de incubación del anticuerpo de captura anti-CXCL7; la concentración y el tiempo de incubación del anticuerpo secundario Biotin-anti-CXCL7, y la concentración y el tiempo de incubación del conjugado AP-Strept. Todas las incubaciones de los inmunorreactivos sobre el SPCE se realizaron en condiciones de humedad y oscuridad a 25 °C. Los resultados obtenidos se describen a continuación, resumiéndose en la Tabla 5.5. los intervalos estudiados para cada variable, así como los valores elegidos como óptimos en cada caso.

Influencia de la concentración y el tiempo de incubación de la caseína

Como se describió en el Apartado 4.4.2.1., la caseína utilizada como agente bloqueante para la preparación del inmunosensor de CXCL7, se incorpora al electrodo modificado con el conjugado IgG-MWCNT de forma previa a la inmovilización del anticuerpo de captura anti-CXCL7. En este estudio se investigó la influencia de la concentración de caseína en el intervalo comprendido entre 0.2 y 1% sobre la respuesta voltamperométrica del inmunosensor con los resultados representados en la Figura 5.32a. Puede observarse que la corriente específica más elevada, así como la mayor relación S/N se obtuvieron empleando una concentración del 0.6 %, por lo que se eligió esta como más adecuada para bloquear las posiciones vacantes del electrodo modificado. Por otro lado, en relación al tiempo de bloqueo (Figura 5.32b), las condiciones óptimas se alcanzaron para un periodo de 30 min, que fue elegido para estudios posteriores. Tiempos inferiores resultan insuficientes para garantizar un bloqueo efectivo, mientras que periodos más largos dan lugar a una considerable disminución de la corriente de pico, debido probablemente a la saturación del electrodo y falta de conductividad. Cabe destacar que, en esta configuración, las corrientes inespecíficas son más altas que las que presentaban los inmunosensores descritos anteriormente.

Probablemente esto es debido a la presencia de los nanotubos de carbono sobre la superficie el electrodo, cuyas propiedades electroquímicas, como la elevada conductividad y la actividad electrocatalítica frente a diversos procesos de oxidación, hace más difícil evitar la aparición de corrientes en el intervalo de potencial barrido, que no correspondan exactamente ni directamente a la reacción de formación del inmunocomplejo. No obstante, como se verá en los siguientes estudios de optimización, la selección cuidadosa de las condiciones de trabajo permitió al menos minimizar las adsorciones no deseadas, alcanzando una buena relación S/N y un buen nivel de sensibilidad.

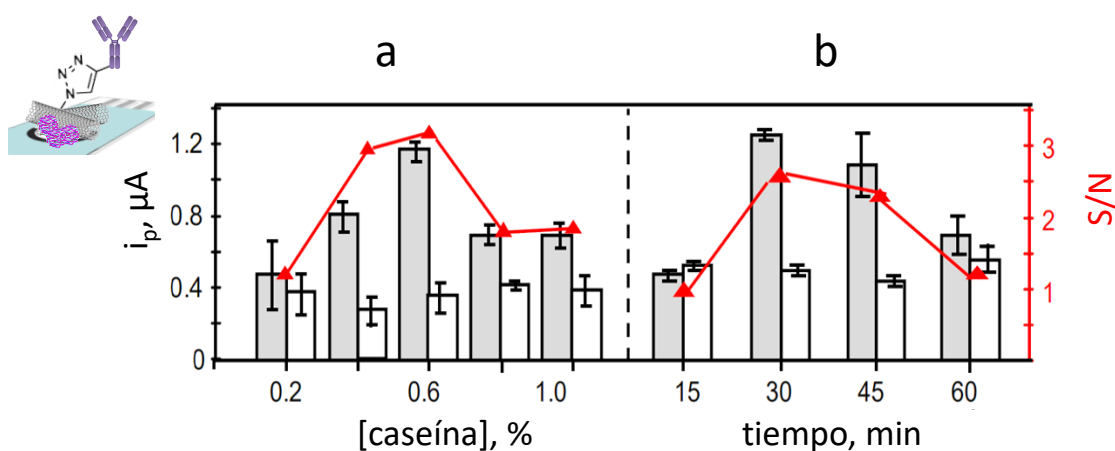


Figura 5.32. Influencia de la concentración (a) y del tiempo de incubación (b) de la caseína sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (gris) de 0.5 ng mL^{-1} de CXCL7; 0.6 % caseína (b), inc. 30 min (a); $2 \text{ } \mu\text{g mL}^{-1}$ anti-CXCL7, inc. 60 min; 100 ng mL^{-1} Biotin-anti-CXCL7, inc. 30 min; $5 \text{ } \mu\text{g mL}^{-1}$ AP-Strept, inc. 30 min. Detección: 1-NPP 5 mM, 5 min reacción; DPV: $-0.2 - +0.7 \text{ V vs. Ag}$; $\Delta E = 50 \text{ mV}$; $v = 10 \text{ mVs}^{-1}$. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Influencia de la concentración y del tiempo de incubación de anti-CXCL7

La influencia de la concentración de anti-CXCL7 sobre la respuesta del inmunosensor se estudió en el intervalo comprendido entre 1 y $10 \text{ } \mu\text{g mL}^{-1}$ con los resultados recogidos en la Figura 5.33a. Como cabía esperar, la corriente específica aumenta con la cantidad de anticuerpo de captura inmovilizada sobre el electrodo IgG-MWCNTs/SPCE hasta una concentración de $2 \text{ } \mu\text{g mL}^{-1}$ disminuyendo ligeramente después debido probablemente al impedimento estérico que produce la presencia de altas concentraciones de este inmunorreactivo, que dificulta el reconocimiento del antígeno. Por otro lado, en la evaluación del tiempo de incubación de anti-CXCL7 (Figura 5.33b),

se observa un valor óptimo en 60 min, en el que se alcanza la mayor relación S/N, siendo este tiempo el elegido para la preparación del inmunosensor.

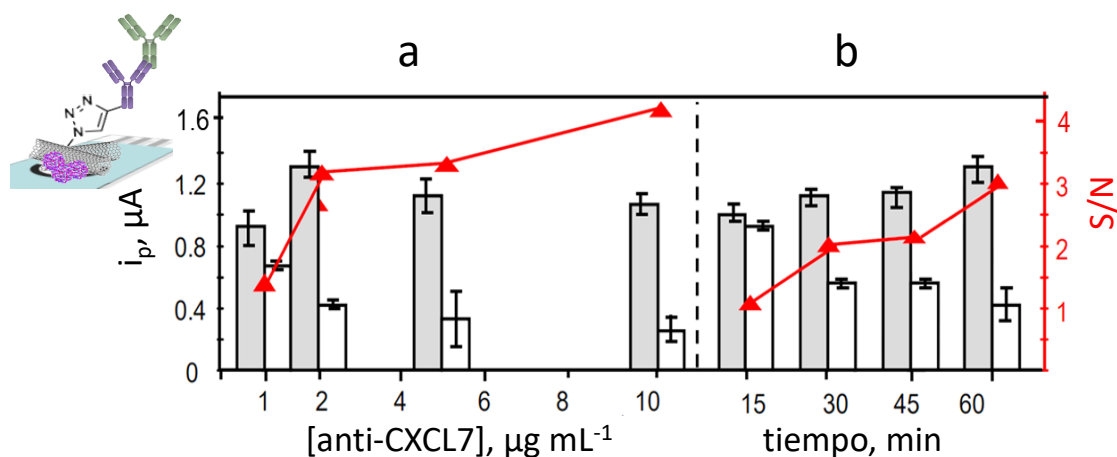


Figura 5.33. Influencia de la concentración (a) y del tiempo de incubación (b) de anti-CXCL7 sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (gris) de 0.5 ng mL^{-1} de CXCL7; 0.6 % caseína, inc. 30 min; $2 \text{ } \mu g mL^{-1}$ anti-CXCL7 (b), inc. 60 min (a); 100 ng mL^{-1} Biotin-anti-CXCL7, inc. 30 min; $5 \text{ } \mu g mL^{-1}$ AP-Strept, inc. 30 min. Otras condiciones como en la Figura 5.32.

Influencia de la concentración y el tiempo de incubación de Biotin-anti-CXCL7

En la Figura 5.34a se han representado los resultados obtenidos en el estudio de la influencia de la concentración del anticuerpo biotinilado Biotin-anti-CXCL7 en el intervalo de 50 a 200 ng mL^{-1} sobre la respuesta voltamperométrica del inmunosensor. Se observa cómo a partir de 100 ng mL^{-1} , la señal específica permanece prácticamente constante, sin embargo, la corriente inespecífica aumenta en todo el intervalo de concentraciones estudiado. De un modo similar, este comportamiento se observó también a partir de 30 min cuando se evaluó el efecto del tiempo de incubación (Figura 5.34b). Probablemente, en presencia de antígeno, concentraciones menores de 100 ng mL^{-1} y tiempos de incubación más breves que el señalado no son suficientes para formar el complejo Biotin-anti-CXCL7-CXCL7, pero una vez alcanzadas las condiciones de saturación, el incremento de la corriente que se produce en ausencia de analito, se debe a la adsorción del anticuerpo sobre el electrodo modificado. Las tendencias observadas proporcionan la máxima relación S/N para los valores indicados: 100 ng mL^{-1} y 30 min, por lo que se escogieron como óptimos para la preparación del inmunosensor.

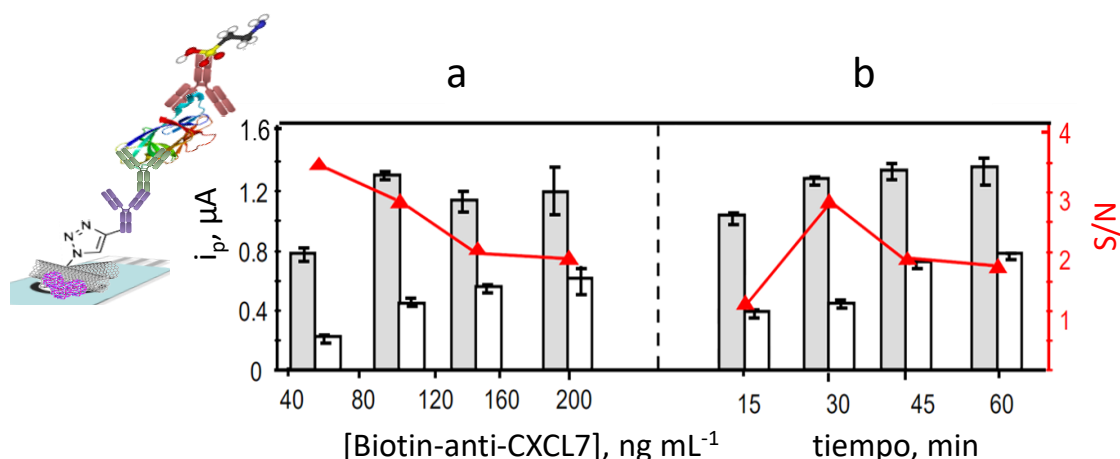


Figura 5.34. Influencia de la concentración (a) y del tiempo de incubación (b) de Biotin-anti-CXCL7 sobre la respuesta del inmunosensor en ausencia (blanco) y en presencia (gris) de 0.5 ng mL^{-1} de CXCL7; 0.6 % caseína, inc. 30 min; $2 \text{ } \mu\text{g mL}^{-1}$ anti-CXCL7, inc. 60 min; Biotin-anti-CXCL7, inc. 30 min (b); 100 ng mL^{-1} (a); $5 \text{ } \mu\text{g mL}^{-1}$ AP-Strept, inc. 30 min. Otras condiciones como en la Figura 5.32.

Influencia de la concentración y el tiempo de incubación de AP-Strept

La optimización de la concentración y del tiempo de incubación del conjugado AP-Strept usado como marcador enzimático en la configuración de tipo sándwich desarrollada, resulta esencial para conseguir un buen funcionamiento del inmunosensor. Como es sabido, se debe alcanzar una cantidad de enzima suficiente como para que la respuesta del inmunosensor dependa de la concentración de la misma y no de la concentración del sustrato. Solo de este modo será posible relacionar la respuesta de corriente con la concentración del analito. Con este objetivo, se evaluó la respuesta voltamperométrica de diferentes inmunosensores preparados con concentraciones de AP-Strept en el intervalo de 3 a $10 \text{ } \mu\text{g mL}^{-1}$ aplicando tiempos de incubación entre 15 y 60 min. Como se aprecia en la Figura 5.35a, la señal específica permanece prácticamente constante para concentraciones de conjugado de 5 y $7 \text{ } \mu\text{g mL}^{-1}$. Por debajo de estos valores no hay suficiente cantidad de conjugado como para obtener la respuesta óptima, mientras que concentraciones más altas dan lugar a un incremento anómalo de la respuesta, ya que viene acompañado de un aumento notable de la señal no específica. Este comportamiento se atribuye, de forma similar a lo explicado anteriormente, a la adsorción sobre el electrodo del AP-Strept sobrante una vez alcanzada la concentración de saturación.

Por otro lado, los resultados obtenidos en el estudio de la influencia del tiempo de incubación (Figura 5.34b) muestran una tendencia análoga. De todo ello se deducen valores óptimos de $5 \mu\text{g mL}^{-1}$ de AP-Strept y un tiempo de incubación de 30 min.

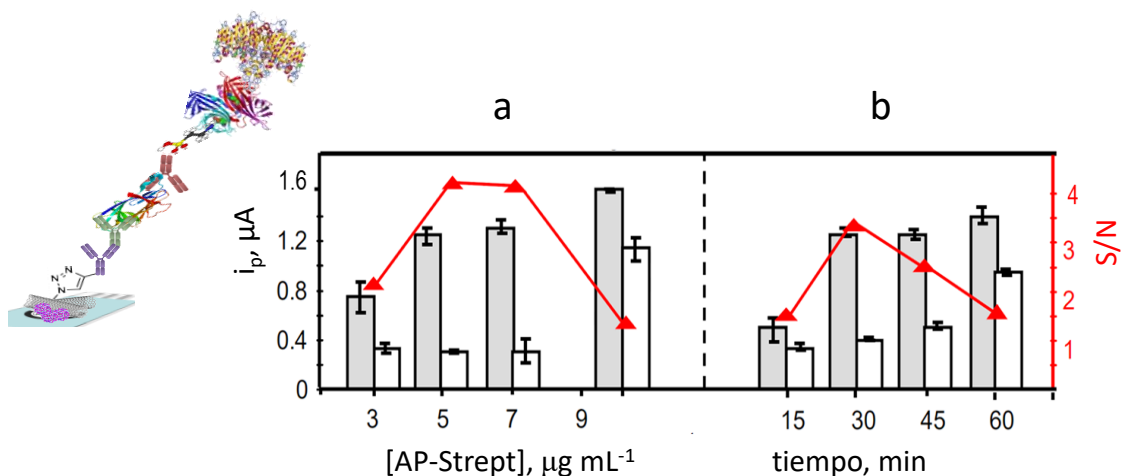


Figura 5.35. Influencia de la concentración (a) y del tiempo de incubación (b) de AP-Strept sobre la respuesta del inmutosensor en ausencia (blanco) y en presencia (gris) de 0.5 ng mL^{-1} de CXCL7; 0.6 % caseína, inc. 30 min; $2 \mu\text{g mL}^{-1}$ anti-CXCL7, inc. 60 min; 100 ng mL^{-1} Biotin-anti-CXCL7, inc. 30 min; 100 ng mL^{-1} (a); AP-Strept, inc. 30 min (b); $5 \mu\text{g mL}^{-1}$ (a). Otras condiciones como en la Figura 5.32.

En la Tabla 5.5 se resumen los estudios de optimización realizados, indicando la variable y el intervalo estudiado, así como el valor elegido en cada caso.

Tabla 5.5. Optimización de las variables experimentales.

Variable	Intervalo estudiado	Valor seleccionado
Concentración de caseína, %	0.2 - 1.0	0.6
Tiempo incubación caseína, min	15 - 60	30
Concentración de anti-CXCL7, $\mu\text{g mL}^{-1}$	1 - 10	2
Tiempo incubación anti-CXCL7, min	15 - 60	60
Concentración de Biotin-anti-CXCL7, ng mL^{-1}	50 - 200	100
Tiempo incubación Biotin-anti-CXCL7, min	15 - 60	30
Concentración de AP-Strept, $\mu\text{g mL}^{-1}$	2.5 - 10	5
Tiempo incubación AP-Strept, min	15 - 60	30

5.2.1.3. Estudios de caracterización

Los electrodos modificados en las condiciones anteriormente descritas se caracterizaron mediante las técnicas de espectroscopia de impedancia electroquímica (EIS) y voltamperometría cíclica (CV). Para llevar a cabo este estudio, se registraron las curvas de Nyquist y los voltamperogramas del electrodo sin modificar (SPCE, curva 1) y modificado con los nanotubos de carbono funcionalizados con el grupo azida (azida-MWCNTs/SPCE, curva 2), así como con el producto de la reacción "click" (IgG-MWCNTs/ SPCE, curva 3) y con el anticuerpo de captura (anti-CXCL7-IgG-MWCNTs/SPCE, curva 4). Como sonda redox se utilizó una disolución de $\text{Fe}(\text{CN})_6^{3-/4-}$ 5.0 mM en KCl 0.1 M. Como puede observarse en la Figura 5.36.a, el electrodo sin modificar presenta mayor resistencia a la transferencia de carga, con $R_{CT} = 868 \, \Omega$. Sin embargo, esta se reduce de forma drástica ($R_{CT} = 155 \, \Omega$) al modificar su superficie con azida-MWCNTs, debido a la elevada conductividad de los nanotubos de carbono. Posteriormente, y como consecuencia del carácter aislante de la biomolécula IgG, el valor de R_{CT} aumenta hasta $285 \, \Omega$. Finalmente, la incubación del anticuerpo de captura anti-CXCL7 intensifica notablemente este aumento hasta un valor de $865 \, \Omega$. Hay que señalar que este incremento de R_{CT} no se debe solo a la presencia del anticuerpo, ya que este se inmoviliza después de bloquear la superficie del electrodo con caseína, de modo que se suman dos efectos que contribuyen a crear una barrera aislante sobre dicha superficie.

Es importante mencionar que este último espectro (curva 4) presenta dos semicírculos seguidos de un dominio lineal, lo que se atribuye a la presencia de dos capas diferenciadas sobre el electrodo [Uygun, 2014]. Este comportamiento ha sido observado anteriormente en otros inmunosensores preparados con caseína como agente bloqueante [Serafin, 2018] y puede deberse al recubrimiento no uniforme del anticuerpo de captura sobre la superficie bloqueada. Para describir este fenómeno se han tenido en cuenta los datos aportados por Uygun [Uygun, 2014] sobre resultados similares, y se han considerado dos circuitos equivalentes como más apropiados. El circuito que aparece en la parte izquierda de la Figura 5.36a representa el comportamiento de las superficies electródicas que proporcionan las curvas 1 a 3 [Lysdat, 2008], y el de la derecha, más complejo, de tipo $R1(R2Q1)(Q2[R3W1])$ permite explicar la forma de la curva 4, ya que se ajusta mejor al comportamiento de una superficie electródica sobre la que se aprecian dos capas, en este caso, aparentemente, la de caseína y la del anticuerpo de captura. Este circuito incluye la resistencia óhmica, R_s , de la disolución electrolítica, la resistencia de

transferencia de carga electrónica, RCT, en serie con la impedancia de difusión de Warburg, W, y en paralelo con la capacitancia de doble capa. El semicírculo de alta frecuencia del diagrama de Nyquist corresponde a la resistencia de transferencia de carga en paralelo a la capacitancia anterior y el semicírculo de baja frecuencia, que se puede atribuir a una segunda capa sobre la superficie electrodoica, contiene la impedancia de Warburg debido a la difusión de la sonda redox.

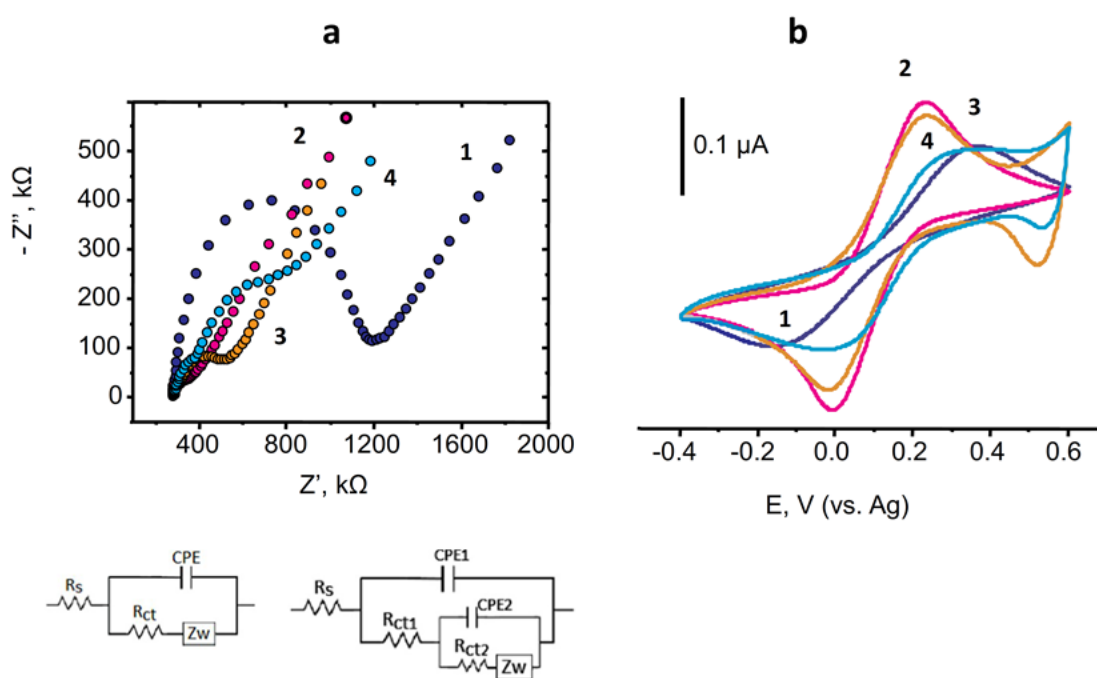


Figura 5.36. Diagramas de Nyquist con los circuitos equivalentes (a) y voltamperogramas cíclicos (b) obtenidos utilizando $\text{Fe}(\text{CN})_6^{3-/4-}$ 5.0 mM en KCl 0.1 M correspondientes al proceso de funcionalización y construcción del inmunosensor para CXCL7: SPCE sin modificar (1); azida-MWCNT/SPCE (2); IgG-MWCNTs/SPCE (3); anti-CXCL7-IgG-MWCNTs/SPCE (4). Condiciones: EIS: número de frecuencia, 50; intervalo de frecuencia, 105 - 0.04 Hz; amplitud 0.01 V. CV: $v = 100 \text{ mV s}^{-1}$.

Los resultados obtenidos mediante voltamperometría cíclica están de acuerdo con los descritos anteriormente. Así, en la Figura 5.36b se puede observar cómo el voltamperograma correspondiente al electrodo modificado con azida-MWCNTs es el que proporciona las mayores corrientes de pico, con $i_{pa} = 164 \text{ nA}$ e $i_{pc} = 168 \text{ nA}$. La semejanza entre los valores de las corrientes anódica y catódica indica el comportamiento cuasi-

reversible sobre este electrodo del sistema electroactivo empleado. Por otro lado, los potenciales de pico aparecen a valores de 224 mV (E_{pa}) y -16 mV (E_{pc}), con una diferencia entre ellos (ΔE) de 240 mV. Esta diferencia es notablemente inferior a la obtenida sobre el electrodo SPCE sin modificar ($\Delta E = 537$ mV). Puede verse también cómo el voltamperograma cíclico del electrodo IgG-MWCNTs/SPCE muestra corrientes de pico menores ($i_{pa} = 136$ nA e $i_{pc} = 132$ nA), como resultado de la menor conductividad de la superficie electródica, y un valor similar de la separación de los potenciales de pico, con $\Delta E = 259$ mV. Finalmente, como era de esperar, la inmovilización posterior de anti-CXCL7 sobre el electrodo bloqueado con caseína dio lugar a una importante disminución de las corrientes de pico, siendo: $i_{pa} = 86$ nA e $i_{pc} = 91$ nA. Por tanto, estos resultados son consistentes con lo encontrado en los estudios mediante EIS y en conjunto demuestran el éxito en la incorporación de los inmunorreactivos sobre el electrodo, así como también la utilidad de la reacción "click" CuAAC para la preparación de plataformas electródicas.

5.2.1.4. Calibrado y características analíticas

En las condiciones optimizadas previamente, se obtuvo el calibrado para la determinación de CXCL7 que se ha representado en la Figura 5.37. Como era de esperar, la respuesta obtenida mediante voltamperometría diferencial de impulsos es directamente proporcional a la concentración de la quimioquina, de forma que la relación entre la corriente de pico y dicha concentración se ajusta a la ecuación: $\Delta i_p, \mu A = (2.49 \pm 0.06) [CXCL7, ng mL^{-1}] + (0.02 \pm 0.02)$ ($R^2 = 0.994$), donde Δi_p es la corriente obtenida tras restar la corriente de fondo de la respuesta voltamperométrica en ausencia de CXCL7. El intervalo lineal abarca desde 0.5 hasta 600 pg mL⁻¹ y el valor de LOD, obtenido con el criterio $3s_b$, donde s_b , es la desviación estándar en unidades de concentración calculada a partir de la medida de 10 respuestas voltamperométricas del inmunosensor en ausencia de proteína, fue de 0.1 pg mL⁻¹.

Las características analíticas del método establecido con el inmunosensor se compararon con las de otros métodos basados en técnicas de biodetección haciendo uso de los datos resumidos en el Apartado 3.5.3. de la Introducción. Al no haberse encontrado ningún biosensor descrito en la bibliografía para CXCL7, se consideraron algunos kits ELISA comerciales. A la vista de los datos que se recogen en la Tabla 3.5.3, puede decirse que el empleo del inmunosensor proporciona mejores resultados. Por ejemplo, el kit de

Abcam (ab100613) da lugar a un calibrado semilogarítmico no lineal en el intervalo de concentraciones de CXCL7 entre 4.1 y 1000 pg mL^{-1} , así como un valor de MDD (dosis mínima detectable), calculado como la media de medidas repetidas de disoluciones en ausencia de analito más dos veces la desviación estándar, de 8.5 pg mL^{-1} .

Puede verse que el intervalo lineal del calibrado obtenido con el inmunosensor alcanza concentraciones notablemente más bajas y, por otra parte, el límite de detección es más de un orden de magnitud inferior al del kit ELISA. Además, el tiempo de ensayo de este kit se extiende por encima de las 4h, mientras que el método puesto a punto en este trabajo requiere solo 2h, aproximadamente, en ambos casos contando a partir de la inmovilización del anticuerpo de captura.

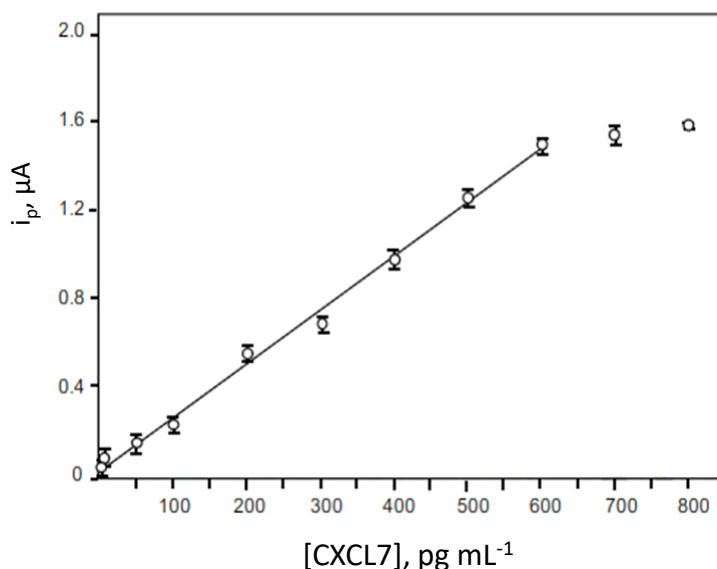


Figura 5.37. Calibrado para la determinación de CXCL7 con el inmunosensor AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE. Las barras de error se han estimado como el triple de la desviación estándar ($n=3$).

La reproducibilidad de las respuestas voltamperométricas se evaluó a partir de la medida repetida de disoluciones de 300 pg mL^{-1} de CXCL7 empleando diferentes inmunosensores preparados el mismo día. El resultado obtenido, expresado como el valor de la desviación estándar relativa (RSD) fue del 3.7 % ($n=10$). También se estudió la reproducibilidad en ausencia y en presencia de 500 pg mL^{-1} de CXCL7 empleando diez inmunosensores distintos construidos en días diferentes, obteniendo valores de RSD del 1.5 % y el 4.4 %, respectivamente. Estos resultados ponen de manifiesto la buena precisión de los métodos de preparación del inmunosensor y de detección electroquímica.

5.2.1.5. Estabilidad y selectividad

Para estudiar la estabilidad de almacenamiento, se preparó en el mismo día una serie de plataformas anti-CXCL7-IgG-MWCNTs/SPCE, que se mantuvieron a 4 °C en ambiente húmedo, a partir de las cuales, cada día de control se medían disoluciones de 500 pg mL⁻¹ de CXCL7 con el inmunosensor completo. Los resultados obtenidos se han representado en la Figura 5.38, en la que se muestra un gráfico de control con los límites correspondientes a los valores $\pm 3s$, siendo s la desviación estándar de las intensidades de pico de diez medidas realizadas el primer día de trabajo (día 0). Como puede observarse, las respuestas obtenidas con el inmunosensor permanecen dentro de dichos límites durante al menos siete días, que fue el periodo de tiempo máximo estudiado.

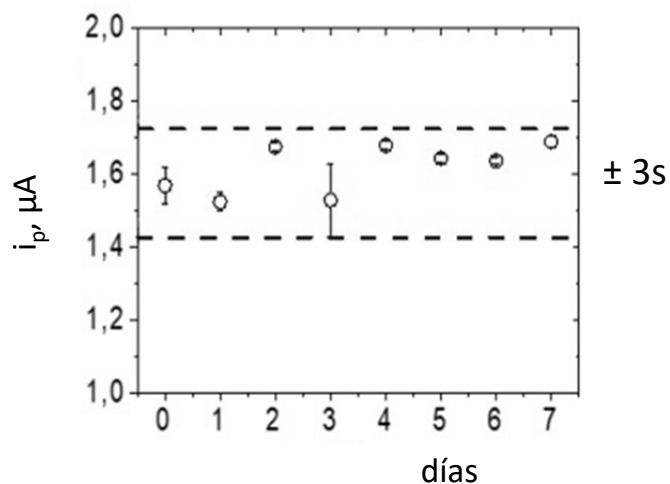


Figura 5.38. Gráfico de control para evaluar la estabilidad de los conjugados anti-CXCL7-IgG-MWCNTs/SPCE en condiciones de humedad a 4°C. Ver el texto para más información.

El efecto sobre la respuesta del inmunosensor de otras especies que pueden estar presentes en las muestras de CXCL7, se estudió midiendo disoluciones preparadas en ausencia de antígeno que contenían albúmina de suero bovino (BSA), inmunoglobulina G humana (IgG), hemoglobina (Hb), factor de crecimiento transformante $\beta 1$ (TGF- $\beta 1$), interleucina 6 (IL-6), interleucina 1 β (IL-1 β), E-cadherina (E-cad), receptor tirosina quinasa (ERBB2), proteína supresora de tumores P53 (p53), cadherina 17 (Cad-17) o receptor del factor de crecimiento de fibroblastos 4 (FGFR4), cada uno de ellos presente a los niveles de concentración normales en suero humano de individuos sanos.

En la Figura 5.39 se han representado los resultados obtenidos, situándolos sobre un control con los límites establecidos a $\pm 3s$, donde s es la desviación estándar de diez medidas de la corriente de pico no específica del inmunosensor. Como puede verse, las respuestas de todas las especies ensayadas aparecen entre esos límites, lo que demuestra la ausencia de interferencia en el método desarrollado, que se debe principalmente a la elevada selectividad de los inmunorreactivos utilizados.

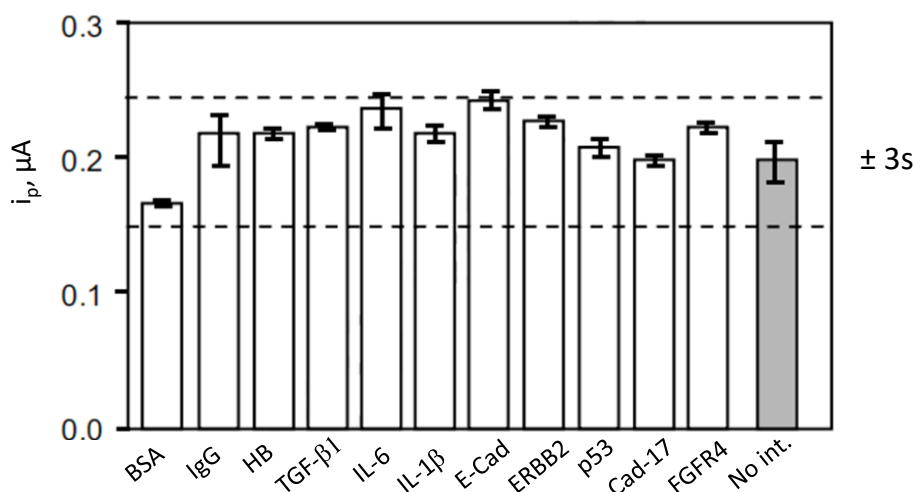


Figura 5.39. Efecto de la presencia de 5 mg mL⁻¹ de BSA, 0.9 mg mL⁻¹ de IgG, 200 ng mL⁻¹ de Hb, 180 ng mL⁻¹ de TGF- β 1, 300 pg mL⁻¹ de IL-6, 55 ng mL⁻¹ de IL-1 β , 55 ng mL⁻¹ de E-cad, 500 μ g mL⁻¹ de ERBB2, 200 ng mL⁻¹ de p53, 100 μ g mL⁻¹ de Cad-17 y 90 ng mL⁻¹ de FGFR4 sobre la respuesta del inmunosensor AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE.

5.2.1.6. Determinación de CXCL7 en suero

Los datos bibliográficos indican que las concentraciones de CXCL7 normales en muestras de suero se sitúan en torno a los 850 ng mL⁻¹ en el caso de individuos sanos [Du, 2018], mientras que en los pacientes diagnosticados de artritis reumatoide pueden alcanzarse concentraciones de algunos miles de ng mL⁻¹ [Yeo, 2015]. Por otra parte, como se ha visto, el intervalo de concentraciones del calibrado, entre 0.5 y 600 pg mL⁻¹, y el límite de cuantificación, LOQ = 0.33 pg mL⁻¹, resultan adecuados para la aplicación del método desarrollado a la determinación de CXCL7 en este tipo de muestras clínicas. Teniendo esto en cuenta, la utilidad analítica del inmunosensor AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE se demostró aplicando el método desarrollado al análisis de CXCL7 en sueros de pacientes diagnosticados de artritis

reumatoide obtenidas en el Hospital Clínico San Carlos de Madrid con consentimiento informado aprobado por los pacientes y el Comité Ético de la Institución.

En primer lugar, se investigó la existencia de efecto matriz en dichas muestras, construyendo un calibrado por adición de CXCL7 entre 0.5 y 600 pg mL⁻¹ sobre suero control en una dilución 1/2500. En la Figura 5.40 se compara el calibrado obtenido con el de las disoluciones patrón observándose que ambos son prácticamente superponibles. La ecuación de regresión para la recta obtenida en el suero es: $\Delta i_p, \mu A = (2.8 \pm 0.5) [CXCL7, ng mL^{-1}] + (0.28 \pm 0.05)$ ($R^2 = 0.995$), en la que la pendiente parece no ser estadísticamente diferente a la de patrones: $(2.49 \pm 0.06) \mu A mL ng^{-1}$. Con el fin de comprobar esta afirmación se realizó un análisis estadístico de ambas pendientes aplicando el test t de Student obteniendo un valor de $t_{exp} = 0.62$, menor que el valor tabulado, $t_{tab} = 2.14$. Este resultado pone de manifiesto la ausencia de efecto matriz, por lo que la determinación en las muestras puede realizarse por interpolación en el calibrado de patrones de la corriente de pico en DPV medida con el inmunosensor en el suero diluido en las condiciones indicadas.

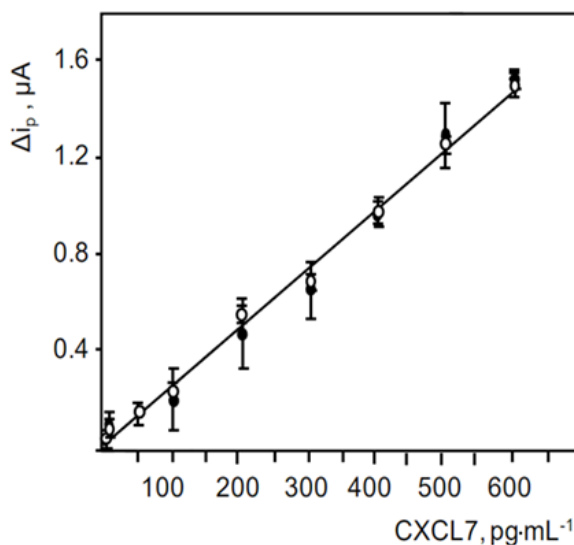


Figura 5.40. Comparación de los calibrados obtenidos con el inmunosensor AP-Strept-Biotin-anti-CXCL7-CXCL7-antiCXCL7-IgG-MWCNTs/SPCE para disoluciones patrón de CXCL7 (blanco) y muestras de suero en dilución 1/2500 (negro). Las barras de error se han estimado como el triple de la desviación estándar (n=3)

En la Tabla 5.6 se comparan los resultados obtenidos con los que proporciona un kit ELISA comercial de la marca R&D Systems (DY393), empleado como método de

validación del análisis. Como puede observarse, existe una excelente concordancia entre las concentraciones encontradas por ambas técnicas, tal como demuestra la comparación estadística, que proporcionó valores de t_{exp} menores que el teórico, $t_{tab} = 2.447$, en todos los casos. Por otro lado, los resultados obtenidos están de acuerdo con lo previsto para este tipo de pacientes, en los que el nivel de CXCL7 es superior al valor estimado en pacientes sanos, de 850 ng mL^{-1} citado anteriormente.

Tabla 5.6. Determinación de CXCL7 en muestras de suero de pacientes diagnosticados de artritis reumatoide

Muestra	Inmunosensor, ng mL^{-1} (n=5)*	ELISA, ng mL^{-1} (n=3)*	t_{exp}
Suero 1	1205 ± 57	1204 ± 90	0.84
Suero 2	1195 ± 95	1132 ± 67	1.2
Suero 3	1488 ± 52	1478 ± 96	1.4

* valor medio $\pm$ ts/ $\sqrt{n}$

5.2.1.7. Conclusiones

En este trabajo se puso a punto el primer inmunosensor para la determinación de la quimioquina CXCL7, un biomarcador de la artritis reumatoide y otras enfermedades autoinmunes. Para su desarrollo se siguió una estrategia innovadora basada en las herramientas de la química "click", haciendo uso de la reacción de cicloadición azida-alquino, catalizada por Cu(I) (CuAAC) para preparar el conjugado IgG-alquino-azida-MWCNTs empleado para inmovilizar el anticuerpo de captura anti-CXCL7 y establecer la configuración de tipo sándwich AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE. El empleo de la técnica de voltamperometría diferencial de impulsos midiendo la señal de oxidación del producto de la reacción enzimática del 1-NPP, sustrato de la fosfatasa alcalina, 1-NP, hizo posible obtener una alta sensibilidad y una excelente precisión, así como otras características analíticas mejoradas respecto a las de los kits ELISA, con la importante ventaja del ahorro en el tiempo de ensayo, de al menos dos horas. Finalmente, los resultados obtenidos en el análisis de suero de pacientes con artritis reumatoide demostraron la utilidad del dispositivo y del método desarrollado para la determinación de esta quimioquina en muestras clínicas.

5.2.2. Inmunosensor para la determinación de IL-1 β

5.2.2.1. Configuración del inmunosensor

La plataforma electroquímica diseñada para la preparación de este inmunosensor se basa en el empleo de la metodología "electroclick", en la que el catalizador de la reacción de cicloadición CuAAC, Cu(I), se sintetiza por reducción amperométrica a partir de una sal cúprica. Las etapas del procedimiento seguido se describieron en el Apartado 4.4.2.2. y se representaron en la Figura 4.12 de la Parte Experimental. Como se observa en la Figura 5.41, la configuración desarrollada es similar a la descrita en el capítulo anterior, utilizando un SPCE modificado con azida-MWCNTs unidos a etinil-IgG por reacción "click" en presencia del catalizador, que se empleó como plataforma para la inmovilización del anticuerpo de captura anti-IL1 β , estableciéndose un esquema de tipo sándwich con un anticuerpo biotinilado y el conjugado AP-Strept. Con respecto al inmunosensor anterior hay que decir que el protocolo "electroclick" es significativamente más rápido, ya que el Cu(I) se obtiene en 300 s frente a toda la noche necesaria para la síntesis química.

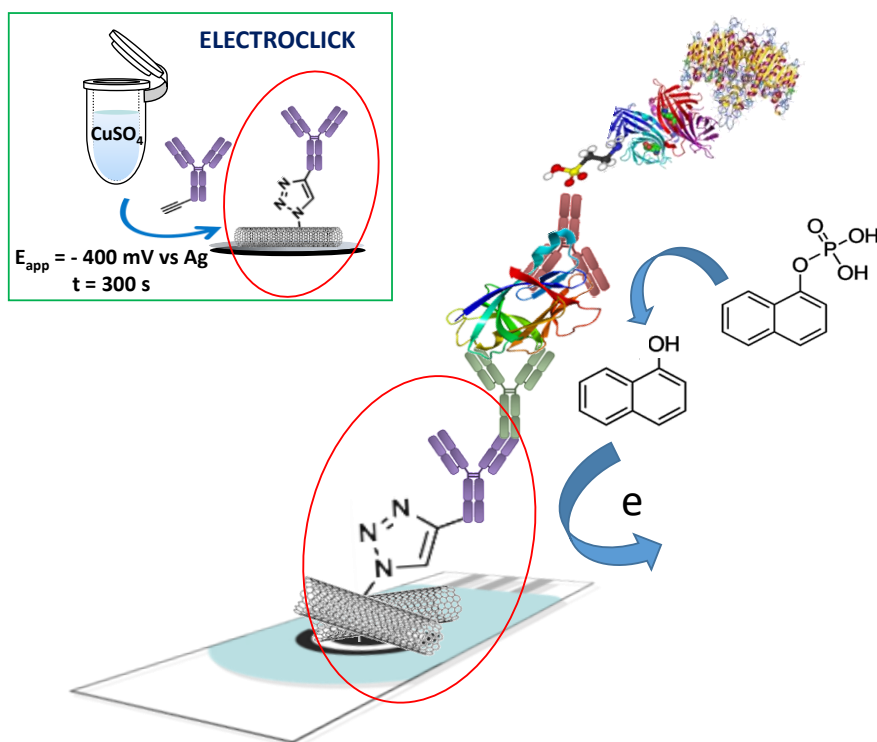


Figura 5.41. Esquema del inmunosensor AP-Strept-Biotin-anti-IL-1 β - IL-1 β -anti-IL-1 β -IgG-MWCNTs/SPCE y las reacciones implicadas en la detección. Inserto: preparación del conjugado IgG-etinil-azida-MWCNTs.

5.2.2.2. Optimización de las variables experimentales

La aplicación de la metodología "electroclick" empleada para la preparación de la plataforma electródica sobre la que se inmovilizan los inmunorreactivos, requiere que se optimicen las variables implicadas en la reacción electroquímica de síntesis del catalizador Cu(I), además de las variables de las que depende la preparación del inmunosensor propiamente dicho y su funcionamiento. Como se señaló en el capítulo anterior, los procedimientos de síntesis de los reactivos azida-MWCNTs y etinil-IgG en las condiciones optimizadas previamente [Sánchez-Tirado, 2016] se describieron en la Parte Experimental. Por tanto, en este caso, los estudios de optimización se centraron en evaluar la influencia del tipo de sal de Cu(II) y su concentración, el pH y la disolución reguladora utilizada para la reducción electroquímica, el potencial de reducción aplicado y el tiempo de electrolisis. Además, se evaluó la influencia de la concentración y el tiempo de incubación de la caseína empleada como agente bloqueante; la concentración y el tiempo de incubación del anticuerpo de captura anti-IL1 β sobre la plataforma IgG-MWCNTs / SPCE; la concentración y el tiempo de incubación del anticuerpo secundario Biotin-anti-IL1 β sobre el conjugado IL1 β -anti-IL1 β -IgG-MWCNTs/SPCE, y la concentración y el tiempo de incubación del conjugado AP-Strept sobre Biotin-anti-IL1 β -IL1 β -anti-IL1 β -IgG-MWCNTs/SPCE.

Los resultados obtenidos en los estudios de optimización se describen a continuación, recogándose en la Tabla 5.7 los intervalos estudiados y los valores óptimos seleccionados para cada variable.

Influencia del tipo de sal de Cu (II) y su concentración

Para evaluar el efecto sobre la respuesta del inmunosensor del tipo de sal cúprica utilizado para obtener el catalizador Cu(I) de la reacción CuAAC por reducción electroquímica, se midieron las corrientes específica (S) e inespecífica (N) utilizando las sales inorgánicas sulfato de cobre (II) (CuSO₄), cloruro de cobre (II) (CuCl₂), nitrato de cobre (II) (Cu(NO₃)₂) y acetato de cobre (II) (Cu(Ac)₂), con los resultados representados en la Figura 5.42a. Puede verse cómo la sal sulfato es la que proporciona la mayor relación S/N como resultado de la elevada corriente específica del inmunosensor y la débil señal en ausencia de antígeno. Sin embargo, cuando se utiliza la sal cloruro se obtiene una corriente específica mucho menor. Estos resultados están de acuerdo con los reportados

por Hein et al. [Hein, 2010], que recomiendan las sales sulfato y acetato para la reducción "in situ" de Cu(II) mientras que argumentan que el anión cloruro es perjudicial debido a su efecto inhibitorio sobre la reacción.

Una vez elegida la sal, se estudió el efecto de su concentración en el intervalo de $5 \cdot 10^{-4}$ a 5 mM, sobre la respuesta voltamperométrica del inmunosensor, con los resultados representados en la Figura 5.42b. En este caso, la mejor relación S/N se obtuvo empleando una disolución 0.005 M de CuSO_4 , que fue elegida para la obtención del catalizador.

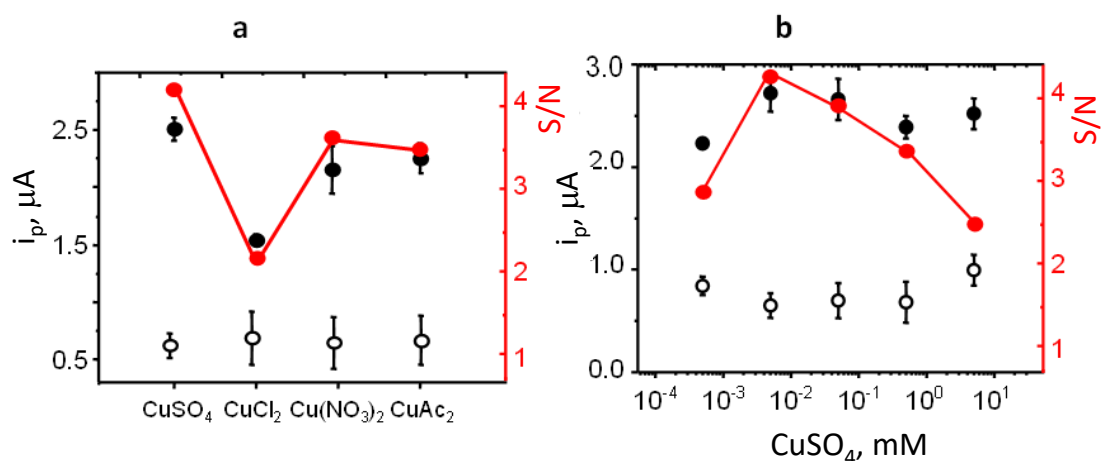


Figura 5.42 Influencia del tipo de sal de Cu (II) (a) y de la concentración de CuSO_4 (b) sobre la respuesta del inmunosensor en ausencia (○) y en presencia de 1.0 ng mL^{-1} de IL-1 β , (●). Condiciones "click": sal, 0.005 mM (a); pH=7.0; 10 mM MES; $E_{\text{red}} = -400 \text{ mV}$ vs Ag; $t_{\text{red}}=300 \text{ s}$. Condiciones inmunosensor: 1% caseína; $10 \mu\text{g mL}^{-1}$ anti-IL1 β ; $0.5 \mu\text{g mL}^{-1}$ Biotin- anti-IL1 β ; $5 \mu\text{g mL}^{-1}$ AP-Strept. Detección: 1-NPP 5 mM, 5 min reacción; DPV: $-0.2 - +0.7 \text{ V}$ vs. Ag; $\Delta E = 50 \text{ mV}$; $v = 10 \text{ mVs}^{-1}$. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Influencia del pH de la reacción "click"

Con el fin de optimizar el medio en el que se realiza la reducción de Cu(II) para obtener el catalizador de la reacción CuAAC, se estudió, en primer lugar, la influencia del pH en el intervalo de 5.0 a 8.0, regulado con disolución MES 10 mM, midiendo las respuestas del inmunosensor preparado de acuerdo con las condiciones elegidas. Los resultados obtenidos (Figura 5.43) demuestran claramente que las condiciones óptimas se alcanzan a un pH de 7.0, donde la corriente específica es máxima y también lo es la relación S/N. Este valor coincide, por otra parte, con el recomendado para llevar a cabo reacciones de bioconjugación haciendo uso de las herramientas de la química "click" [Presolski, 2011].

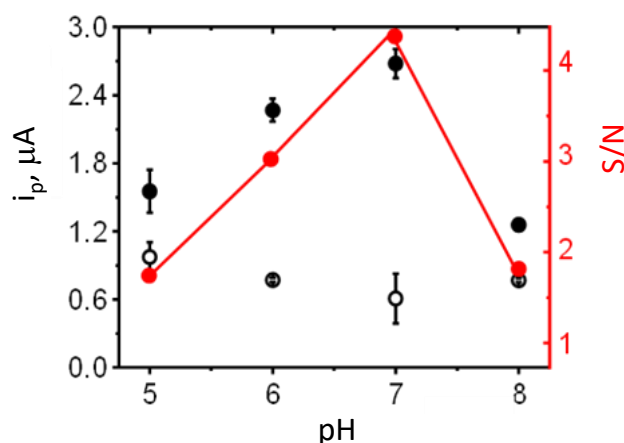


Figura 5.43. Influencia del pH de la reacción "click" sobre la respuesta del inmunosensor en ausencia ($\circ$) y en presencia de 1.0 ng mL^{-1} de IL-1 β , ($\bullet$). Condiciones "click": CuSO_4 0.005 mM ; 10 mM MES ; $E_{\text{red}} = -400 \text{ mV vs Ag}$; $t_{\text{red}}=300 \text{ s}$. Otras condiciones como en la Figura 5.42.

Influencia de la naturaleza y la concentración de la disolución reguladora

Una vez elegido el pH, se estudió la influencia sobre la respuesta del inmunosensor del tipo y concentración del regulador empleado en la reacción de cicloadición etinil-azida catalizada por el ion cuproso electrogenerado. La Figura 5.44a muestra los resultados obtenidos en la evaluación de distintas disoluciones reguladoras de pH 7.0: fosfato (PB), MES, citrato y Tris, observándose que la mayor corriente específica se alcanzaba en presencia de MES. Hay que tener en cuenta, por un lado, las posibles reacciones de los iones fosfato y citrato con el Cu(II) , que pueden dar lugar a complejos o formar especies insolubles y, por otro, la presencia perjudicial del cloruro en el regulador Tris, preparado a partir de su sal clorhidrato. Es importante señalar que, al igual que en los estudios anteriores, este resultado también está de acuerdo con los datos publicados, que recomiendan el empleo del regulador MES, o el equivalente, del ácido propano sulfónico (MOPS) como los más adecuados ya que no contienen especies que puedan interaccionar con el ion cobre [Mash, 2003]. Finalmente, respecto a la concentración de la disolución reguladora, la Figura 5.43b muestra claramente que el valor óptimo es 10 mM .

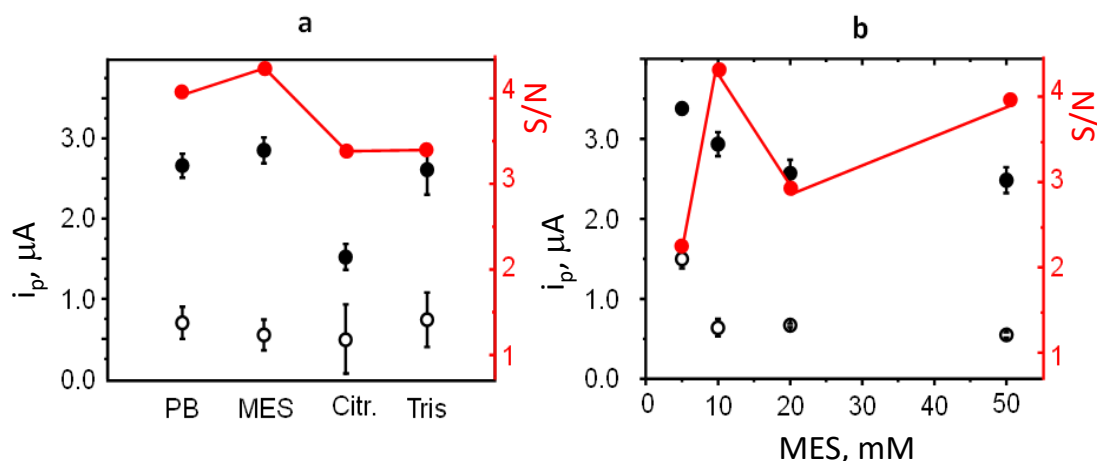


Figura 5.44. Influencia del tipo (a) y la concentración (b) de la disolución reguladora de pH de la reacción "click" sobre la respuesta del inmunosensor en ausencia (○) y en presencia de 1.0 ng mL $^{-1}$ de IL-1 β , (●). Condiciones "click": CuSO $_4$ 0.005 mM; regulador 10 mM (a); E_{red} = -400 mV vs Ag; t_{red} = 300 s. Otras condiciones como en la Figura 5.42.

Influencia del potencial de reducción y el tiempo de aplicación

Con el fin de optimizar las condiciones experimentales en las que llevar a cabo la reducción electroquímica de Cu(II) para obtener el catalizador, Cu(I), de la reacción de cicloadición etinil-azida, se estudió la influencia del potencial aplicado al electrodo y el tiempo de aplicación del mismo sobre la respuesta del inmunosensor, con los resultados que se han representado en la Figura 5.45. En el caso del potencial, se observa que tanto las corrientes específicas como las inespecíficas permanecen prácticamente constantes en el intervalo de 0.0 a -0.4 V vs Ag (Figura 5.45a) y tienden a disminuir a potenciales más negativos. Al mismo tiempo, la relación S/N es estable hasta llegar a -0.6 V. Teniendo en cuenta estos resultados, se eligió un potencial de -0.4 V como más adecuado para asegurar la formación de Cu(I) sobre el electrodo. Nuevamente, este valor concuerda bastante bien con los datos encontrados en la bibliografía. Por ejemplo, Devaraj et al. [Devaraj, 2006] aplicaron un potencial de -300 mV vs Ag/AgCl para la síntesis electroquímica de Cu(I) usado como catalizador de la reacción "click" del etilferroceno sobre un electrodo de oro modificado con una monocapa autoensamblada (SAM) funcionalizada con un grupo azida. Por otro lado, los resultados representados en la Figura 5.44b permiten elegir un tiempo de electrorreducción óptimo de 5 min, que asegura una buena respuesta del inmunosensor.

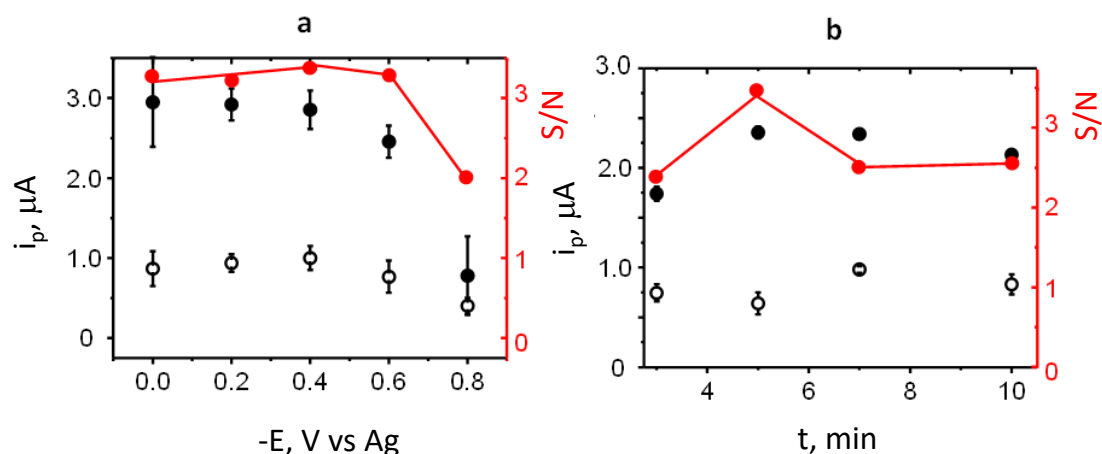


Figura 5.45. Influencia del potencial de reducción para la síntesis de Cu(I) (a) y del tiempo de aplicación del mismo (b) en la respuesta del inmunosensor en ausencia ($\circ$) y en presencia de 1.0 ng mL^{-1} de IL-1 β , ($\bullet$). Condiciones "click": CuSO_4 0.005 mM ; 10 mM MES pH 7.0; $t_{\text{red}} = 300 \text{ s}$ (a); $E_{\text{red}} = -400 \text{ mV vs Ag}$ (b). Otras condiciones como en la Figura 5.42.

Influencia de la concentración de azida-MWCNTs y etinil-IgG

Según se indica en el procedimiento descrito en la Parte Experimental, la preparación de los electrodos modificados se llevó a cabo depositando la suspensión de nanotubos funcionalizados con un grupo azida (azida-MWCNTs) sobre el SPCE, que se cubría después, una vez seco, con una disolución de reacción conteniendo etinil-IgG y CuSO_4 en regulador MES 10 mM de pH 7.0. En primer lugar, se procedió a la optimización de la cantidad de azida-MWCNTs depositado, estudiando la influencia de su concentración, en el intervalo de 0.25 a 2 mg mL^{-1} , sobre la respuesta del inmunosensor. Puede verse (Figura 5.46a) cómo una concentración de 0.5 mg mL^{-1} de nanotubos funcionalizados proporciona la mejor respuesta, por lo que se eligió para preparar la superficie azida-MWCNTs/SPCE. Por otro lado, para optimizar la cantidad de etinil-IgG, se evaluaron diferentes diluciones comprendidas entre $1/500$ y $1/50$, encontrándose (Figura 5.46b) los mejores resultados para una dilución $1/100$.

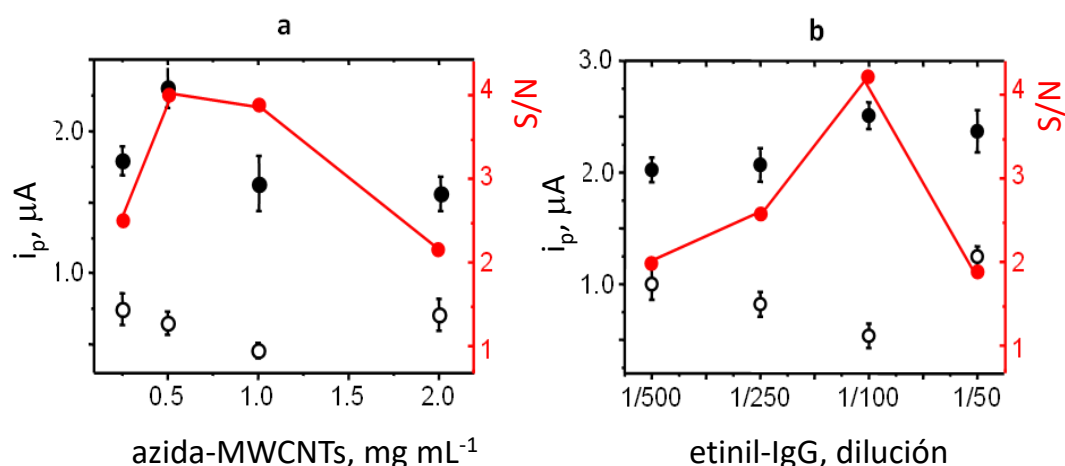


Figura 5.46. Influencia de la concentración de azida-MWCNTs (a) y de la dilución de etinil-IgG (b) en la respuesta del inmunosensor en ausencia ($\circ$) y en presencia de 1.0 ng mL^{-1} de IL-1 β , ($\bullet$). Condiciones "click": CuSO_4 0.005 mM ; $10 \text{ mM MES pH } 7.0$; $t_{\text{red}} = 300 \text{ s}$ (a); $E_{\text{red}} = -400 \text{ mV vs Ag}$ (b); 0.5 mg mL^{-1} azida-MWCNTs (b); etinil-IgG dilución $1/100$ (a). Otras condiciones como en la Figura 5.42.

Influencia de la concentración y tiempo de incubación del agente bloqueante

Una vez optimizadas las variables involucradas en la reacción "electroclick" CuAAC, se estudió el efecto de las variables de las que depende el funcionamiento del inmunosensor para IL-1 β . En primer lugar, se evaluó la influencia de la concentración de caseína usada como bloqueante sobre la corriente de pico, depositando sobre el electrodo modificado IgG-MWCNTs/SPCE alícuotas de la disolución de bloqueo preparada a diferentes niveles de concentración, entre el 0.2 y el 1.0% . Los resultados representados en la Figura 5.47a indican que por debajo de un 0.4% de caseína, el bloqueo es insuficiente, permaneciendo prácticamente constante la relación S/N a partir de este valor con un máximo apreciable para un 1.0% , que es la concentración escogida. Por otro lado, para esta concentración de caseína, el tiempo de incubación apropiado para un correcto bloqueo de la superficie del electrodo es de 30 min (Figura 5.47b).

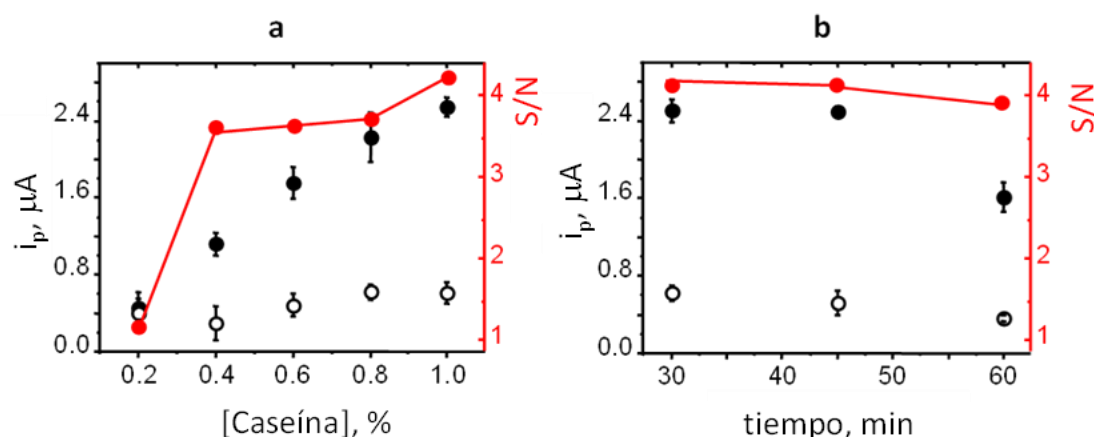


Figura 5.47. Influencia de la concentración de caseína (a) y del tiempo de incubación (b) en la respuesta del inmunosensor en ausencia ($\circ$) y en presencia de 1.0 ng mL $^{-1}$ de IL-1 β , ($\bullet$). Condiciones "click": como en la Figura 5.35. Condiciones inmunosensor: 1.0% caseína (b); 30 min (a). Otras condiciones como en la Figura 5.42.

Influencia de la concentración y tiempo de incubación de los inmunorreactivos

En este apartado se describen los estudios de optimización de los anticuerpos anti-IL1 β y Biotin-anti-IL1 β utilizados para la captura y la detección de la citoquina, así como del conjugado AP-Strept empleado como marcador enzimático para la detección. Los resultados obtenidos se han representado en la Figura 5.48.

En primer lugar, la optimización de la concentración de anti-IL1 β se llevó a cabo estudiando su efecto, sobre la respuesta voltamperométrica del inmunosensor. En la Figura 5.48a puede apreciarse que la corriente específica prácticamente no varía con la concentración de anticuerpo, mientras que sí lo hace la señal no específica a la mayor de las concentraciones estudiada. Así, la máxima relación S/N se obtuvo para 10 μg mL $^{-1}$ de anti-IL1 β . En cuanto al tiempo de incubación, la Figura 5.48b muestra claramente cómo un periodo de 60 min es el más adecuado. Además, en este estudio se pone de manifiesto que la aplicación de tiempos de incubación muy largos (en este caso 90 min) hacen que se inmovilice una gran cantidad de anticuerpo, con el consiguiente impedimento estérico y la caída de la señal. La influencia de la concentración de Biotin-anti-IL1 β sobre la respuesta del inmunosensor se estudió en el intervalo de 0.25 a 1.0 μg mL $^{-1}$, observándose cómo la señal específica aumenta ligeramente con la concentración del anticuerpo biotinilado entre 0.25 y 0.5 μg mL $^{-1}$ (Figura 5.48c), manteniéndose constante para concentraciones superiores. Por tanto, esta última concentración fue elegida para la preparación del inmunosensor. Como muestra la Figura 5.48d, una tendencia similar se

encontró en el estudio del tiempo de incubación de este anticuerpo, con un valor óptimo a 60 min. Finalmente, el estudio del efecto de la concentración y el tiempo de incubación del conjugado AP-Strept sobre la corriente de pico del inmunosensor proporcionó los resultados representados en la Figura 5.48e y f, que llevaron a elegir $5 \mu\text{g mL}^{-1}$ y 30 min para alcanzar una unión efectiva con el anticuerpo biotinilado.

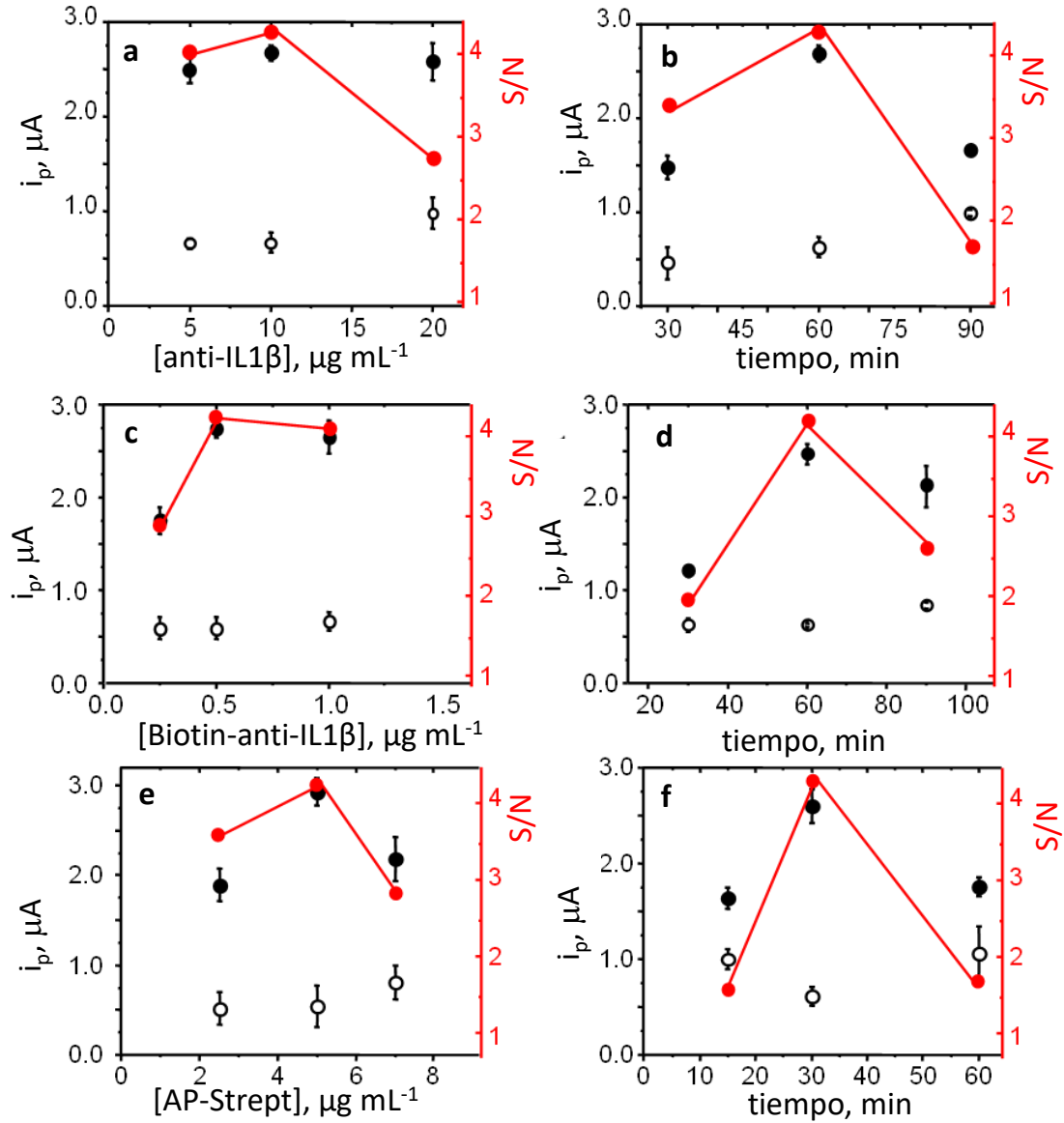


Figura 5.48. Influencia de la concentración y el tiempo de incubación de: anti-IL1 β (a, b), Biotin-anti-IL1 β (c, d) y AP-Strept (e, f) en la respuesta del inmunosensor en ausencia ($\circ$) y en presencia de 1.0 ng mL^{-1} de IL-1 β , 1% caseína, inc. 30 min; anti-IL-1 β inc. 60 min (a); $10 \mu\text{g mL}^{-1}$ anti-IL-1 β (b); Biotin-anti-IL-1 β inc. 60 min (c); $0.5 \mu\text{g mL}^{-1}$ Biotin-anti-IL-1 β (d); AP-Strept 30 min (e); $5 \mu\text{g mL}^{-1}$ AP-Strept (f). Otras condiciones como en la Figura 5.42.

En la Tabla 5.7 se resumen los estudios de optimización realizados, indicando la variable y el intervalo estudiado, así como el valor elegido en cada caso.

Tabla 5.7. Optimización de las variables experimentales.

Variable	Intervalo estudiado o compuesto	Valor seleccionado
Sal de Cu(II)	CuSO ₄ , CuCl ₂ , Cu(NO ₃) ₂ , CuAc ₂	CuSO ₄
Concentración de CuSO ₄ , mM	5 x 10 ⁻⁴ - 5	0.005
pH	5 - 8	7
Disolución reguladora de pH	PB, MES, Cit, Tris	MES
Concentración de regulador, mM	1 - 50	10
Potencial de reducción, E _{red} , mV vs Ag	-800 - 0	-400
Tiempo de aplicación de E _{red} , s	180 - 600	300
Concentración de azida-MWCNTs, mg mL ⁻¹	0.25 - 2.0	0.5
Dilución de etinil-IgG	1/500 - 1/50	1/100
Concentración de caseína, %	0.2 - 1.0	1.0
Tiempo incubación caseína, min	30 - 60	30
Concentración de anti-IL1 β , μ g mL ⁻¹	5 - 20	10
Tiempo incubación anti-IL1 β , min	30 - 90	60
Concentración de Biotin-anti-IL1 β , μ g mL ⁻¹	0.25 - 1.0	0.5
Tiempo incubación Biotin-anti-IL1 β , min	30 - 90	60
Concentración de AP-Strept, μ g mL ⁻¹	2.5 - 7.0	5.0
Tiempo incubación AP-Strept, min	15 - 60	30

5.2.2.3. Estudios de caracterización

Para caracterizar la superficie de los electrodos modificados y su comportamiento electroquímico frente a un sistema electroactivo modelo, como el Fe(CN)₆^{3-/4-}, se emplearon las técnicas de espectroscopia de impedancia electroquímica (EIS) y voltamperometría cíclica (CV). En la Figura 5.49 se han representado los espectros de Nyquist y los voltamperogramas de los electrodos en las sucesivas fases de modificación

incluida la inmovilización del anticuerpo de captura. Como se observa en la Figura 5.49a, la resistencia a la transferencia de carga medida con el electrodo sin modificar, $R_{CT} = 1654 \Omega$, es mucho mayor que la del electrodo modificado con azida-MWCNTs, $R_{CT} = 460 \Omega$, debido a la elevada conductividad de los nanotubos. Posteriormente, tras la reacción "click", se observa un ligero incremento de R_{CT} , hasta 515Ω , como corresponde al electrodo IgG-MWCNTs/SPCE debido a la presencia de la biomolécula. Finalmente, la inmovilización del anticuerpo de captura produce un aumento de la resistencia hasta alcanzar 985Ω causada por la presencia de una barrera parcialmente aislante sobre la superficie del electrodo. En este caso, teniendo en cuenta nuevamente los diferentes modelos presentados por Uygun [Uygun, 2014] para superficies biosensoras, se ha observado un mejor ajuste de las curvas obtenidas al circuito de Randles representado en la Figura 5.38a, en el que aparecen tres resistencias, R_1 correspondiente a la disolución, R_2 , la resistencia entre la disolución y el electrodo modificado con las biomoléculas, y R_3 , la resistencia entre la disolución y el propio electrodo. Por otro lado, Q_1 es la capacitancia del recubrimiento y Q_2 es la capacitancia de la doble capa.

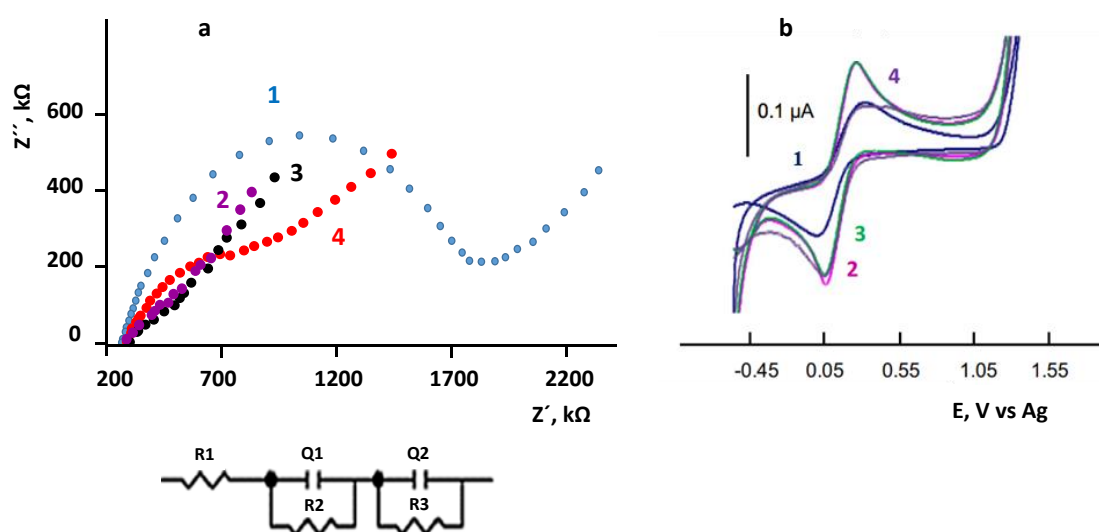


Figura 5.49. Diagramas de Nyquist con los circuitos equivalentes (a) y voltamperogramas cíclicos (b) obtenidos utilizando $\text{Fe}(\text{CN})_6^{3-/4-}$ 5.0 mM en KCl 0.1 M correspondientes al proceso de funcionalización y construcción del inmunosensor para IL-1 β : SPCE sin modificar (1); azida-MWCNT/SPCE (2); IgG-MWCNTs/SPCE (3); anti-IL1 β -IgG-MWCNTs/SPCE (4). Condiciones EIS: número de frecuencia, 50; intervalo de frecuencia, 105-0.04 Hz; amplitud 0.01 V. Condiciones CV: $v=100 \text{ mV s}^{-1}$.

Los resultados obtenidos en el estudio por voltamperometría cíclica están de acuerdo con los anteriores (Figura 5.49b). Así, el electrodo modificado con azida-MWCNTs proporciona corrientes de oxidación y reducción notablemente superiores a las del electrodo sin modificar, con valores de $i_{pa} = 120 \text{ nA}$ e $i_{pc} = 135 \text{ nA}$, frente a las obtenidas sobre el SPCE: $i_{pa} = 70 \text{ nA}$ e $i_{pc} = 80 \text{ nA}$. Por otro lado, la diferencia de los potenciales de pico es menor ($\Delta E = 200 \text{ mV}$) sobre el electrodo modificado que, sobre el electrodo sin modificar, $\Delta E = 325 \text{ mV}$. Finalmente, los voltamperogramas registrados con el electrodo IgG-MWCNTs/SPCE presentan menores corrientes de pico, disminuyendo estas de nuevo cuando se inmoviliza el anticuerpo de captura anti-IL1 β sobre el electrodo, siendo $i_{pa} = 70 \text{ nA}$ e $i_{pc} = 100 \text{ nA}$.

Los resultados obtenidos por ambas técnicas demuestran que el uso de la reacción de cicloadición empleando azida-MWCNTs y etinil-IgG combinada con la electrosíntesis del catalizador Cu(I) es adecuada para la preparación de plataformas electroquímicas para la construcción de inmunosensores.

5.2.2.4. Calibrado y características analíticas

En la Figura 5.50 se ha representado el calibrado obtenido para disoluciones patrón de IL-1 β en las condiciones experimentales optimizadas previamente, así como algunos de los voltamperogramas en DP registrados para diferentes concentraciones de antígeno. Como puede observarse, las corrientes de pico varían proporcionalmente a la concentración del analito ajustándose a dos intervalos lineales que vienen definidos por las ecuaciones: $i_p, \mu\text{A} = (4.1 \pm 0.1) [\text{IL-1}\beta], \text{ ng mL}^{-1} + (0.62 \pm 0.01)$, con $R^2 = 0.998$, entre 10 y 200 pg mL^{-1} , así como $i_p, \mu\text{A} = (2.3 \pm 0.1) [\text{IL-1}\beta], \text{ ng mL}^{-1} + (0.90 \pm 0.03)$, con $R^2 = 0.998$, entre 200 y 1200 pg mL^{-1} . Una posible explicación de la aparición de los dos tramos rectos a bajas y altas concentraciones de antígeno es la cantidad limitada de anticuerpo de captura disponible para complejar la citoquina. En efecto, al aumentar la concentración de IL-1 β , las posiciones activas del anticuerpo llegan a escasear haciendo que disminuya la pendiente del calibrado [Afsharad, 2016]. El límite de detección se calculó de acuerdo con el criterio usual, $3s_b/m$, donde m es la pendiente del calibrado obtenido a bajas concentraciones y s_b se estimó como la desviación estándar de diez medidas de la intensidad de pico en DPV del inmunosensor preparado en ausencia de analito. El valor obtenido fue $\text{LOD} = 5.2 \text{ pg mL}^{-1}$.

Las características analíticas del método desarrollado se compararon con las de otros métodos basados en técnicas de bioensayo para la determinación de IL-1 β . En relación a los kits ELISA comerciales (ver Introducción, Tabla 3.5.2), se aprecia que, por ejemplo, los de Biogen (CC) 584410 o Abcam ab214025 proporcionan valores de MDD (dosis mínima detectable) del mismo orden o ligeramente superior al del inmunosensor, pero en este caso, el intervalo lineal del calibrado es más amplio. Además, aunque existen kits ELISA más sensibles, estos requieren tiempos de ensayo notablemente superiores al del inmunosensor. Por ejemplo, el kit de Ray Biotech ELH-IL1b-1, con una MDD de 0.3 pg mL⁻¹ IL-1 β , requiere 4h 45 min frente a las 2h 30 min del método propuesto, extendiéndose el calibrado hasta una concentración mucho menor, de 100 pg mL⁻¹.

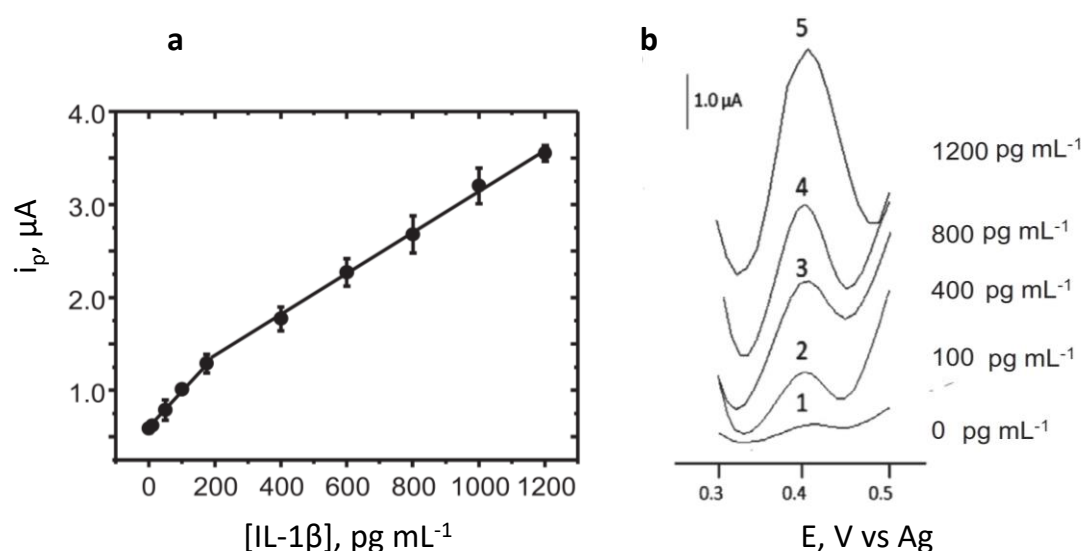


Figura 5.50. Calibrado (a) y voltamperogramas en DP (b) para la determinación de IL-1 β con el inmunosensor AP-Strept-Biotin-anti-IL1 β -IL-1 β -anti-IL1 β -IgG-MWCNTs/SPCE. Las barras de error se han estimado como el triple de la desviación estándar (n=3).

Los estudios de reproducibilidad de las medidas voltamperométricas realizadas con diez inmunosensores preparados el mismo día, en ausencia y en presencia de 1 ng mL⁻¹ de IL-1 β , proporcionaron valores de la RSD del 2.4 % y 5.2 %, respectivamente. Para inmunosensores preparados en días diferentes, los valores de RSD obtenidos fueron del 3.7% y 6.2%, respectivamente (n=10). Estos resultados demuestran la buena precisión alcanzada tanto en la medida electroquímica como en la preparación del inmunosensor para la determinación de IL-1 β .

5.2.2.5. Estabilidad y selectividad

Se investigó la estabilidad de almacenamiento de las bioplataformas anti-IL-1 β -IgG-MWCNTs/SPCE preparando en el mismo día una batería de ellas y conservándolas a 4 °C en ambiente húmedo. Posteriormente, a partir de estos conjugados se prepararon inmunosensores completos con los que se midieron disoluciones de 1 ng mL⁻¹ de IL-1 β durante diferentes días con los resultados representados en la Figura 5.51, en la que se ha incluido unos límites de control situados a $\pm 3s$, donde s es la desviación estándar de las medidas ($n=10$) realizadas el primer día de trabajo (día 0). Puede verse cómo las respuestas obtenidas se mantienen dentro de los límites de control durante al menos 10 días, que fue el máximo periodo estudiado.

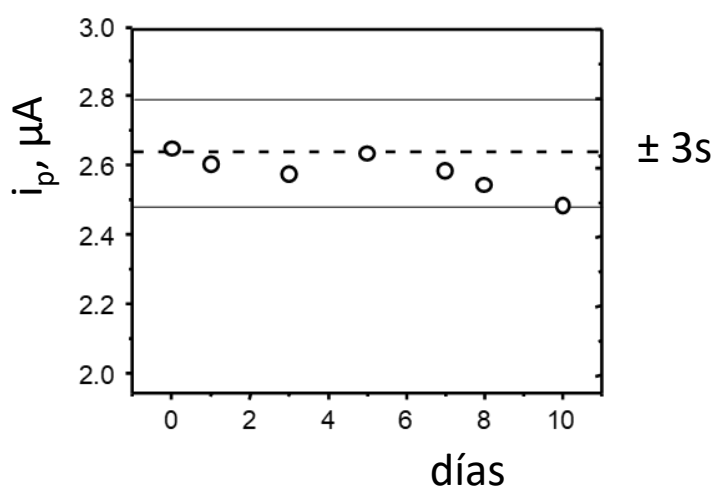


Figura 5.51. Gráfico de control para evaluar la estabilidad de los conjugados anti-IL1 β -IL-1 β -anti IL1 β -IgG-MWCNTs/SPCE en condiciones de humedad a 4°C. Ver el texto para más información.

Se llevó a cabo un estudio de la selectividad del método establecido con el inmunosensor midiendo la respuesta voltamperométrica en presencia de proteínas que pueden encontrarse junto al analito IL-1 β en sus muestras. Las especies potencialmente interferentes fueron: bilirrubina (BIL), factor de crecimiento transformante β 1 (TGF), leptina (LEP), interleucina 8 (IL-8), interleucina 6 (IL-6), hemoglobina (HB), colesterol (CHL), albúmina de suero bovino (BSA), grelina (GHRL) y factor de necrosis tumoral (TNF). Todas ellas se ensayaron a los niveles de concentración esperables en suero de individuos sanos.

En la Figura 5.52 se muestran los resultados obtenidos. Como puede apreciarse, ninguna de las especies ensayadas da lugar a interferencia significativa, apareciendo sus respuestas entre los límites situados a $\pm 3s$, donde ahora, s es la desviación estándar de diez medidas de la corriente de pico del inmunosensor en ausencia de antígeno. Estos resultados demuestran la elevada selectividad del método desarrollado, debido fundamentalmente a la práctica especificidad de los inmunorreactivos utilizados.

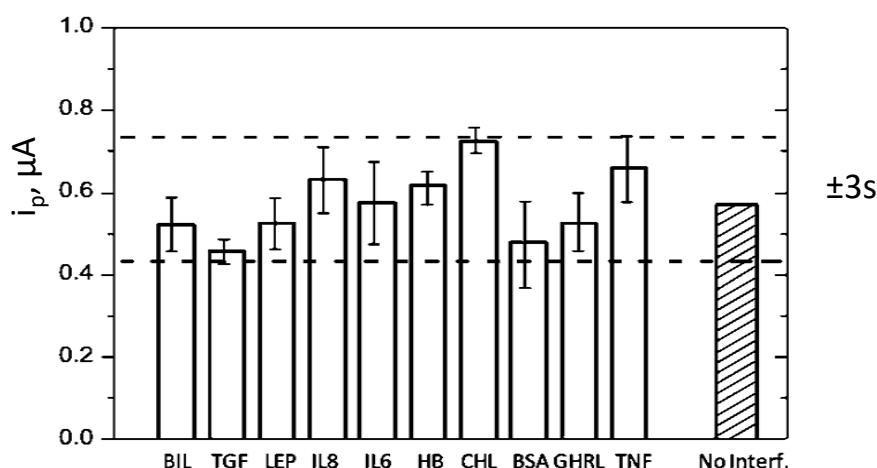


Figura 5.52. Efecto de la presencia de $190 \mu g mL^{-1}$ BIL; $500 pg mL^{-1}$ TGF; $30 pg mL^{-1}$ LEP; $30 pg mL^{-1}$ IL-8; $50 pg mL^{-1}$ IL-6; $50 \mu g mL^{-1}$ HB; $50 \mu g mL^{-1}$ CHL; $5 mg mL^{-1}$ BSA; $500 pg \mu g mL^{-1}$ GHRL y $100 pg mL^{-1}$ TNF sobre la respuesta del inmunosensor AP-Strept-Biotin-anti-IL1 β -IL-1 β -anti-IL1 β -IgG-MWCNTs/SPCE.

5.2.2.6. Determinación de IL-1 β en saliva

Las características analíticas del calibrado obtenido con el inmunosensor para IL-1 β son adecuadas para la determinación de esta citoquina en muestras de saliva, en la que la concentración esperable en individuos sanos es de unas pocas decenas de $pg mL^{-1}$ [Mirrieles, 2010] [Rathnayake, 2013]. Para esta aplicación se utilizó el intervalo de concentraciones más bajo, de 10 a $200 pg mL^{-1}$ de IL-1 β , realizando la determinación del contenido endógeno de IL-1 β sobre alícuotas de $5 \mu L$ de saliva sin diluir recolectada con el dispositivo Salivette®. Con el fin de investigar la existencia de efecto matriz en las muestras, se comparó dicho calibrado de patrones con el de adiciones de IL-1 β sobre una muestra de saliva en el mismo intervalo de concentraciones (Figura 5.53). La pendiente del calibrado de la muestra: $(4.6 \pm 0.2) 10^3 \mu A mL pg^{-1}$, resultó muy parecida a la obtenida con disoluciones patrón de IL-1 β : $(4.1 \pm 0.1) 10^3 \mu A mL pg^{-1}$, similitud que fue demostrada a nivel estadístico por aplicación del test t de Student. En efecto, el valor de $t_{exp} = 2.06$, es inferior al teórico, $t_{tab} = 2.306$, por lo que estadísticamente no existen

diferencias significativas entre ambas pendientes, lo que supone la ausencia de efecto matriz y la posibilidad de la determinación de IL-1 β en muestras de saliva por interpolación directa en el calibrado de patrones.

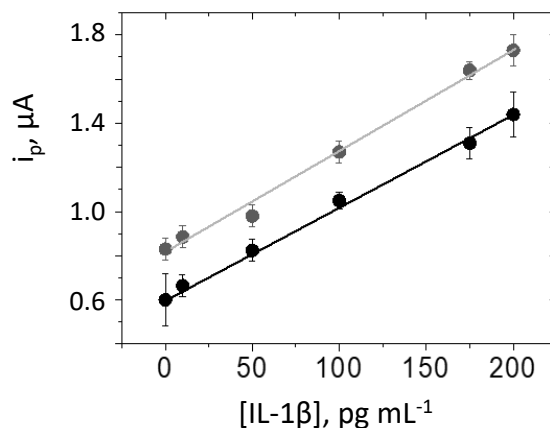


Figura 5.53. Comparación de los calibrados obtenidos con el inmunosensor AP-Strept-Biotin-anti-IL1 β -IL1 β -anti-IL1 β -IgG-MWCNTs/SPCE para disoluciones patrón de IL1 β (negro) y muestras de saliva sin diluir (gris). Las barras de error se han estimado como el triple de la desviación estándar (n=3).

Se analizaron seis muestras de saliva de voluntarios sanos por triplicado con los resultados que se resumen en la Tabla 5.8. La concentración media de IL-1 β determinada en dichas muestras, 54 ± 2 pg mL $^{-1}$, se comparó con la encontrada con el kit ELISA comercial de referencia usado como método de validación: 51.8 ± 0.5 (n=4). Estos valores, además, concuerdan con los reportados en la bibliografía para individuos sanos, en los que pueden encontrarse niveles entre 33 y 61 pg mL $^{-1}$ de IL-1 β [Mirrieles, 2010] [Rathnayake, 2013].

Tabla 5.8. Determinación de IL-1 β en muestras de saliva*.

Muestra	IL-1 β , pg mL $^{-1}$
S1	51.0 $\pm$ 0.5
S2	53.0 $\pm$ 0.3
S3	54.8 $\pm$ 0.3
S4	53.0 $\pm$ 0.2
S5	54.8 $\pm$ 0.5
S6	56.7 $\pm$ 0.5

* $\bar{x} \pm ts/\sqrt{n}$

5.2.2.7. Conclusiones

En este trabajo se introdujo por primera vez la técnica de "electroclick" para preparar un inmunosensor electroquímico usando la citoquina IL-1 β como objetivo analítico. La metodología utilizada implica la unión de la IgG etinilada a MWCNTs funcionalizados con un grupo azida mediante reacción de cicloadición catalizada por Cu(I) (CuAAC). Como novedad relevante, el ion cuproso se obtuvo mediante reducción electroquímica de una sal de Cu(II) aplicando un potencial de -0.4 V vs Ag, durante 300 s, lo que significa una considerable reducción de tiempo con respecto a la síntesis química del catalizador. Además de ser más rápida, esta estrategia participa de las ventajas de la llamada "Química Verde", y es respetuosa con el medio ambiente al no requerir el uso de reactivos. Por otro lado, la unión IgG-anticuerpo de captura permite la inmovilización orientada de este, lo que junto al diseño de una configuración de inmunoensayo tipo sándwich y el empleo de DPV como técnica de transducción, hace que se alcance una elevada sensibilidad.

Las características analíticas de método puesto a punto en este trabajo mejoran las de los kits ELISA comerciales, destacando el tiempo de ensayo, que es más de una hora más breve. Finalmente, se ha demostrado un excelente comportamiento en la determinación de IL-1 β en saliva sin diluir, sin necesidad de tratamiento alguno, y por simple interpolación en el calibrado de patrones.

5.3. BIOPLATAFORMAS ELECTROQUÍMICAS BASADAS EN MICROPARTÍCULAS MAGNÉTICAS FUNCIONALIZADAS

Las micropartículas magnéticas (MBs) funcionalizadas han demostrado ampliamente su utilidad en numerosas aplicaciones para la construcción de bioplataformas electroquímicas por su capacidad para la inmovilización de biomoléculas, entre otras ventajas. En particular, en el caso de los métodos de inmunodetección, la elevada área superficial y el alto número de funcionalidades de estas partículas las convierte en sustratos idóneos para la incorporación de anticuerpos (o antígenos), dando lugar a bioconjugados magnéticos para la detección del analito que, al prepararse fuera del electrodo, evitan o minimizan el efecto matriz y/o el riesgo de interferencias [Yáñez-Sedeño, 2016]. Además, como ya se ha señalado, las propiedades magnéticas de las MBs facilitan enormemente la preparación de estos dispositivos analíticos, especialmente las etapas de optimización de las condiciones experimentales y la detección. La combinación de estos materiales con el uso de electrodos planos proporciona las mejores condiciones para la medida electroquímica, ya que es posible concentrar los bioconjugados sobre la superficie del electrodo de trabajo logrando una transferencia electrónica eficiente que da lugar a corrientes de elevada magnitud.

En esta Tesis Doctoral se han puesto a punto cuatro plataformas electroquímicas para el desarrollo de inmunosensores asistidos por MBs funcionalizadas con diferentes grupos enlazantes, aplicados a la determinación de seis biomarcadores de enfermedades autoinmunes. El objetivo principal de estos trabajos ha sido disponer de medios de biodetección sensibles, selectivos y estables para poderlos aplicar al análisis de muestras clínicas complejas de pacientes diagnosticados con estas enfermedades.

Los objetivos específicos establecidos al inicio de estas investigaciones fueron:

1. Construir los primeros diseños de inmunoensayo electroquímico sensibles y seguros para autoanticuerpos biomarcadores de artritis reumatoide (RA) y esclerosis múltiple (MS).
2. Preparar bioplataformas electroquímicas basadas en MBs como soporte de inmovilización de las biomoléculas sobre electrodos serigrafiados duales para la determinación simultánea de dos autoanticuerpos y de dos proteínas en suero de pacientes diagnosticados con RA.

3. Utilizando una estrategia similar sobre electrodos simples, poner a punto dos esquemas de inmunodetección para la determinación de un autoanticuerpo y de una quimioquina en suero de pacientes diagnosticados con MS.

5.3.1. Bioplataforma inmunosensora para la determinación simultánea de autoanticuerpos biomarcadores de artritis reumatoide: RF y CCPA

5.3.1.1. Configuración de los bioconjugados

En el Apartado 4.4.3.1. de la Parte Experimental se describió el procedimiento de preparación de esta plataforma electroquímica, mostrándose en la Figura 4.13. un esquema de etapas implicadas en dicha preparación, así como el método de determinación simultánea de los autoanticuerpos factor reumatoide humano (RF) y anti-péptido cíclico citrunilado (CCPA), ambos biomarcadores de la enfermedad de artritis reumatoide (RA).

En la Figura 5.54 se ha representado el esquema de dicha bioplataforma en la que se muestran los inmunoconjugados magnéticos preparados para la determinación de ambos autoanticuerpos. Se emplearon partículas magnéticas modificadas con grupos carboxilo (cMBs) y con neutravidina (Neutr-MBs), respectivamente, así como electrodos serigrafiados de carbono duales, SPdCEs. La configuración desarrollada para RF se basa en la inmovilización de un fragmento de fracción constante (Fc) de inmunoglobulina G humana, Fc(IgG), sobre las cMBs, seguido del diseño de un esquema de inmunoensayo tipo sándwich que implica la incorporación del autoanticuerpo RF (analito) y un anticuerpo de detección IgM marcado con peroxidasa (HRP-IgM). Para la determinación de CCPA se inmovilizó el péptido citrunilado cíclico biotinilado (CCP-Biotin) sobre las partículas Neutr-MBs incorporando después el analito CCPA seguido de un anticuerpo de detección IgG marcado con peroxidasa (HRP-IgG). Señalar que se eligió el empleo de MBs funcionalizadas con neutravidina debido a su elevada afinidad por la biotina [Nguyen, 2012], así como por su carga casi neutra, que minimiza las interacciones no específicas, tal como se señaló en la Introducción. La determinación de ambos biomarcadores se realizó por amperometría en disolución agitada con H₂O₂ como sustrato enzimático e hidroquinona (HQ) como mediador redox, aplicando un potencial constante de - 0.2 V vs. Ag, al que se detecta la reducción electroquímica de la quinona formada como producto de la reacción catalizada por la peroxidasa.

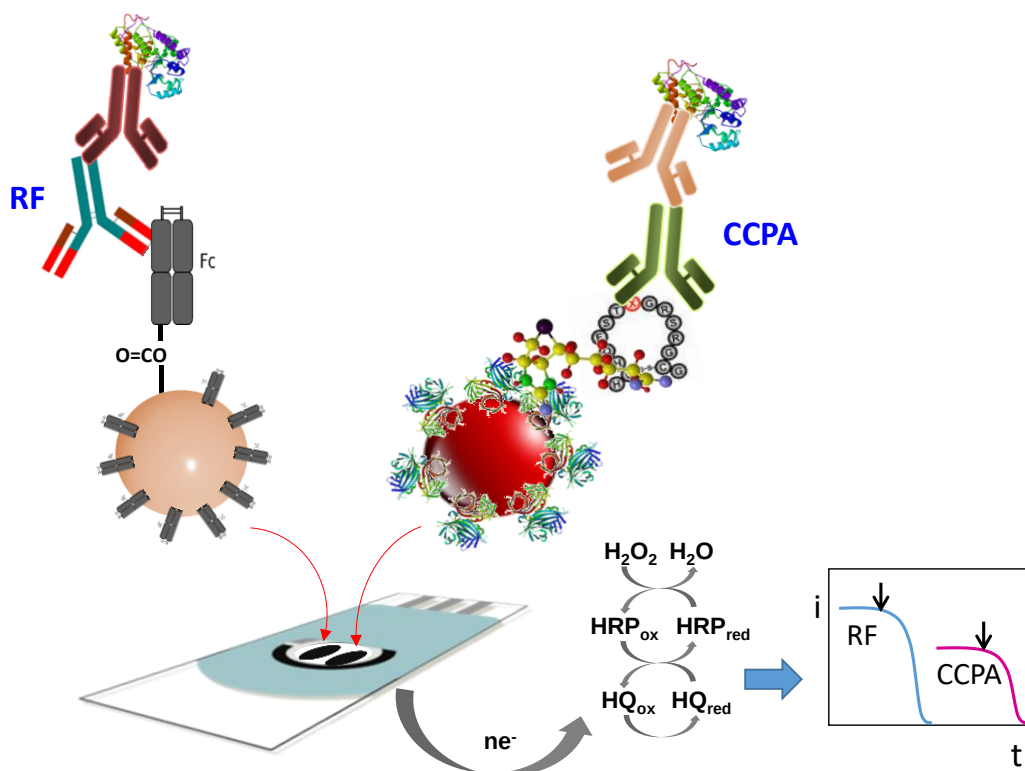


Figura 5.54. Esquema de la plataforma dual para la preparación de los inmunosensores asistidos magnéticamente: HRP-IgM-RF-Fc(IgG)-cMBs/SPdCE y HRP-IgG-CCPA-CCP-Biotin-Neutr-MBs/SPdCE, y de la reacción implicada en la detección.

5.3.1.2. Optimización de las variables experimentales

En los apartados que siguen se describen los estudios realizados para la optimización de las variables implicadas en la preparación de los bioconjugados magnéticos y en las repuestas electroquímicas de la plataforma inmunosensora construida de forma individual sobre electrodos SPdCE para cada biomarcador. Para la determinación de RF, las variables evaluadas fueron: la concentración y el tiempo de incubación de Fc(IgG) sobre las cMBs, y la dilución y el tiempo de incubación de HRP-IgM sobre el bioconjugado RF-Fc(IgG)-MBs. En el caso de CCPA, se optimizó la concentración y el tiempo de incubación del péptido biotinilado CCP-Biotin sobre las Neutr-MBs, y la concentración y el tiempo de incubación del conjugado HRP-IgG sobre CCPA-CCP-Biotin-Neutr-MBs. También se investigó la posibilidad de incorporar el anticuerpo CCPA (analito) y el conjugado HRP-IgG en una sola etapa. Otras variables experimentales como el pH y el potencial de detección adecuados para la medida amperométrica con el sistema $\text{H}_2\text{O}_2/\text{HQ}$ fueron las mismas optimizadas en trabajos anteriores [Eguílaz, 2010].

Al igual que en los capítulos anteriores, tras la descripción de los estudios de optimización, en la Tabla 5.9 se resumen los intervalos estudiados para cada variable y el valor elegido como óptimo.

Influencia de la concentración y el tiempo de incubación de Fc(IgG)

La concentración del fragmento Fc(IgG) inmovilizado sobre las partículas magnéticas carboxiladas juega un papel decisivo en el intervalo lineal de los calibrados y la sensibilidad de los inmunoensayos. Para optimizar esta variable se estudió su influencia en la respuesta del inmunosensor usando valores comprendidos entre 5 y 35 $\mu\text{g mL}^{-1}$ en disolución reguladora MES 25 mM de pH 5.0, con los resultados representados en la Figura 5.55a. Como puede apreciarse, la señal específica aumenta con la concentración del fragmento Fc hasta una concentración de 25 $\mu\text{g mL}^{-1}$, disminuyendo ligeramente para una concentración superior debido probablemente a la saturación de los grupos carboxilo de las cMBs. Por otro lado, se obtienen corrientes inespecíficas de muy baja magnitud en todo el intervalo estudiado, siendo esta una característica del uso de MBs funcionalizadas, lo que llevó a elegir la concentración citada por ser la que proporciona también la mayor relación S/N.

En cuanto al tiempo de incubación del fragmento Fc(IgG), los resultados representados en la Figura 5.55b permiten elegir un periodo de 30 min como más adecuado para estudios posteriores.

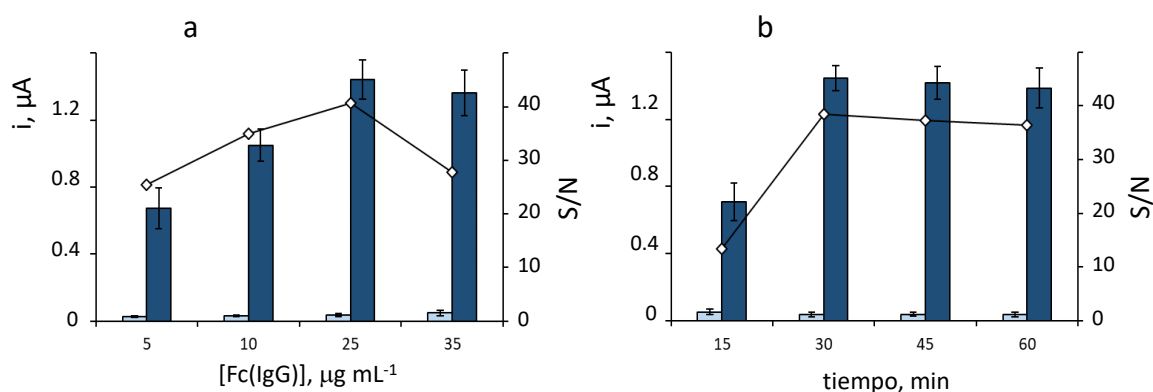


Figura 5.55. Influencia de la concentración (a) y del tiempo de incubación (b) de Fc(IgG) sobre la respuesta amperométrica en ausencia (azul claro) y presencia (azul oscuro) de 300 IU mL^{-1} RF. Fc(IgG), inc. 60 min (a); 25 $\mu\text{g mL}^{-1}$ Fc(IgG) (b); HRP-IgM sin diluir, inc. 60 min. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Influencia de la dilución y el tiempo de incubación de HRP-IgM

La concentración de HRP-IgM afecta a las respuestas amperométricas del biosensor según las tendencias que se muestran en la Figura 5.56a. Se observa que la respuesta específica disminuye al aumentar el factor de dilución mientras que la corriente inespecífica permanece prácticamente constante en todo el intervalo estudiado. De este modo, la mayor relación S/N se alcanzó usando la disolución del conjugado sin diluir. Por otro lado, el tiempo óptimo de incubación para asegurar una unión efectiva de HRP-IgM sobre el bioconjugado RF-Fc(IgG)-MBs, se alcanza a los 60 min (Figura 5.56b).

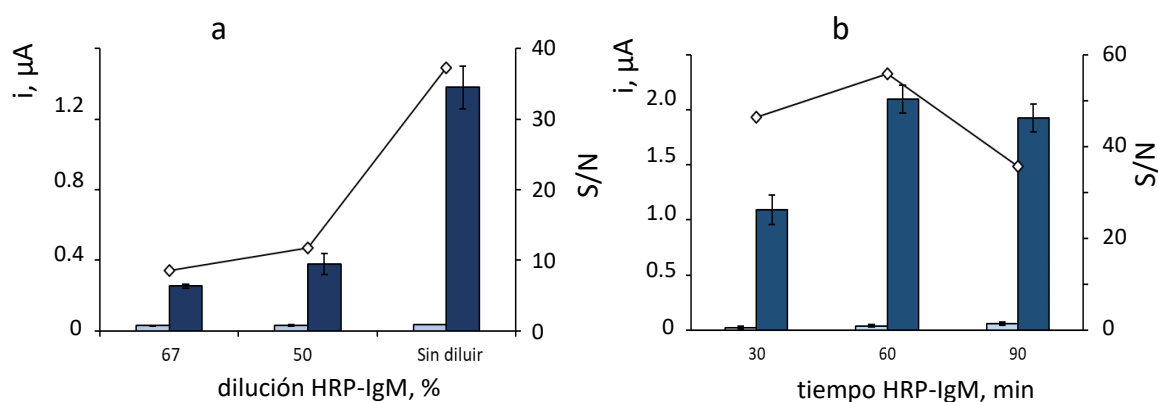


Figura 5.56. Influencia de la dilución (a) y del tiempo de incubación (b) de HRP-IgM sobre la respuesta amperométrica en ausencia (azul claro) y presencia (azul oscuro) de 300 IU mL^{-1} de RF. $25 \mu\text{g mL}^{-1}$ Fc(IgG), inc. 30 min; HRP-IgM, 60 min (a); HRP-IgM sin diluir (b). Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Influencia de la concentración y el tiempo de incubación de CCP-Biotin

El efecto de la cantidad de péptido biotinilado inmovilizado sobre las Neutr-MBs se estudió evaluando la respuesta amperométrica obtenida en ausencia y en presencia de 20 IU mL^{-1} de CCPA, tras el empleo de concentraciones de CCP-Biotin en el intervalo de 5 a 100 ng mL^{-1} . Como se observa en la Figura 5.57, es necesario utilizar una concentración de al menos $10 \mu\text{g mL}^{-1}$ para obtener una buena corriente específica. Por otro lado, las respuestas inespecíficas permanecen prácticamente constantes y con una magnitud muy baja en todo el intervalo estudiado, alcanzándose la mayor relación S/N para $25 \mu\text{g mL}^{-1}$, que fue la que se eligió como óptima. Una tendencia similar se encuentra en el estudio del tiempo de incubación del conjugado CCP-Biotin, requiriéndose un periodo de 30 min para alcanzar la mayor relación S/N.

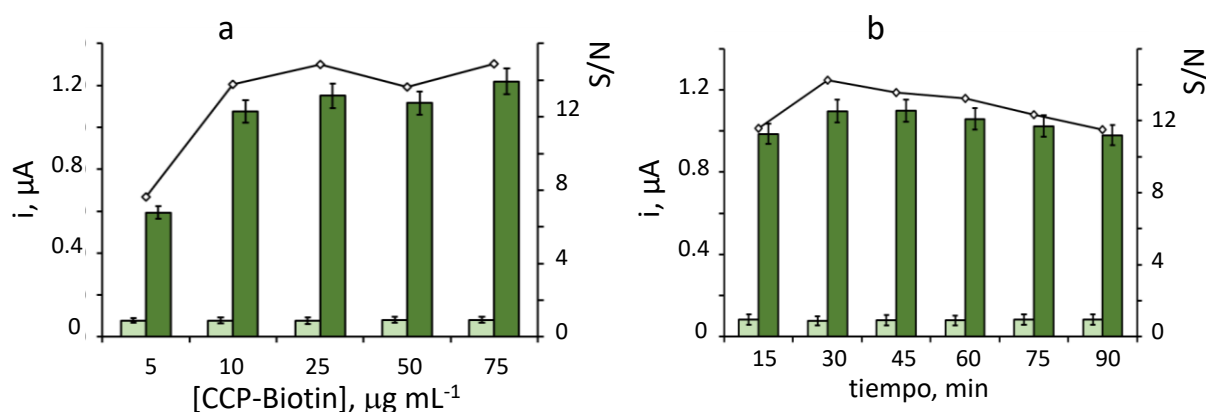


Figura 5.57. Influencia de la concentración (a) y del tiempo de incubación (b) de CCP-Biotin sobre la respuesta amperométrica en ausencia (verde claro) y presencia (verde oscuro) de 20 IU mL⁻¹ de CCPA. CCP-Biotin, inc. 30 min (a); 25 $\mu\text{g mL}^{-1}$ CCP-Biotin (b); HRP-IgG sin diluir, inc. 60 min. Barras de error estimadas como el triple de la desviación estándar (n=3).

Influencia de la dilución del conjugado HRP-IgG

En la Figura 5.58 se han representado los resultados obtenidos en la optimización de la concentración del anticuerpo secundario marcado con peroxidasa, HRP-IgG. Como se puede observar, sin apenas observarse cambios en la repuesta inespecífica, la señal específica va disminuyendo a medida que aumenta la dilución del conjugado. Por tanto, con el fin de alcanzar la máxima sensibilidad, se eligió utilizar la disolución comercial de HRP-IgG sin diluir. En cuanto al tiempo de incubación, puede verse que la corriente específica aumenta a medida que lo hace dicho periodo en el intervalo de 30 a 90 min, disminuyendo después debido a un efecto de saturación en presencia de un exceso de bioconjugado. Por otro lado, las respuestas inespecíficas aumentan muy ligeramente con el tiempo de incubación, por lo que la mayor relación S/N se obtuvo para 60 min, que se eligió como periodo óptimo para estudios posteriores.

Una vez optimizadas las variables que afectan a las respuestas amperométricas en la determinación de RF y CCPA, cabe destacar la elevada magnitud de la relación S/N obtenida en ambos casos, con valores en torno a 50 y superiores a 12, respectivamente, para los inmunosensores HRP-IgM-RF-Fc(IgG)-cMBs/SPdCE y HRP-IgG-CCPA-CCP-Biotin-Neutr-MBs/SPdCE, lo que pone de manifiesto, por un lado, la práctica ausencia de señales inespecíficas que proporciona el empleo de MBs funcionalizadas y, por otro, la elevada sensibilidad de las bioplataformas desarrolladas para detectar la reacción de inmunoensayo.

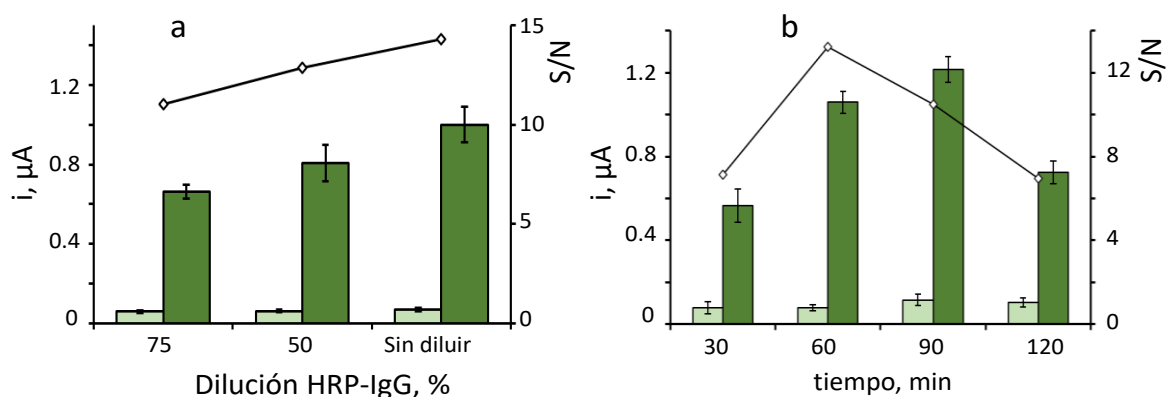


Figura 5.58. Influencia de la dilución (a) y del tiempo de incubación (b) de HRP-IgG sobre la respuesta amperométrica en ausencia (verde claro) y presencia (verde oscuro) de 20 IU mL^{-1} de CCPA. $25 \mu\text{g mL}^{-1}$ de CCP-Biotin, inc. 30 min; HRP-IgG, 60 min (a); HRP-IgG sin diluir (b). Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Influencia de la incubación de CCPA y HRP-IgG en una o dos etapas

Con el propósito de reducir en lo posible el tiempo de ensayo, se realizó un estudio comparativo de las respuestas obtenidas mediante incubación de CCPA y HRP-IgG en una sola etapa, durante 60 min, usando una mezcla de ambos inmunorreactivos, o en dos etapas de 60 min, empleando disoluciones de cada uno de ellos. En la Figura 5.59 se muestran los resultados obtenidos en ambas situaciones, observándose una importante pérdida de señal específica cuando los anticuerpos se incuban en la misma etapa. Este hecho se atribuye a la menor eficiencia de biorreconocimiento entre el CCPA y el péptido CCP-Biotin inmovilizado sobre las Neutr-MBs debido al impedimento producido por la presencia de HRP-IgG.

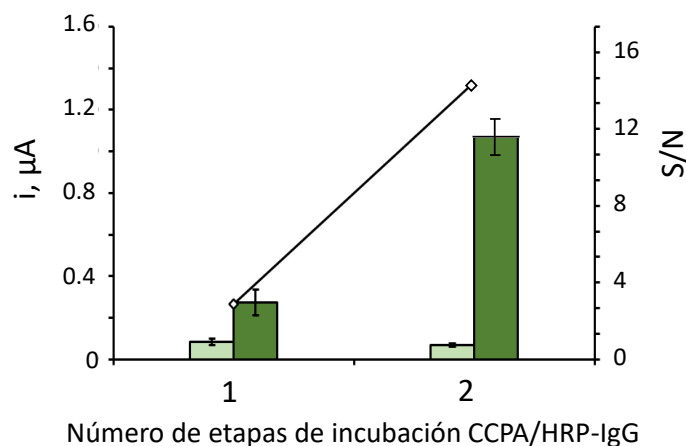


Figura 5.59. Influencia de la incubación de CCPA y HRP-IgG en una o dos etapas sobre la respuesta amperométrica. Otras condiciones como la Figura 5.57.

Tabla 5.9. Optimización de las variables experimentales.

Variable	Intervalo estudiado	Valor seleccionado
Concentración de Fc(IgG), $\mu\text{g mL}^{-1}$	5 - 35	25
Tiempo incubación Fc(IgG), min	15 - 60	30
Dilución HRP-IgM, %	sin diluir, 50, 67	sin diluir
Tiempo incubación HRP-IgM, min	30 - 90	60
Concentración de CCP-Biotin, $\mu\text{g mL}^{-1}$	5 - 100	25
Tiempo incubación CCP-Biotin, min	15 - 90	30
Dilución HRP-IgG, %	sin diluir, 50, 75	sin diluir
Tiempo HRP-IgG, min	30 - 120	60
Etapas incubación CCPA/HRP-IgG	1, 2	2

5.3.1.3. Calibrados y características analíticas

En la Figura 5.60 se han representado los calibrados obtenidos para RF y CCPA con la bioplataforma desarrollada sobre el electrodo SPdCE en las condiciones óptimas elegidas anteriormente, mostrándose en la Figura 5.61 algunas señales amperométricas registradas para cada biomarcador.

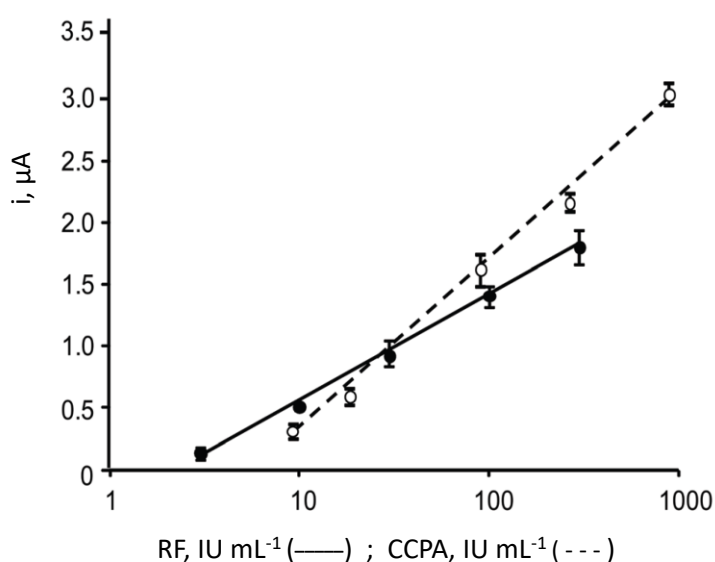


Figura 5.60. Calibrados obtenidos para la determinación de RF (línea continua) y CCPA (línea discontinua) utilizando la inmunoplateforma dual: HRP-IgM-RF-Fc(IgG)-cMBs/SPdCE y HRP-IgG-CCPA-CCP-Biotin-Neutr-MBs/SPdCE. Barras de error estimadas como el triple de la desviación estándar (n=3)

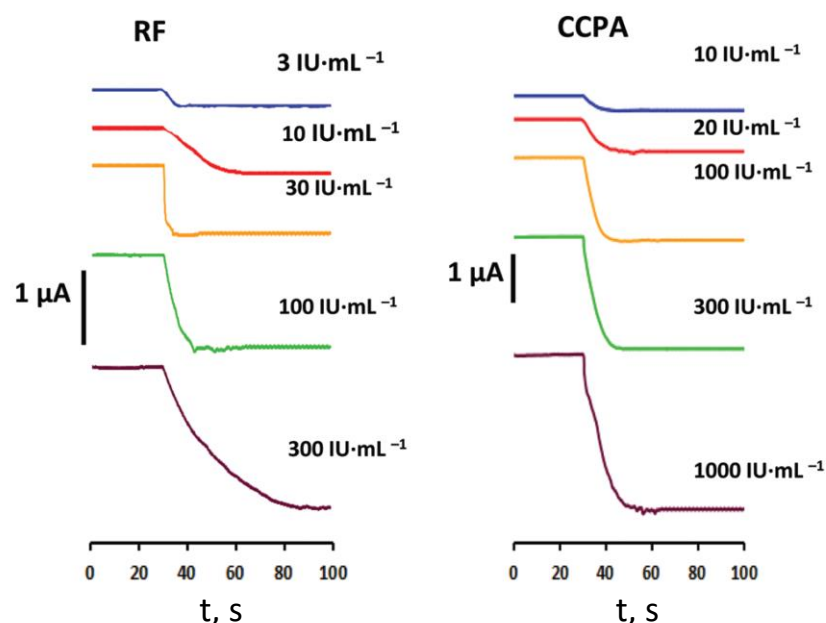


Figura 5.61. Señales amperométricas registradas para la obtención de los calibrados de RF y CCPA por medida a -0.2 V vs Ag.

El calibrado para la determinación de RF se extiende en el intervalo lineal comprendido entre 3 y 300 IU mL⁻¹, con $R^2 = 0.995$, ajustándose a la ecuación: $i, \text{nA} = (848 \pm 36) \log ([\text{RF}], \text{IU mL}^{-1}) - (315 \pm 59)$. El límite de detección se calculó con el criterio habitual de $3 s_b/m$, donde s_b es la desviación estándar de diez respuestas amperométricas registradas en ausencia de analito. El valor obtenido fue: LOD = 0.8 IU mL⁻¹. Por otro lado, el intervalo lineal del calibrado para CCPA abarca desde 10 hasta 1000 IU mL⁻¹ ($R^2 = 0.995$), y viene regido por la ecuación: $i, \text{nA} = (1409 \pm 54) \log ([\text{CCPA}], \text{IU mL}^{-1}) - (1179 \pm 113)$. En este caso, se obtuvo: LOD = 2.5 IU mL⁻¹.

Estas características se compararon con las de los métodos existentes para la determinación individual de los biomarcadores (ver Tablas 3.5.6 y 3.5.7 en la Introducción). Por ejemplo, entre los kits ELISA comerciales, los de la marca Eagle Biosciences (RFM31-K01 y CCP31-K01) alcanzan, respectivamente, una concentración mínima detectable de RF de 1 IU mL⁻¹ y de 2 IU mL⁻¹ CCPA, esta última expresada como sensibilidad funcional. Estos valores son muy parecidos a los límites de detección obtenidos en la determinación simultánea utilizando la bioplataforma electroquímica descrita en este trabajo. Por otro lado, cuando se establece la comparación con los pocos biosensores existentes en la bibliografía se encuentra, en algún caso, un límite de detección muy superior, como el del biosensor descrito por Pan et al. [Pan, 2015], con un

LOD de 40 IU mL⁻¹ RF. Sin embargo, los demás biosensores de RF y de CCPA muestran límites de detección del mismo orden de magnitud o inferiores. Obviamente, la principal ventaja de la bioplataforma desarrollada es la posibilidad de determinar simultáneamente los dos biomarcadores de artritis reumatoide, lo que no es posible usando los kits ELISA y no ha sido descrito hasta ahora para ningún biosensor. Además, el volumen de muestra necesario para hacer esta determinación es de tan solo 25 µL de cada biomarcador para su determinación simultánea, frente a los 100 µL que se requieren en los test ELISA para la medida individual.

La repetibilidad de las respuestas amperométricas se evaluó por medida de disoluciones en ausencia y en presencia de 300 IU mL⁻¹ de RF o 20 IU mL⁻¹ de CCPA con diez bioplataformas duales diferentes preparadas el mismo día. Los valores de RSD para las medidas en ausencia de RF y CCPA fueron del 6.1% y el 5.4%, respectivamente, mientras que los valores de RSD calculados a partir de las respuestas obtenidas en presencia de RF y de CCPA fueron del 6.8% y el 5.7%. Valores similares de RSD se obtuvieron para las medidas realizadas con bioplataformas preparadas en diferentes días, lo que confirma la adecuada reproducibilidad del procedimiento experimental seguido incluyendo la preparación de los magnetoconjugados, su captura sobre los SPdCEs y la detección amperométrica simultánea.

5.3.1.4. Estabilidad, selectividad y reactividad cruzada

Como en otras configuraciones, en la preparada para la determinación simultánea de RF y CCPA se estudió, respectivamente, la estabilidad de almacenamiento de los conjugados Fc(IgG)-cMBs y CCP-Biotin-Neutr-MBs. Para ello, siguiendo el procedimiento experimental optimizado anteriormente, se preparó, en el mismo día, una batería de cada tipo de bioconjugados que se almacenaron a 8°C en una disolución de PBS. A partir de ellos, cada día de trabajo, se desarrollaron las correspondientes configuraciones de tipo sándwich con 300 IU mL⁻¹ de RF o 1000 IU mL⁻¹ de CCPA, disponiéndolas sobre el SPdCE. Los resultados obtenidos, recogidos en la Figura 5.62, muestran cómo las respuestas de la plataforma dual se mantuvieron durante 20 días dentro de los límites de control establecidos en $\pm 2s$, siendo s la desviación estándar de las medidas ($n=3$) realizadas el primer día del estudio.

Estos resultados tienen una gran importancia desde el punto de vista de la utilidad práctica del inmunosensor porque demuestran que es posible preparar varios conjugados, almacenarlos y, posteriormente, cuando se requiera, construir la bioplataforma dual para su uso inmediato.

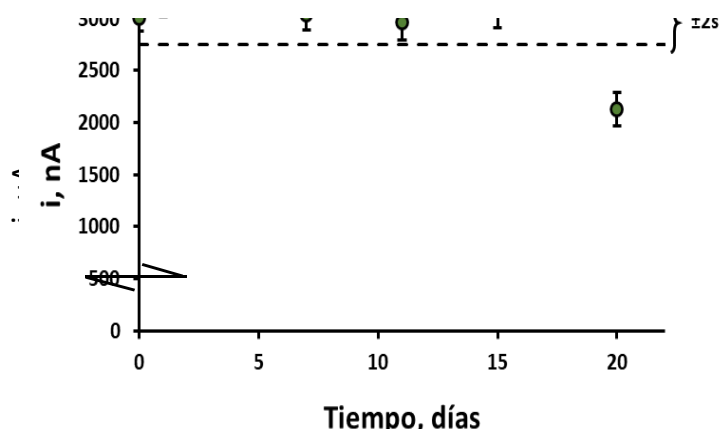


Figura 5.62. Gráfico de control para evaluar la estabilidad de los conjugados Fc(IgG)-cMBs (azul) y CCP-Biotin-Neutr-MBs (verde) en PBS a 8 °C. Ver el texto para más información.

La selectividad de la inmunoplataforma dual se investigó midiendo las respuestas amperométricas de disoluciones preparadas en ausencia y en presencia de 30 IU mL⁻¹ de RF y de 20 IU mL⁻¹ de CCPA, que contenían distintas proteínas que pueden estar presentes en las muestras de estos biomarcadores a las concentraciones esperables en suero de individuos sanos: 1 mg mL⁻¹ de inmunoglobulina humana (hIgG); 50 mg mL⁻¹ de albúmina de suero humana (HSA); 5 mg mL⁻¹ de hemo-globina (HB); 100 pg mL⁻¹ de interferón γ (IFN); 50 pg mL⁻¹ de ligando de receptor activador para el factor nuclear κ B (RANKL); 50 pg mL⁻¹ de interleucina 1 β (IL-1 β); 300 pg mL⁻¹ de interleucina 6 (IL-6); 300 pg mL⁻¹ de interleucina 8 (IL-8); 200 pg mL⁻¹ de factor de necrosis tumoral α (TNF- α); 200 pg mL⁻¹ de C-X-C ligando 4 (CXCL4), y 50 ng mL⁻¹ de C-X-C ligando 7 (CXCL7). La Figura 5.63 muestra los resultados obtenidos, observándose cómo ninguna de las proteínas estudiadas interfiere en la determinación de RF o CCPA en las condiciones experimentales utilizadas, reflejando así la alta selectividad del fragmento Fc(IgG) y del péptido CCP-Biotin utilizados en la captura de los analitos de interés.

Por otro lado, con el fin de evaluar la posible reactividad cruzada entre los electrodos de trabajo adyacentes, W1 y W2, se midieron las respuestas amperométricas de mezclas de RF y CCPA preparando conjugados HRP-IgM-X-Fc(IgG)-cMBs y HRP-IgG-X-CCP-Biotin-Neutr-MBs donde X representa: a) 0 IU mL⁻¹ de RF y de CCPA ; b) 1000 IU mL⁻¹ de CCPA en W1 y 300 IU mL⁻¹ de RF en W2, y c) 1000 IU mL⁻¹ de CCPA en W2 y 300 IU mL⁻¹ de RF en W1. Como muestra la Figura 5.64, no existe interferencia cruzada significativa entre los electrodos de trabajo, lo que demuestra nuevamente la utilidad de la bioplataforma dual para la determinación simultánea de los biomarcadores.

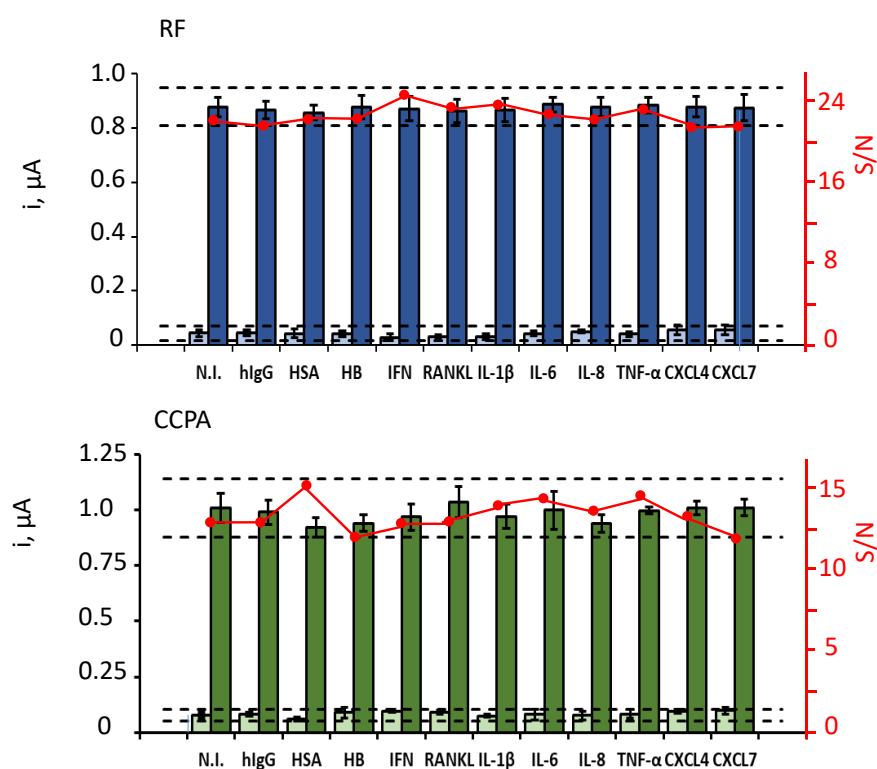


Figura 5.63. Respuestas amperométricas obtenidas con la bioplataforma dual en ausencia de RF (azul claro) o de CCPA (verde claro), y en presencia de 30 IU mL⁻¹ de RF (azul oscuro) o 20 IU mL⁻¹ de CCPA (verde oscuro), y en disoluciones preparadas en ausencia (N.I.) o en presencia de hIgG, HSA, HB, IFN, RANKL, IL-1 β , IL-6, IL-8, TNF- α , CXCL4 o CXCL7. Barras de error estimadas como tres veces la desviación estándar de tres replicados. Ver el texto para más información.

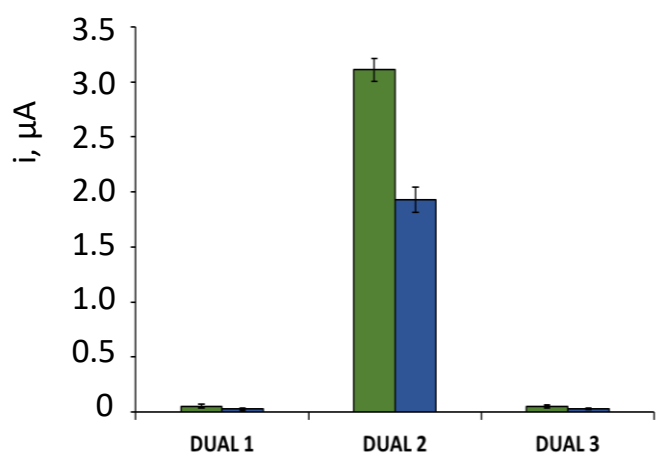


Figura 5.64. Respuestas amperométricas obtenidas simultáneamente: a) en ausencia de CCPA (verde) y RF (azul) (DUAL 1), b) en presencia de 1000 U mL^{-1} de CCPA y 300 U mL^{-1} de RF utilizando HRP-IgG-CCPA-CCP-Biotin-Neutr-MBs en W1 y HRP-IgM-RF-Fc(IgG)-cMBs en W2 (DUAL 2), y c) HRP-IgG-RF-CCP-Biotin-Neutr-MBs en W1 y HRP-IgM-CCPA-Fc(IgG)-cMBs en W2 (DUAL 3). Barras de error estimadas como tres veces la desviación estándar de tres replicados. Ver el texto para más información.

5.3.1.5. Determinación simultánea de RF y CCPA en suero

Como se ha visto, los intervalos lineales de los calibrados para la determinación de RF ($3 - 300 \text{ IU mL}^{-1}$) y de CCPA ($10 - 1000 \text{ IU mL}^{-1}$) abarcan amplios márgenes de concentración, alcanzando respectivamente límites de cuantificación (LOQs) de 2.7 IU mL^{-1} y 8.3 IU mL^{-1} . Trasladando estos resultados a la aplicación a muestras reales de interés clínico, hay que considerar los datos existentes acerca de los niveles de corte de ambos biomarcadores en suero para diferenciar individuos sanos de pacientes con artritis reumatoide. En este punto es importante señalar que la información al respecto es numerosa, pero los datos no son coincidentes. Por ejemplo, se ha publicado el criterio de $\text{RF} > 40 \text{ IU mL}^{-1}$ y $\text{CCPA} > 50 \text{ IU mL}^{-1}$ como adecuado para el diagnóstico de la enfermedad en pacientes con artritis temprana [Jansen, 2002]. Más recientemente, las concentraciones de corte de ambos biomarcadores se han extendido a los intervalos de 6 a 30 IU mL^{-1} para RF y 5 a 25 IU mL^{-1} CCPA [Zhu, 2019]. Como puede verse, el método desarrollado es apropiado para determinar los autoanticuerpos en muestras de suero humano, en los que cabe esperar niveles de concentración similares o superiores a los indicados.

La utilidad del biosensor dual se demostró mediante su aplicación a la determinación de RF y CCPA en controles séricos positivos y negativos proporcionados por las casas comerciales Abcam (ab178653) y Demeditec (DE7760), respectivamente. La existencia de efecto matriz se evaluó comparando las pendientes de los calibrados de disoluciones estándar de RF o CCPA con las obtenidas por adiciones patrón de los autoanticuerpos sobre el suero sin diluir. La aplicación del test t de Student para un nivel de significación de 0.05 y 5 grados de libertad proporcionó valores de t_{exp} de 2.219 para RF y 0.689 para CCPA, ambos inferiores al $t_{\text{tab}} = 2.571$, por lo que estadísticamente no se aprecian diferencias significativas entre las pendientes estudiadas. Por lo tanto, la determinación de los autoanticuerpos RF y CCPA se pudo realizar mediante la interpolación directa de las señales obtenidas en el calibrado de patrones. En la Tabla 5.10 se comparan los resultados del análisis de las muestras de suero para la determinación simultánea de los dos biomarcadores empleando la bioplataforma dual desarrollada en este trabajo con los que proporcionan los kits ELISA, en este caso de forma individual para cada uno de ellos siguiendo los protocolos correspondientes basados en la detección colorimétrica con el sistema H_2O_2 / TMB.

Tabla 5.10. Determinación simultánea de RF y CCPA en suero humano*.

Suero	RF, IU mL⁻¹		CCPA, IU mL⁻¹	
	Bioplataforma dual (n=6)	ELISA (n=3)	Bioplataforma dual (n=6)	ELISA (n=3)
Control negativo	4.3 ± 0.5	4 ± 1	9 ± 1	9 ± 5
Control positivo	62 ± 6	64 ± 9	263 ± 5	265 ± 10

* Valor medio ± ts

Puede observarse una buena concordancia entre los resultados obtenidos empleando ambas metodologías. Cabe señalar que las concentraciones encontradas en los controles negativos están al nivel de lo que sería de esperar de acuerdo con la bibliografía [Zhu, 2019]. Por otro lado, los controles positivos contienen un nivel medio de RF similar al encontrado en otros estudios dedicados al seguimiento de pacientes con artritis reumatoide (77 IU mL⁻¹) y del mismo orden de magnitud, en el caso del CCPA, que el encontrado en esos mismos estudios (169 IU mL⁻¹) [Turesson, 2007].

5.3.1.6. Conclusiones

En este trabajo se ha desarrollado la primera inmunoplateforma electroquímica dual para la determinación simultánea de factor reumatoide (RF) y anticuerpo anti-péptido cíclico citrunilado (CCPA), dos autoanticuerpos biomarcadores clave en el diagnóstico y seguimiento clínico de la artritis reumatoide. Para su construcción se aprovechó la afinidad del anticuerpo RF por el fragmento Fc de la IgG y por la IgM, así como la fortaleza y práctica especificidad de la unión neutravidina-biotina junto a la capacidad de las micropartículas magnéticas (MBs) para la preparación de bioconjugados estables, para inmovilizar, respectivamente, HRP-IgM-RF-Fc(IgG) e IgG-CCPA-CCP-Biotin sobre MBs funcionalizadas con grupos carboxilo y con neutravidina e implementar dos configuraciones de inmunoensayo tipo sándwich sobre electrodos serigrafiados duales de carbono (SPdCEs), empleando anticuerpos de detección marcados con peroxidasa. Entre las ventajas del método propuesto cabe señalar la elevada sensibilidad y amplio intervalo lineal de los calibrados, así como el tiempo de ensayo, de dos horas para la determinación simultánea de los dos biomarcadores, requiriéndose tan solo 25 μ L de cada muestra para su determinación simultánea.

5.3.2. Bioplateforma inmunosensora para la determinación simultánea de biomarcadores de inflamación en artritis reumatoide: CXCL7 y MMP3

5.3.2.1. Configuración de los bioconjugados

En la Figura 5.65 se ha representado un esquema de la plataforma electroquímica dual desarrollada para la determinación simultánea de los biomarcadores CXCL7 (quimioquina C-X-C ligando 7) y MMP3 (enzima metaloproteinasa de matriz 3), basada en el uso de micropartículas magnéticas carboxiladas (cMBs) y electrodos serigrafiados duales de carbono (SPdCE). Las etapas implicadas en su preparación y el procedimiento seguido se describieron en el Apartado 4.4.3.2. de la Parte Experimental y se representaron en la Figura 4.14. Como ya se informó en la Introducción, estas proteínas están involucradas en los procesos inflamatorios asociados a la artritis reumatoide. En el caso de la CXCL7, se encuentran niveles elevados en las fases iniciales de la enfermedad, mientras que la enzima MMP3, debido a su actividad gelatinasa, también participa en el deterioro de las articulaciones, por lo que su detección permite apreciar el progreso degradativo y observar la eficacia de los tratamientos.

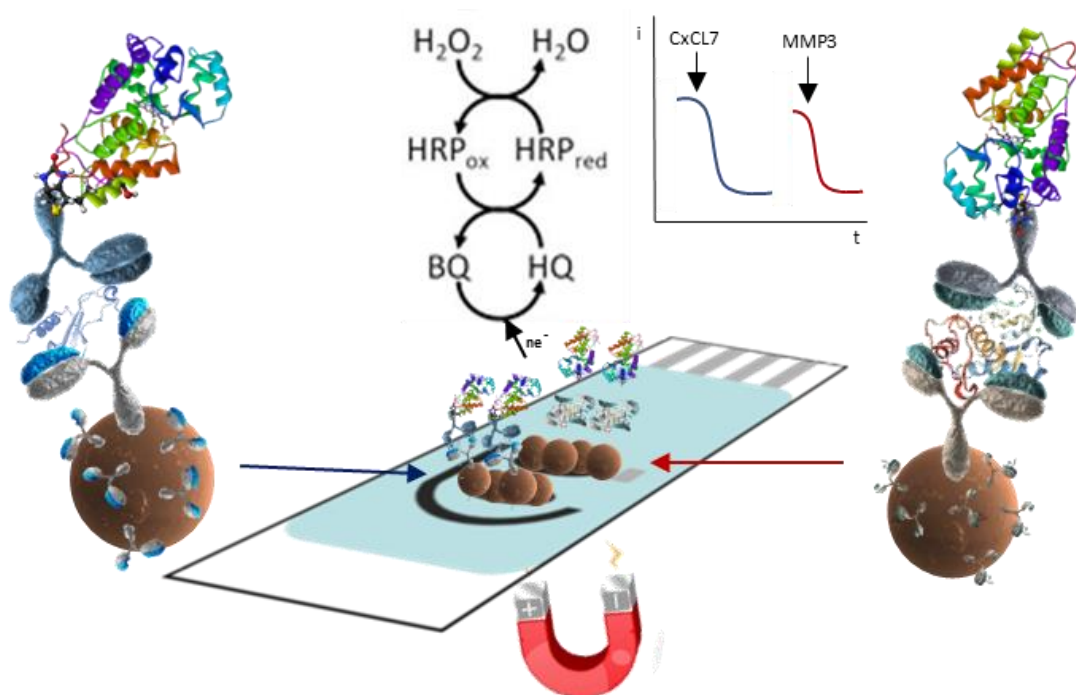


Figura 5.65. Esquema de la plataforma dual HRP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-cMBs/ SPdCE y HRP-Strept-Biotin-anti-MMP3-MMP3-anti-MMP3-cMBs/SPdCE, y de la reacción implicada en la detección.

Como puede verse en la figura, los bioconjugados preparados sobre las cMBs son equivalentes para ambos biomarcadores, desarrollándose esquemas de inmunoensayo de tipo sándwich con los correspondientes anticuerpos de detección modificados con biotina, Biotin-anti-CXCL7 y Biotin-anti-MMP3, y un conjugado enzimático HRP-Strept. Al igual que en el capítulo anterior, la determinación electroquímica de estas dos proteínas se realizó mediante amperometría a un potencial de reducción -0.2 V vs Ag, empleando H_2O_2 como sustrato enzimático e hidroquinona (HQ) como mediador redox.

5.3.2.2. Optimización de las variables experimentales

Para la puesta a punto de esta bioplataforma dual, se optimizaron las variables que afectan a su preparación y a la respuesta electroquímica, tomando como criterio de selección la mayor relación de corrientes entre las señales amperométricas obtenidas en ausencia (N) y en presencia (S) de 1 ng mL^{-1} de CXCL7 o de 2 ng mL^{-1} de MMP3. Para mayor facilidad, los estudios de optimización se realizaron sobre plataformas individuales, usando electrodos serigrafiados de carbono con una única superficie de

trabajo (SPCEs). Las variables estudiadas fueron la concentración y tiempo de incubación de los anticuerpos de captura (anti-CXCL7 o anti-MMP3) y de detección (Biotin-anti-CXCL7 o Biotin-anti-MMP3), la concentración del conjugado de estreptavidina marcada con HRP (HRP-Strept) y el tiempo de incubación de la etanolamina usada como agente bloqueante. Otras variables, como la cantidad de partículas magnéticas carboxiladas y la concentración de etanolamina fueron las recomendadas en los protocolos de utilización de las cMBs. En cuanto en la activación de los grupos carboxilo con EDC/NHSS y la medida amperométrica, se utilizaron las condiciones optimizadas en trabajos anteriores.

Influencia de la concentración y el tiempo de incubación de anti-CXCL7

En la Figura 5.66 se han representado los resultados obtenidos en el estudio de estas variables empleando bioplateformas preparadas en ausencia (verde claro) y en presencia de 1 ng mL^{-1} de CXCL7, así como diferentes concentraciones de anticuerpo de captura anti-CXCL7 en el intervalo de 2.5 a $25 \text{ } \mu\text{g mL}^{-1}$ (Figura 5.66a) y tiempos de incubación de 30 a 75 min (Figura 5.66b).

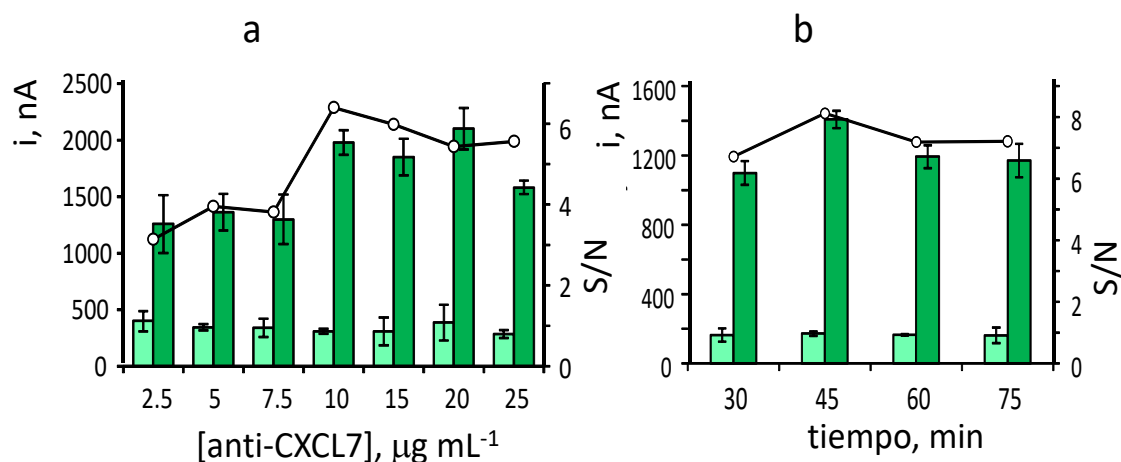


Figura 5.66. Influencia de la concentración (a) y del tiempo de incubación (b) de anti-CXCL7 sobre la respuesta amperométrica en ausencia (verde claro) y presencia (verde oscuro) de 1 ng mL^{-1} de CXCL7, inc. 60 min ; anti-CXCL7, inc. 45 min (a); $10 \text{ } \mu\text{g mL}^{-1}$ anti-CXCL7 (b); etanolamina 1 M , inc. 30 min ; $0.25 \text{ } \mu\text{g mL}^{-1}$ Biotin-anti-CXCL7, inc. 60 min ; HRP-Strept dil. $1/2000$, inc. 20 min . Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Como puede observarse, la concentración de anticuerpo que proporciona la mejor relación S/N es la de $10 \text{ } \mu\text{g mL}^{-1}$. Concentraciones menores proporcionan una menor respuesta específica al disminuir el número de sitios de inmovilización, mientras que por

encima de dicha concentración se aprecia una tendencia a disminuir la magnitud de dicha respuesta, probablemente como consecuencia de un efecto de saturación. En cuanto a la corriente inespecífica, esta permanece prácticamente constante en todo el intervalo de concentraciones estudiado. Por otro lado, los resultados obtenidos en el estudio del tiempo de incubación (Figura 5.66b) muestran una mejor relación S/N para 45 min, que fue el tiempo elegido para el trabajo posterior.

Influencia del tiempo de bloqueo con etanolamina

La etanolamina es el agente bloqueante recomendado en el protocolo de empleo de las cMBs para evitar adsorciones inespecíficas [<https://www.thermofisher.com/es/es/home/references/protocols.html>], y ha sido utilizada para la preparación de plataformas inmunosensoras en trabajos anteriores, usándose en concentración 1M [Ruiz-Valdepeñas Montiel, 2015] en medio regulador fosfato de pH 8.0 [Sánchez-Tirado, 2017]. En este trabajo se optimizó el tiempo de bloqueo, con los resultados que se muestran en la Figura 5.67. Puede verse cómo, según lo esperado, tanto la corriente específica como la no específica disminuyen con el tiempo de incubación en la disolución bloqueante debido a la pasivación parcial de la superficie electródica. Sin embargo, esta disminución afecta de forma desigual a los dos tipos de respuestas, encontrándose la mayor relación S/N para 30 minutos, que fue seleccionado para estudios posteriores.

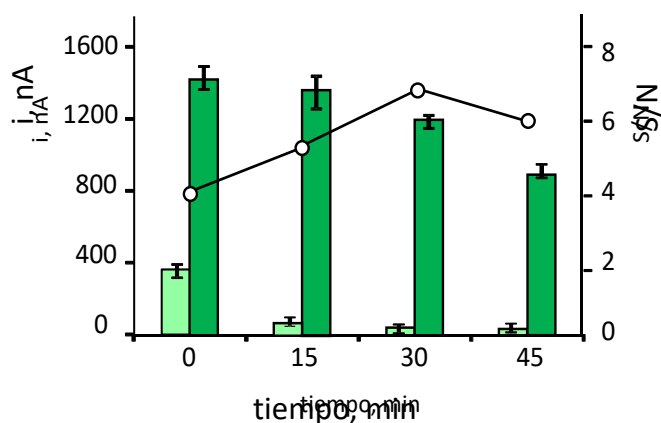


Figura 5.67. Influencia del tiempo de bloqueo con etanolamina 1M sobre la respuesta amperométrica en ausencia (verde claro) y presencia (verde oscuro) de 1 ng mL^{-1} de CXCL7, inc. 60 min. Otras condiciones, como en la Figura 5.66.

Influencia de la concentración y el tiempo de incubación de Biotin-anti-CXCL7

La influencia de estas variables sobre la respuesta electroquímica de la bio-plataforma HRP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-MBs/SPCE se estudió en las condiciones optimizadas previamente, en el intervalo de 0.05 y 0.5 $\mu\text{g mL}^{-1}$ de anticuerpo biotinilado y empleando tiempos de 30 a 90 min. En la Figura 5.68a se observa el aumento progresivo de la respuesta específica con la concentración de Biotin-anti-CXCL7, como corresponde a un incremento de los puntos de unión con el conjugado HRP-Strept. Sin embargo, la concentración más alta del intervalo estudiado, 0.5 $\mu\text{g mL}^{-1}$ da lugar a una mayor respuesta inespecífica y, como consecuencia, la relación S/N disminuye significativamente. Esto se debe probablemente a la presencia de un exceso de anticuerpo de captura que se uniría al conjugado enzimático originando corrientes no específicas sobre la superficie del electrodo. Estos resultados llevaron a elegir 0.25 $\mu\text{g mL}^{-1}$ de anticuerpo biotinilado como la concentración más adecuada para monitorizar la reacción de inmunoensayo. En lo que respecta al tiempo de incubación de este conjugado, la Figura 5.68b muestra claramente que la mejor opción es la de 60 min.

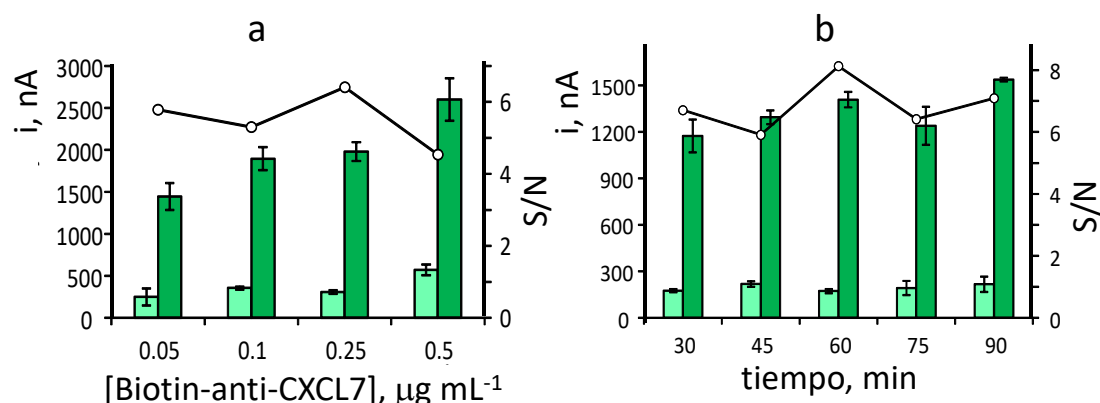


Figura 5.68. Influencia de la concentración (a) y del tiempo de incubación (b) de Biotin-anti-CXCL7 sobre la respuesta amperométrica en ausencia (verde claro) y presencia (verde oscuro) de 1 ng mL^{-1} de CXCL7, inc. 60 min; Biotin-anti-CXCL7, inc. 60 min (a); 0.25 $\mu\text{g mL}^{-1}$ Biotin-anti-CXCL7 (b). Otras condiciones, como en la Figura 5.66.

Influencia de la concentración de HRP-Strept

Para estudiar la influencia de la concentración del conjugado enzimático sobre la respuesta amperométrica se prepararon diluciones del mismo en el margen comprendido entre 1/500 y 1/4000, mostrándose los resultados obtenidos en la Figura 5.69. En ella se observa la disminución de las señales específica e inespecífica a medida que lo hace la concentración de HRP-Strept debido a la menor cantidad de enzima disponible para la

reacción. Por tanto, de acuerdo con el criterio de la máxima relación S/N se escogió una dilución 1/2000. Finalmente, una vez optimizadas todas las variables anteriores, el tiempo de incubación de HRP-Strept se mantuvo en el usado hasta este punto, 20 min, que ha demostrado ser un periodo adecuado para la detección del inmunoensayo.

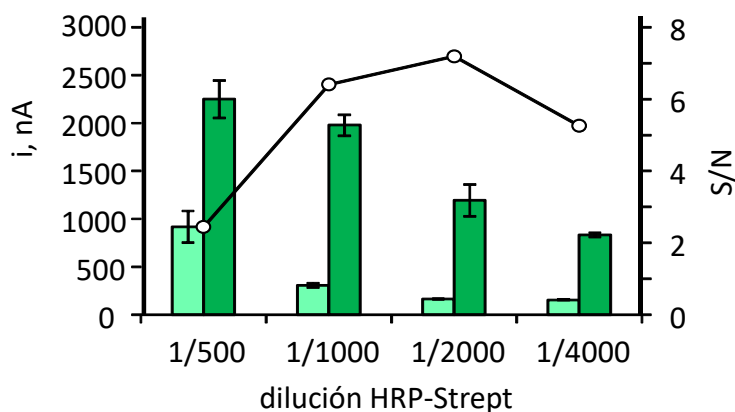


Figura 5.69. Influencia de la dilución de HRP-Strept sobre la respuesta amperométrica en ausencia (verde claro) y presencia (verde oscuro) de 1 ng mL^{-1} de CXCL7, inc. 60 min; Otras condiciones, como en la Figura 5.66.

Influencia de la concentración y el tiempo de incubación de anti-MMP3

Los resultados obtenidos en estos estudios de optimización son similares a los encontrados para la proteína anterior. Así, en el caso de la concentración de anti-MMP3, evaluada en el intervalo de 5 a $20 \text{ } \mu\text{g mL}^{-1}$ (Figura 5.70a) se observó el aumento de la respuesta específica con dicha concentración hasta alcanzar $15 \text{ } \mu\text{g mL}^{-1}$, disminuyendo dicha respuesta cuando se utiliza una concentración superior. La explicación de esta tendencia es similar a la descrita anteriormente, y se atribuye al mayor número de posiciones de unión con el antígeno, hasta que se aprecian efectos de saturación en presencia de un exceso de anticuerpo de captura. Por otro lado, la respuesta inespecífica prácticamente no varía en el intervalo estudiado, por lo que la relación S/N máxima se alcanza a la concentración de anti-MMP3 citada, que fue la elegida para estudios posteriores. En lo que respecta al tiempo de incubación del anticuerpo de captura (Figura 5.70b), los resultados obtenidos ponen de manifiesto que es necesario mantener un periodo de incubación superior a 30 min con el fin de alcanzar respuestas específicas de mayor magnitud. Como se observa, a partir de 45 min, tanto las corrientes específicas

como las no específicas permanecen prácticamente constantes, por lo que se eligió este tiempo como óptimo para el inmunoensayo.

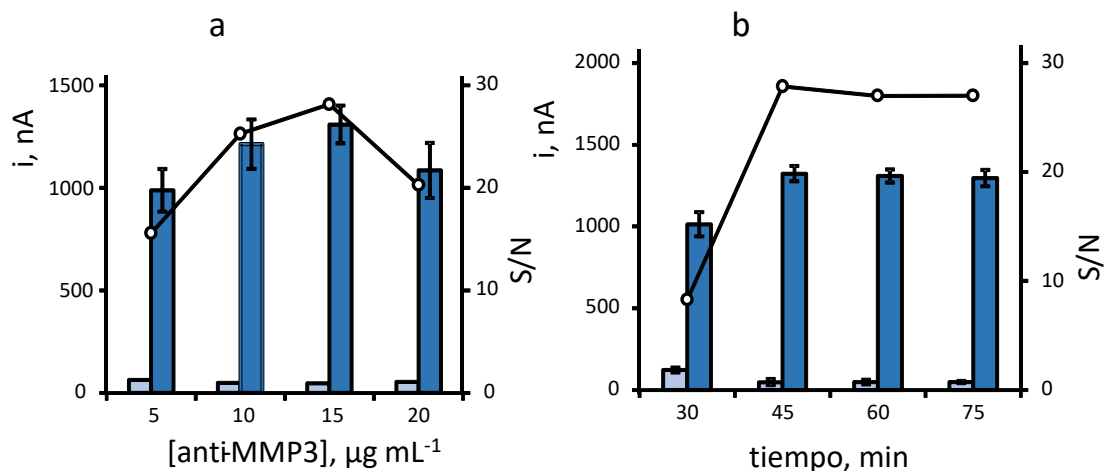


Figura 5.70. Influencia de la concentración (a) y del tiempo de incubación (b) de anti-MMP3 sobre la respuesta amperométrica en ausencia (azul claro) y presencia (azul oscuro) de 2 ng mL^{-1} de MMP3, inc. 60 min; anti-MMP3, inc. 45 min (a); $15 \mu\text{g mL}^{-1}$ anti-MMP3 (b); etanolamina 1M, inc. 30 min; $1 \mu\text{g mL}^{-1}$ Biotin-anti-MMP3, inc. 45 min; HRP-Strept dil. 1/500, inc. 20 min. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Influencia del tiempo de bloqueo con etanolamina

Trabajando en las mismas condiciones que en el estudio anterior, se observó un comportamiento similar al evaluar el efecto de esta variable sobre la respuesta amperométrica.

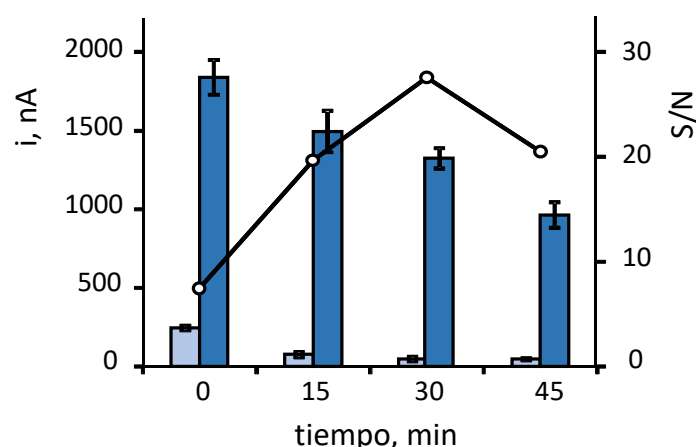


Figura 5.71. Influencia del tiempo de bloqueo con etanolamina 1M sobre la respuesta amperométrica en ausencia (azul claro) y presencia (azul oscuro) de 2 ng mL^{-1} de MMP3, inc. 60 min. Otras condiciones, como en la Figura 5.69.

Como era de esperar (Figura 5.71), la mayor duración del tiempo de bloqueo da lugar a una menor conductividad del electrodo, originando la disminución de las corrientes específica e inespecífica. Esta variación proporciona un máximo de la relación S/N a los 30 min, que fue el tiempo de bloqueo elegido.

Influencia de la concentración y el tiempo de incubación de Biotin-anti-MMP3

El estudio del efecto de la concentración del anticuerpo de detección biotinilado sobre las respuestas amperométricas se realizó en las condiciones experimentales anteriores, empleando inmunoconjugados preparados con disoluciones de Biotin-anti-MMP3 que contenían entre 0.5 y 2 $\mu\text{g mL}^{-1}$. La Figura 5.72a muestra los resultados obtenidos, que permiten apreciar el incremento de la señal específica entre 0.5 y 1.5 $\mu\text{g mL}^{-1}$ de conjugado y su posterior estabilización. La explicación de esta tendencia coincide con lo descrito anteriormente. Por otra parte, en este estudio también se observa un ligero aumento de las señales inespecíficas a las concentraciones más elevadas de Biotin-anti-MMP3, lo que proporciona un valor máximo de la relación S/N para 1 $\mu\text{g mL}^{-1}$ de dicho anticuerpo. Por otro lado, según revela la Figura 5.72b, en estas condiciones experimentales, el tiempo de incubación óptimo para este conjugado es de 45 min.

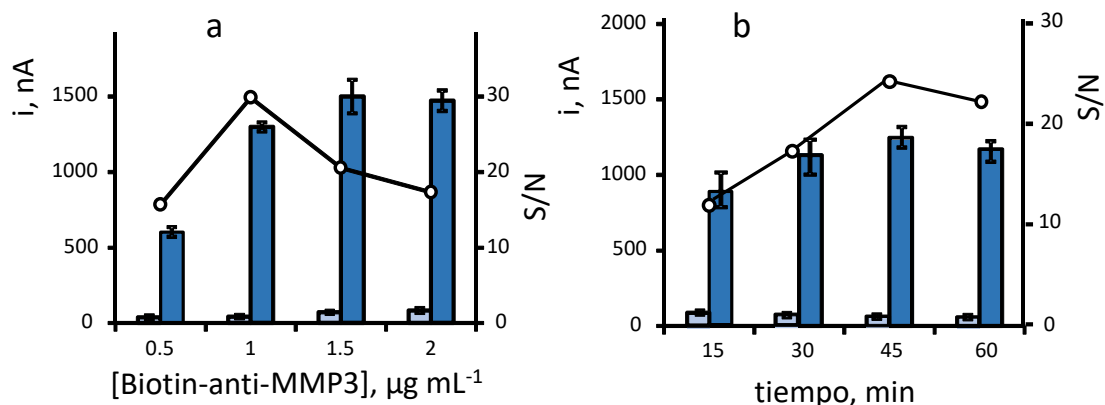


Figura 5.72. Influencia de la concentración (a) y del tiempo de incubación (b) de Biotin-anti-MMP3 sobre la respuesta amperométrica en ausencia (azul claro) y presencia (azul oscuro) de 2 ng mL^{-1} de MMP3, inc. 60 min; Biotin-anti-MMP3, inc. 45 min (a); 1 $\mu\text{g mL}^{-1}$ Biotin-anti-MMP3 (b). Otras condiciones, como en la Figura 5.69.

Influencia de la concentración de HRP-Strept

Como se observa en la Figura 5.73, el efecto de la variación de la concentración del conjugado enzimático sobre la respuesta amperométrica se estudió empleando disoluciones de HRP-Strept preparadas a diferentes grados de dilución comprendidos entre 1/250 y 1/1500. Puede apreciarse una disminución paulatina de las corrientes al aumentar el grado de dilución como consecuencia de una menor cantidad de enzima disponible para catalizar la reacción enzimática, obteniéndose la mayor relación S/N para una dilución 1/500. Por otro lado, también en este caso se eligió un tiempo de incubación de 20 min para el conjugado enzimático por ser adecuado para la obtención de señales específicas de elevada intensidad.

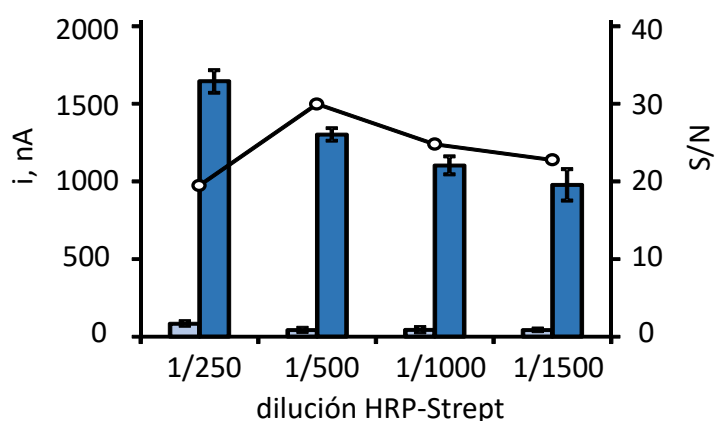


Figura 5.73. Influencia de la dilución de HRP-Strept sobre la respuesta amperométrica en ausencia (azul claro) y presencia (azul oscuro) de 2 ng mL^{-1} de MMP3, inc. 60 min. Otras condiciones, como en la Figura 5.69.

Al finalizar los estudios de optimización, es importante destacar que las respuestas inespecíficas obtenidas con los inmunoconjugados HRP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-MBs y HRP-Strept-Biotin-anti-MMP3-MMP3-anti-MMP3-MBs preparados en las condiciones experimentales optimizadas en el conjunto de estos estudios representan menos de un 14% y de un 4%, respectivamente, de la respuesta específica. Esta característica es atribuible no solo a la adecuada optimización de variables llevada a cabo en dichos estudios, sino también a las ventajas del empleo de micropartículas magnéticas que permiten la preparación de los inmunoconjugados fuera del electrodo facilitando además la aplicación de etapas de lavado que minimizan las adsorciones no deseadas responsables de las corrientes de fondo no debidas específicamente a interacciones entre los inmunorreactivos.

Tabla 5.11. Optimización de las variables experimentales para la determinación simultánea de CXCL7 y MMP3.

Variable	Intervalo estudiado	Valor seleccionado
Concentración de anti-CXCL7, $\mu\text{g mL}^{-1}$	2.5 - 25	10
Tiempo incubación anti-CXCL7, min	30 - 75	45
Tiempo bloqueo etanolamina, min	0 - 45	30
Concentración de Biotin-anti-CXCL7, $\mu\text{g mL}^{-1}$	0.05 - 0.5	0.25
Tiempo incubación Biotin-anti-CXCL7, min	30 - 75	45
Dilución HRP-Strept	1/500 - 1/4000	1/2000
Concentración de anti-MMP3, $\mu\text{g mL}^{-1}$	5 - 20	15
Tiempo incubación anti-MMP3, min	30 - 75	45
Tiempo bloqueo etanolamina, min	0 - 45	30
Concentración de Biotin-anti-MMP3, $\mu\text{g mL}^{-1}$	0.5 - 2	1
Tiempo incubación Biotin-anti-MMP3, min	30 - 90	60
Dilución HRP-Strept	1/250 - 1/1500	1/500

5.3.2.3. Calibrado y características analíticas

En la Figura 5.74 se han representado los calibrados obtenidos para los biomarcadores CXCL7 y MMP3 en las condiciones de trabajo previamente optimizadas, observándose la variación lineal creciente de la respuesta amperométrica con el logaritmo de la concentración de antígeno en ambos casos. Para la quimioquina CXCL7, la tendencia observada se ajustó a la ecuación $i, \text{nA} = (2041 \pm 47) \log ([\text{CXCL7}], \text{ng mL}^{-1}) + (746 \pm 52)$ ($R^2 = 0.997$), con un intervalo lineal a concentraciones comprendidas entre 0.5 y 75 ng mL^{-1} (Figura 5.73a). Con respecto al calibrado obtenido para MMP3, la ecuación correspondiente es: $i, \text{nA} = (297 \pm 5) \log ([\text{MMP3}], \text{pg mL}^{-1}) + (135 \pm 10)$, con $R^2 = 0.998$ y un intervalo lineal de concentración comprendido entre 1.0 y 2000 pg mL^{-1} (Figura 5.73b). Los límites de detección se calcularon de acuerdo con el criterio $3s_b/m$, donde s_b es la desviación estándar de las medidas amperométricas ($n=10$) obtenidas para la

concentración más baja de los calibrados, y m la pendiente del calibrado correspondiente. Los valores calculados fueron: LOD = 0.3 ng mL⁻¹ de CXCL7 y 0.8 pg mL⁻¹ de MMP3.

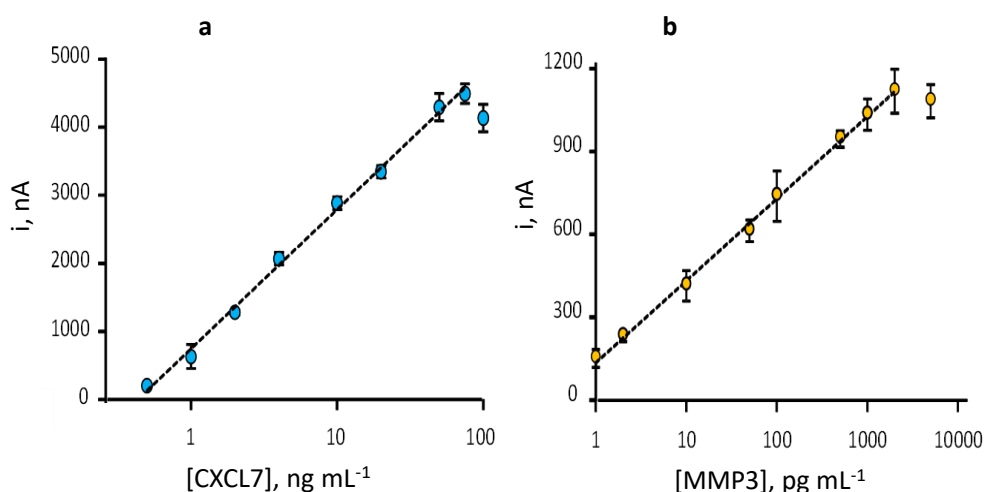


Figura 5.74. Calibrados obtenidos para la determinación de CXCL7 (a) y MMP3 (b) utilizando la inmunoplateforma dual: HRP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-cMBs/SPdCE y HRP-Strept-Biotin-anti-MMP3-MMP3-anti-MMP3-cMBs/SPdCE. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Cuando las características analíticas obtenidas con la bioplateforma dual se comparan con las de otras técnicas de inmunoensayo se aprecian algunas ventajas. Por ejemplo, en el caso de la enzima MMP3, el intervalo lineal del calibrado es más amplio que los que proporcionan los kits ELISA (ver Tabla 3.5.9. en la Introducción) y, además, la concentración más baja medible es del orden de treinta veces menor que la que proporcionan los sistemas comerciales de R&D Systems [https://www.rndsystems.com/products/human-total-mmp-3-duoelisa_dy513] usando los mismos inmunorreactivos, así como cerca de veinte veces menor que la indicada en el kit de Abcam [<https://www.abcam.com/human-mmp3-elisa-kit-ab269371.html>]. Por otro lado, la relación semilogarítmica entre la corriente amperométrica y la concentración de MMP3 obtenida en este calibrado viene definida por una línea recta en lugar de una curva y, además, el límite de detección es del orden de 3.5 veces menor que el del kit ELISA de Abcam. Finalmente, con respecto a la comparación con el único inmunosensor encontrado en la bibliografía para la determinación de MMP3 [Munge, 2010], las características analíticas del método puesto a punto con la bioplateforma dual son también mejores, a pesar de que en aquel se utilizaba una estrategia de amplificación. Por ejemplo, el límite de detección alcanzado es más de trescientas veces menor y el intervalo lineal del calibrado es más amplio, presentando una pendiente cuatro veces mayor.

En el caso de la quimioquina CXCL7, las ventajas son menos evidentes. Por ejemplo, el calibrado obtenido con el inmunosensor desarrollado anteriormente (ver Apartado 5.2.1.) presentaba un intervalo lineal comprendido entre 0.5 y 600 pg mL⁻¹. Sin embargo, en el método desarrollado con la bioplataforma dual, este intervalo se extiende en el margen de concentraciones de CXCL7 de ng mL⁻¹ que, por otra parte, como se verá, es adecuado para el análisis de suero humano sin necesidad de realizar excesivas diluciones. Por otro lado, esta misma diferencia se encuentra cuando se compara el método desarrollado con el del kit ELISA comercial de Abcam (ab100613). Como se muestra en la Tabla 3.5.3. de la Introducción, en este caso se obtiene un calibrado semilogarítmico no lineal en el intervalo de concentraciones de antígeno comprendido entre 4.1 y 1000 pg mL⁻¹ de CXCL7.

En general, puede destacarse como principal ventaja de la bioplataforma desarrollada, que es posible llevar a cabo la determinación simultánea de ambos biomarcadores en un tiempo de ensayo de 2h 50 min, contando los periodos de incubación del antígeno y de los inmunorreactivos siguientes, lo que supone un periodo muy inferior a las casi cinco horas necesarias, evaluando el tiempo del mismo modo, en el inmunoensayo ELISA para la determinación individual de CXCL7. Por último, el volumen de muestra requerido para la llevar a cabo la determinación simultánea es de 25 µL para cada biomarcador, cuatro veces inferior a lo que se necesita en el kit ELISA para cada determinación individual.

Se estudió la reproducibilidad de la metodología desarrollada por comparación de las respuestas amperométricas proporcionadas por distintos inmunosensores individuales preparados el mismo día en ausencia y en presencia de 2 ng mL⁻¹ de CXCL7 o MMP3. Los valores de RSD (n=5) obtenidos fueron del 3.1% y 4.8% para CXCL7 y del 4.7% y 4.9% para MMP3, respectivamente. Siguiendo el mismo procedimiento, se determinó la reproducibilidad de las corrientes proporcionadas por diferentes inmunosensores preparados en distintos días, obteniendo los valores de RSD (n=10) del 3.7% y 4.0% en ausencia y en presencia de 2 ng mL⁻¹ de CXCL7, así como del 5.4 % y 5.6% en ausencia y en presencia de 2 ng mL⁻¹ de MMP3. Los resultados obtenidos demuestran la buena precisión de los procedimientos seguidos para la preparación de los inmunoconjugados y la medida amperométrica.

5.3.2.4. Estabilidad, selectividad y reactividad cruzada

Al igual que en capítulos anteriores, se estudió la estabilidad de almacenamiento de los inmunoconjugados preparados sobre las cMBs. Para ello, una serie de conjugados anti-CXCL7-MBs/SPCEs y anti-MMP3-MBs/SPCEs se mantuvieron a 4°C en ambiente húmedo a partir del momento de su preparación y se usaron cada día de control para medir 2 ng mL⁻¹ de CXCL7 o de MMP3, respectivamente, añadiendo los inmunorreactivos correspondientes. Los resultados obtenidos en este estudio (Figura 5.75) permiten apreciar cómo las respuestas amperométricas permanecen dentro de los límites de control situados a ± 2 veces la desviación estándar de la corriente media proporcionada por 10 inmunoconjugados preparados el primer día (día 0), durante al menos 25 días (máximo tiempo estudiado). Este periodo, relativamente largo, es importante desde el punto de vista de la aplicación del método desarrollado al análisis de rutina de estos biomarcadores en muestras de interés clínico, ya que es posible disponer de una batería de conjugados almacenados en las condiciones indicadas, y utilizarlos posteriormente, para la medida de los antígenos diana en el momento necesario.

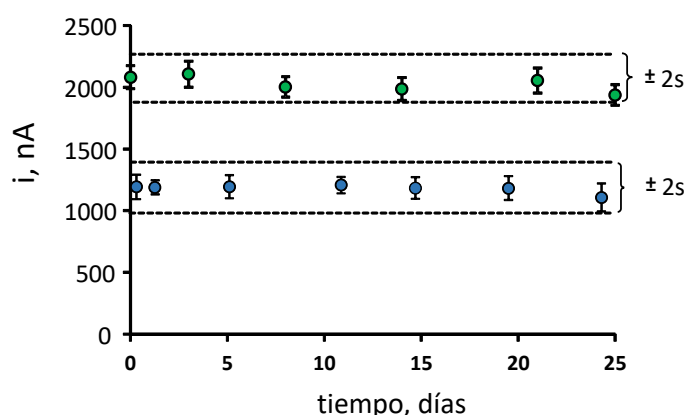


Figura 5.75. Gráfico de control para evaluar la estabilidad de los conjugados anti-CXCL7-cMBs (verde) y anti-MMP3-cMBs (azul) a 4 °C en ambiente húmedo. Ver el texto para más información.

La selectividad del inmunosensor dual se evaluó comparando las respuestas amperométricas medidas en ausencia y en presencia de 2 ng mL⁻¹ de CXCL7 o MMP3 en disoluciones que contenían diferentes especies que pueden coexistir con los analitos en las muestras de suero humano. Los niveles de concentración estudiados fueron los que cabe esperar en el caso de individuos sanos: 7 µg mL⁻¹ de ácido ascórbico (AA); 100 IU mL⁻¹ de anticuerpo anti-péptido cíclico citrunilado (CCPA); 70 pg mL⁻¹ de endoglina

CD105; 50 ng mL⁻¹ de C-X-C ligando 4 (CXCL4); 2 ng mL⁻¹ de metaloproteína 3 (MMP3); 50 ng mL⁻¹ de C-X-C ligando 7 (CXCL7); 5 mg mL⁻¹ de hemoglobina (HB); 1 mg mL⁻¹ de inmunoglobulina G humana (hIgG); 5 mg mL⁻¹ de albúmina de suero humano (HSA); 50 pg mL⁻¹ de ligando de receptor activador para el factor nuclear κ B (RANKL), y 100 IU mL⁻¹ de factor reumatoide (RF). Los resultados obtenidos aparecen representados en la Figura 5.76, y ponen de manifiesto la ausencia de interferencia significativa en la determinación de los analitos diana. En efecto, solo se aprecian ligeras diferencias de corriente en presencia de los diferentes compuestos potencialmente interferentes, observándose además que, en todos los casos, las respuestas específica e inespecífica permanecen dentro de los límites situados en el entorno de ± 2 veces la desviación estándar del valor medio de la señal amperométrica (n=10). Todo ello demuestra una elevada selectividad debida principalmente a la práctica especificidad de los anticuerpos de captura empleados para el reconocimiento de los biomarcadores CXCL7 y MMP3 diana, así como, en el caso del ácido ascórbico, a la excelente selectividad de la transducción amperométrica en las condiciones de trabajo optimizadas.

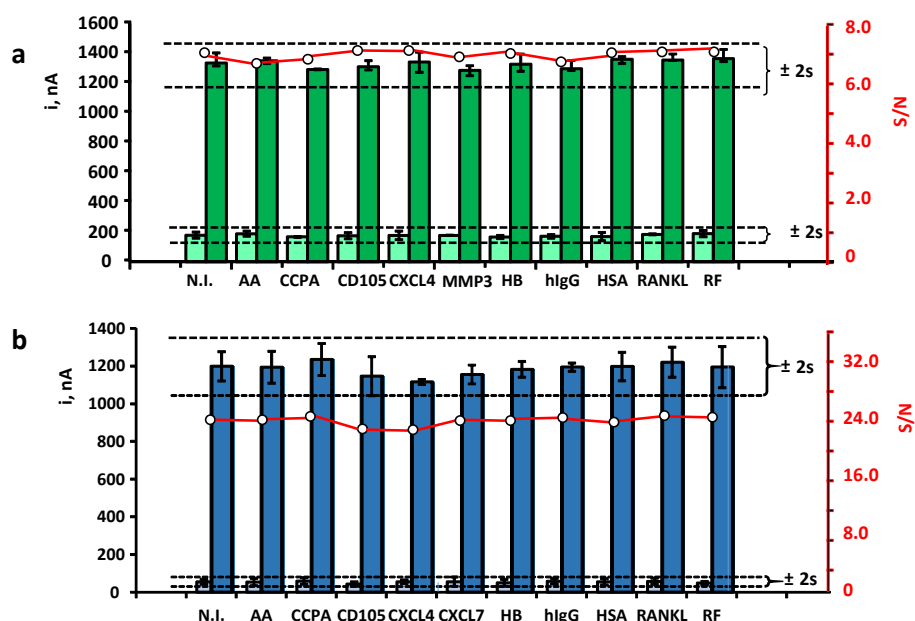


Figura 5.76. Respuestas amperométricas obtenidas con la bioplataforma dual en ausencia de CXCL7 (verde claro) o de MMP3 (azul claro), y en presencia de 2 ng mL⁻¹ de CXCL7 (verde oscuro) o de MMP3 (azul oscuro), y en disoluciones preparadas en ausencia (N.I.) o en presencia de AA, CCPA, CD105, CXCL4, MMP3 (a), CXCL7 (b), HB, hIgG, HSA, RANKL y RF Barras de error estimadas como tres veces la desviación estándar de tres replicados. Ver el texto para más información.

Con el fin de asegurar que no se producía interferencia cruzada entre las superficies próximas del electrodo dual, se llevó a cabo un ensayo en el que se midieron de forma simultánea las respuestas amperométricas para diferentes mezclas de CXCL7 y MMP3, utilizando la configuración HRP-Strept-Biotin-anti-CXCL7-X-anti-CXCL7-cMBs en el electrodo de trabajo 1 (W1) y HRP-Strept-Biotin-anti-MMP3-X-anti-MMP3-cMBs en el electrodo de trabajo 2 (W2). En la Figura 5.77 se recogen los resultados obtenidos a partir de las medidas realizadas en las condiciones que se indican en el pie de la misma. Brevemente: a) en ausencia de CXCL7 en W1 y en ausencia MMP3 en W2; b) en presencia de 2 ng mL⁻¹ de CXCL7 en W1 y 2 ng mL⁻¹ de MMP3 en W2, y c) como en b) pero en los electrodos contrarios. Como puede apreciarse, no se observó interferencia significativa entre las superficies de los dos electrodos de trabajo, confirmando la viabilidad de la plataforma desarrollada para la determinación simultánea de CXCL7 y MMP3.

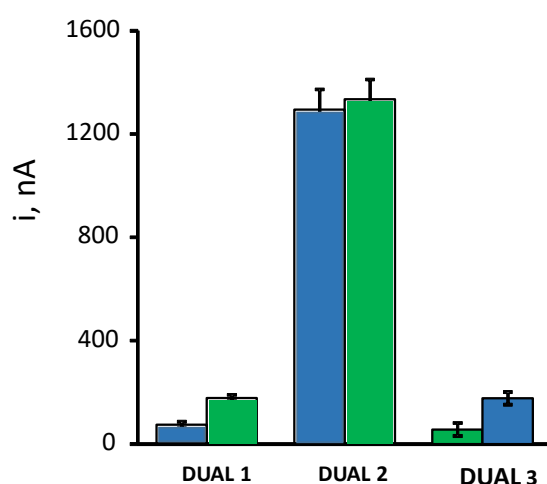


Figura 5.77. Respuestas amperométricas obtenidas simultáneamente: a) en ausencia de CXCL7 (verde) y RF (azul) (DUAL 1), b) en presencia de 2 ng mL⁻¹ de CXCL7 y de 2 ng mL⁻¹ de MMP3 utilizando HRP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-MBs en W1 y HRP-Strept-Biotin-anti-MMP3-MMP3-anti-MMP3-MBs en W2 (DUAL 2), y c) HRP-Strept-Biotin-anti-MMP3-MMP3-anti-MMP3-MBs en W1 y HRP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-MBs en W2 (DUAL 3). Barras de error estimadas como tres veces la desviación estándar de tres replicados. Ver el texto para más información.

5.3.2.5. Determinación simultánea de MMP3 y CXCL7 en suero

La utilidad del inmunosensor dual para el análisis simultáneo de CXCL7 y MMP3 en muestras biológicas se demostró mediante su aplicación a sueros de individuos sanos y de pacientes con artritis reumatoide diagnosticada. Como en casos anteriores, primero

se evaluó la posibilidad de existencia de efecto matriz midiendo los calibrados obtenidos por adición de alícuotas de CXCL7 y MMP3 sobre suero en dilución 1/50 con PBS de pH 7.4. El análisis estadístico aplicando el test de t-Student para la comparación de las pendientes obtenidas mediante los calibrados de disoluciones patrón de CXCL7 (2041 ± 47 nA) y MMP3 (297 ± 5 nA) y las obtenidas para los calibrados de adiciones estándar en suero, 2310 ± 120 nA para CXCL7 y 296 ± 5 nA para MMP3, proporcionó valores de $t_{\text{exp}} = 2.120$ para CXCL7 y 0.657 para MMP3, ambos inferiores al valor teórico tabulado, $t_{\text{tab}} = 2.228$. Estos resultados demostraron que no existen diferencias significativas entre las pendientes y, por tanto, la ausencia de efecto matriz en las condiciones de dilución ensayadas. La determinación simultánea de ambos biomarcadores mediante interpolación directa en el calibrado de patrones de CXCL7 y MMP3 se llevó a cabo en las diferentes muestras reales con los resultados resumidos en la Tabla 5.12.

Como puede verse en dicha Tabla, las concentraciones encontradas con el método desarrollado y las que proporcionan los correspondientes kits ELISA para la determinación individual de ambas proteínas, son prácticamente coincidentes, demostrándose en la Figura 5.78 la excelente correlación existente entre las dos series de resultados, con pendientes muy próximas a la unidad y ordenadas en el origen que incluyen el cero.

Tabla 5.12. Determinación de simultánea de CXCL7 y MMP3 en suero humano.

Grupo	Muestra	CXCL7, ng mL ⁻¹		MMP3, ng mL ⁻¹	
		Bioplataforma*	ELISA**	Bioplataforma*	ELISA**
Individuos sanos	8097	843 ± 70	832 ± 81	2.0 ± 0.5	2.0 ± 0.7
	8118	858 ± 32	860 ± 65	3.1 ± 0.7	3.1 ± 0.8
	8139	823 ± 48	821 ± 73	2.6 ± 0.6	3 ± 1
	8160	883 ± 57	891 ± 58	3.5 ± 0.5	3.4 ± 0.8
Pacientes con RA	4285	1219 ± 47	1226 ± 51	38 ± 4	39 ± 5
	6499	1328 ± 60	1308 ± 65	15 ± 2	17 ± 4
	5650	1521 ± 100	1513 ± 72	40 ± 3	40 ± 6
	6501	1201 ± 69	1195 ± 57	18 ± 2	19 ± 3

Valores medios $\pm$ ts/ $\sqrt{n}$; *n=4; **n=2.

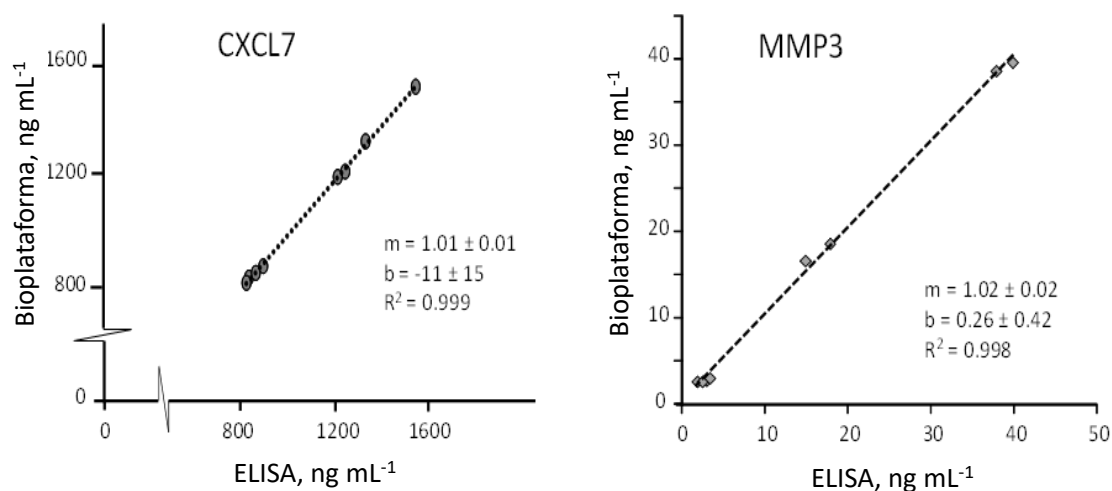


Figura 5.78. Correlación entre las concentraciones de CXCL7 y MMP3 determinadas en suero de individuos sanos y pacientes con artritis reumatoide con la bioplateforma dual desarrollada y con los test ELISA individuales.

Por otro lado, y como dato más relevante a comentar, puede observarse que los resultados obtenidos son consistentes con los niveles de concentración esperados de acuerdo con las características de las muestras. En el caso de la quimioquina CXCL7, recientemente se ha reportado la concentración de 850 ng mL⁻¹ en suero de individuos sanos [Du, 2018]. Esta concentración coincide apreciablemente con la encontrada y, además, se observa claramente que el biomarcador está sobreexpresado en el suero de pacientes con artritis reumatoide a niveles aproximadamente una vez y media superiores. Respecto a la enzima MMP3, los resultados obtenidos también están de acuerdo con los datos de la bibliografía [Ribbens, 2002], que sitúan los niveles en suero de individuos sanos en torno a las unidades de ng mL⁻¹, aumentando a unas pocas decenas de ng mL⁻¹ en los pacientes diagnosticados con artritis reumatoide. En el caso de las muestras analizadas, la concentración en estos pacientes es entre 6 y 14 veces más alta que en los individuos sanos. Con el fin de apreciar mejor las diferencias encontradas, en la Figura 5.79 se han representado los resultados resumidos en la Tabla anterior.

Puede verse que, en el caso de individuos sanos, las concentraciones encontradas no difieren mucho entre ellas. Sin embargo, esto sí ocurre en los pacientes, sobre todo en el caso de la determinación de MMP3.

Un análisis en detalle de las muestras reveló que las concentraciones notablemente inferiores de la enzima en los sueros 6499 y 6501 (muestra 2 y 4) se explicaban debido a

que los pacientes habían recibido un tratamiento clínico y se encontraban en un estado de remisión de la enfermedad. Por el contrario, los sueros 4285 y 5650 pertenecían a pacientes que mostraban una elevada inflamación articular. Estos resultados ponen de manifiesto el excelente valor diagnóstico que posee la determinación de MMP3, que permite monitorizar la existencia de inflamación y, consecuentemente, la aparición o prevalencia de procesos patológicos subyacentes que conducen a la destrucción articular en la artritis reumatoide.

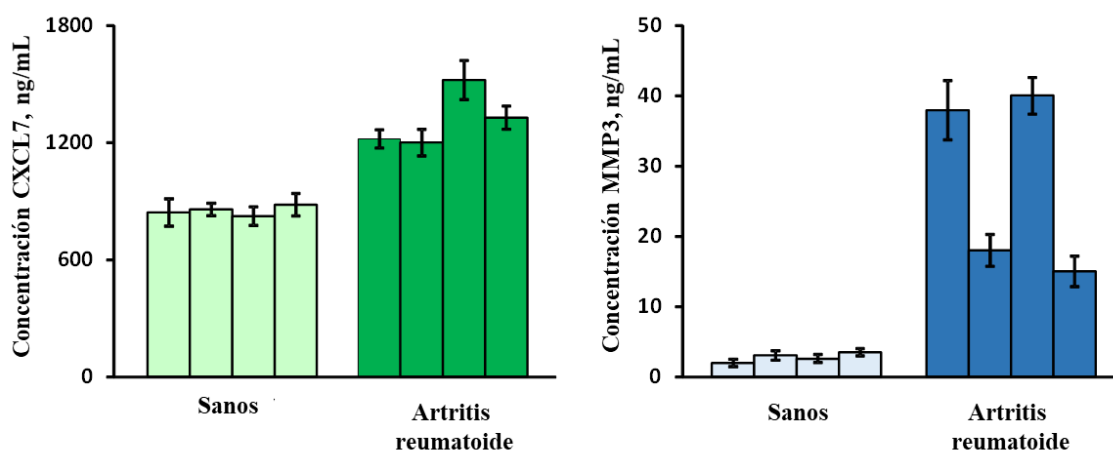


Figura 5.79. Determinación simultánea de CXCL7 (verde) y MMP3 (azul) con la bioplataforma dual desarrollada en suero de individuos sanos y pacientes con artritis reumatoide.

5.3.2.6. Conclusiones

En este trabajo se ha descrito el desarrollo de la primera inmunoplateforma electroquímica que permite la determinación simultánea de CXCL7 y MMP3, ambos biomarcadores clínicamente relevantes de la artritis reumatoide. La metodología puesta a punto implica el establecimiento de dos esquemas de inmunoensayo empleando MBs carboxiladas para la inmovilización de los bioconjugados y anticuerpos específicos de captura para ambas biomoléculas, usando detección amperométrica con el sistema HRP/HQ/H₂O₂. Tras la optimización de todas las variables implicadas, se obtuvieron límites de detección de 0.3 ng mL⁻¹ para CXCL7 y 0.8 pg mL⁻¹ para MMP3. Además de su elevada sensibilidad, la bioplataforma es selectiva y su empleo permitió llevar a cabo la determinación simultánea de ambos compuestos diana en sueros de individuos sanos y pacientes con artritis reumatoide diagnosticada, en un tiempo de ensayo de tan solo 2 h 50 min, poniendo de manifiesto su utilidad para aplicaciones clínicas.

5.3.3. Bioplateformas inmunosensoras para la determinación de biomarcadores de esclerosis múltiple: anti-MBP y CCL5

5.3.3.1. Configuración de los bioconjugados

Como se ha indicado, las bioplateformas para la determinación de estos marcadores: autoanticuerpo anti-proteína básica de mielina (anti-MBP) y β -quimioquina CCL5, se desarrollaron con el objetivo de poner a punto dos esquemas de inmunodetección electroquímica para el diagnóstico y seguimiento de pacientes con esclerosis múltiple (MS). Debido a la similitud entre ambas configuraciones, se ha considerado adecuado describir de forma conjunta los resultados obtenidos en su preparación e implementación. Los procedimientos seguidos y las etapas de construcción de estas plataformas se resumieron en los Apartados 4.4.3.3. y 4.4.3.4. y en las Figuras 4.15 y 4.16 de la Parte Experimental, representándose aquí los diseños correspondientes junto con los esquemas de la reacción enzimática y de detección electroquímica.

En el caso del anticuerpo anti-MBP (Figura 5.80), se utilizaron MBs carboxiladas para unir covalentemente el antígeno MBP seguido del analito, anti-MBP y de un anticuerpo de detección, IgG marcado con HRP.

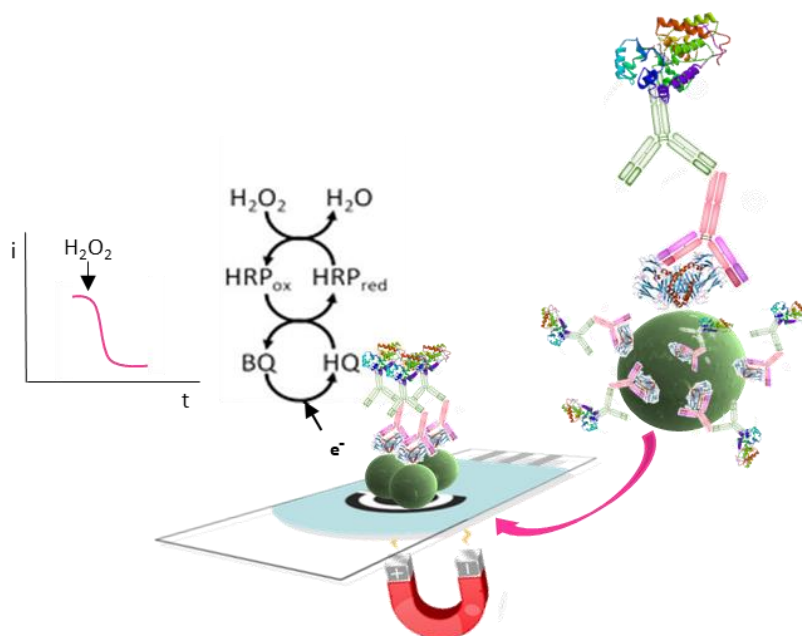


Figura 5.80. Esquema de la bioplateforma HRP-IgG-anti-MBP-MBP-cMBs/SPCE, y de la reacción implicada en la detección.

Por otro lado, la Figura 5.81 muestra el esquema utilizado para la determinación de CCL5 que implica el uso de MBs funcionalizadas con neutravidina (Neutr-MBs) sobre las que se inmovilizó el anticuerpo de captura biotinilado (anti-CCL5-Biotin) para enlazar el antígeno diana seguido del anticuerpo de detección unido a un conjugado enzimático HRP-IgG.

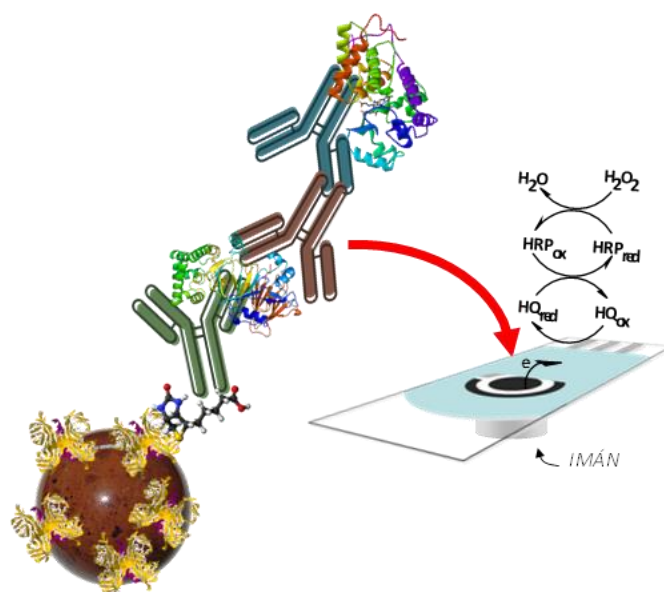


Figura 5.81. Esquema de la bioplataforma HRP-IgG-anti-CCL5-CCL5-anti-CCL5-Biotin-Neutr-MBs/SPCE, y de la reacción implicada en la detección.

5.3.3.2. Optimización de las variables experimentales

Con el objetivo de encontrar las mejores condiciones que proporcionen la mayor sensibilidad y el margen de concentración más amplio para la determinación de los biomarcadores, en estos estudios se investigó la influencia de la concentración y el tiempo de incubación de la proteína MBP sobre las MBs carboxiladas, el tiempo de bloqueo con etilendiamina, y la concentración y el tiempo de incubación del conjugado HRP-IgG sobre anti-MBP-MBP-MBs, así como la concentración y el tiempo de incubación del anticuerpo anti-CCL5-Biotin sobre las Neutr-MBs, la concentración y el tiempo de incubación del anticuerpo anti-CCL5 y la concentración y el tiempo de incubación del conjugado HRP-IgG sobre anti-CCL5-CCL5-anti-CCL5-Neutr-MBs. Como en los

capítulos anteriores, en cada caso, la selección del valor óptimo se realizó siguiendo el criterio de la mayor relación entre la respuesta específica (S) originada por la reacción de reconocimiento antígeno-anticuerpo, y la no específica (N) debida a interacciones que generan corrientes no deseadas. A continuación, se describen los estudios realizados y se justifican los resultados obtenidos, recogiendo finalmente en la Tabla 5.13 un resumen de los mismos indicando los intervalos estudiados y los valores óptimos seleccionados.

Influencia de la concentración y el tiempo de incubación de MBP

Para la determinación del anticuerpo anti-MBP, se inmovilizó la proteína específica MBP sobre las cMBs empleando el procedimiento de activación de los grupos carboxilo basado en el empleo de los reactivos EDC y NHSS en las condiciones optimizadas en trabajos anteriores [Ojeda, 2014]. El efecto de la concentración de esta proteína sobre la respuesta amperométrica se evaluó en el intervalo de 10 a 25 $\mu\text{g mL}^{-1}$ con los resultados representados en la Figura 82^a.

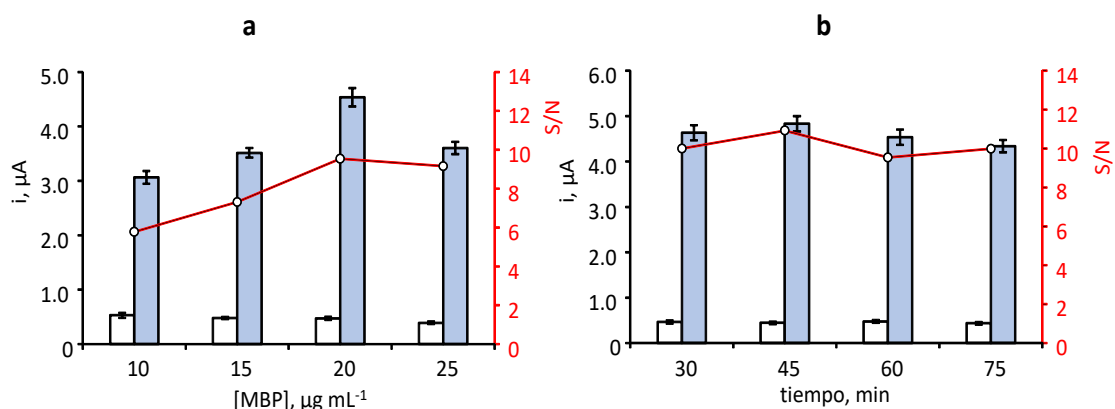


Figura 5.82. Influencia de la concentración (a) y del tiempo de incubación (b) de MBP sobre la respuesta amperométrica en ausencia (blanco) y en presencia de 1 ng mL^{-1} de anti-MBP, inc. 60 min; $20 \mu\text{g mL}^{-1}$ MBP (b), inc. 45 min (a); etanolamina 1 M, inc. 30 min; $0.50 \mu\text{g mL}^{-1}$ de HRP-IgG, inc. 60 min. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

Como puede observarse, la señal inespecífica permanece prácticamente constante mientras que la corriente específica aumenta con la concentración de MBP hasta $20 \mu\text{g mL}^{-1}$. La disminución posterior de esta corriente se atribuye, como en los casos anteriores, a la saturación de las cMBs por un exceso de proteína. Por lo tanto, se eligió una concentración de $20 \mu\text{g mL}^{-1}$ como óptima de MBP para la preparación del bioconjugado.

En cuanto al tiempo de incubación, en la Figura 82b se observa escasa variación de las respuestas específica e inespecífica en el intervalo estudiado. Por ello, se eligió un periodo de 45 min por encontrarse una ligera mejora de la relación S/N en estas condiciones.

Influencia del tiempo de bloqueo

La preparación del bioconjugado para la determinación de anti-MBP requirió del empleo de un agente bloqueante para minimizar las adsorciones inespecíficas sobre las cMBs. En este estudio se ensayaron tiempos de incubación de etanolamina 1M sobre MBP-MBs/SPCE en el intervalo de 30 a 90 min con los resultados que se han representado en la Figura 5.83. Como puede verse, en este caso, no se observó una disminución rápida de la respuesta al aumentar el tiempo de bloqueo incluso utilizando tiempos más largos que en estudios anteriores. Sin embargo, puede apreciarse una menor corriente inespecífica para 60 min, que hace que se incremente la relación S/N, por lo que se escogió este valor para continuar con la optimización del biosensor.

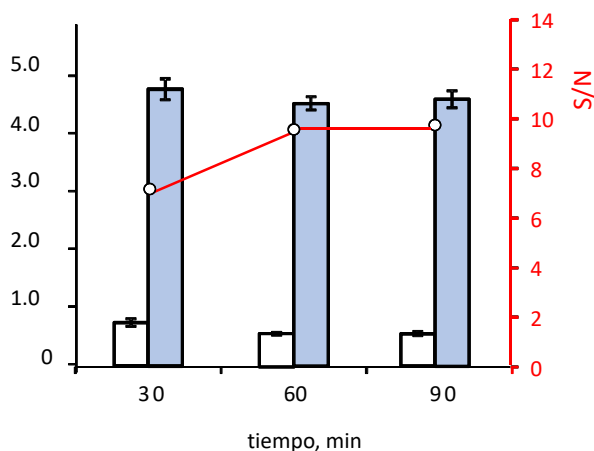


Figura 5.83. Influencia del tiempo de bloqueo con etanolamina 1M sobre la respuesta amperométrica en ausencia (blanco) y presencia (azul) de 1 ng mL⁻¹ de anti-MBP inc. 60 min. Otras condiciones, como en la Figura 5.81.

Influencia del número de etapas en la incubación de HRP-IgG y anti-MBP

Con el fin de asegurar el tiempo mínimo de ensayo para la preparación de la bioplateforma, se estudió la posibilidad de incubación del autoanticuerpo anti-MBP y el conjugado HRP-IgG en una misma etapa de incubación de 60 min, en lugar de en las dos etapas independientes, como se venía realizando en los estudios de optimización anteriores. En la Figura 5.84 se muestran los resultados obtenidos, los cuales reflejan un

considerable aumento de respuesta inespecífica al realizar una única etapa de incubación que conduce a una clara disminución de la relación S/N. Estos resultados se deben probablemente a la menor eficiencia de captura del anticuerpo anti-MBP cuando este se conjuga primero con el anticuerpo secundario HRP-IgG. Por ese motivo, se decidió continuar con la incubación independiente en dos etapas diferenciadas.

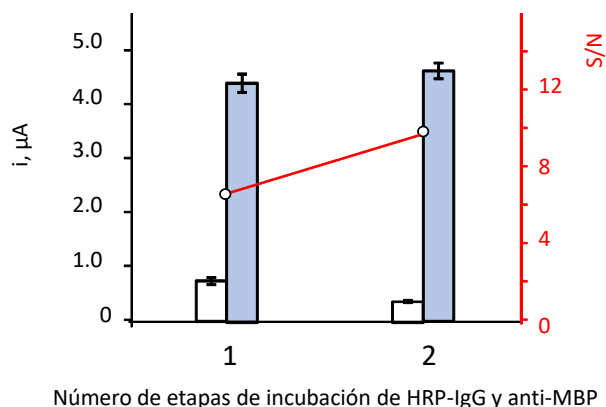


Figura 5.84. Influencia de la incubación de anti-MBP y HRP-IgG en una o dos etapas sobre la respuesta amperométrica. Otras condiciones como la Figura 5.81.

Influencia de la concentración y el tiempo de incubación de HRP-IgG

El efecto en la respuesta amperométrica de la cantidad de HRP-IgG inmovilizada sobre el conjugado anti-MBP-MBP-cMBs se investigó en el intervalo de 0.25 a 1.0 $\mu\text{g mL}^{-1}$. Como era de esperar, las corrientes específicas aumentan paulatinamente con la concentración de bioconjugado. Al mismo tiempo, aunque las señales inespecíficas incrementan también su magnitud, este aumento es más ligero y las corrientes son mucho menores (Figura 5.85a).

Como consecuencia, se encuentra una elevada relación S/N en todo el margen estudiado, que es máxima para 0.50 $\mu\text{g mL}^{-1}$, por lo que fue seleccionada como óptima para estudios posteriores. Por otro lado, respecto al tiempo de incubación del anticuerpo marcado con peroxidasa, en la Figura 5.85b se aprecia claramente que es suficiente con un periodo de 15 min para alcanzar los mejores resultados.

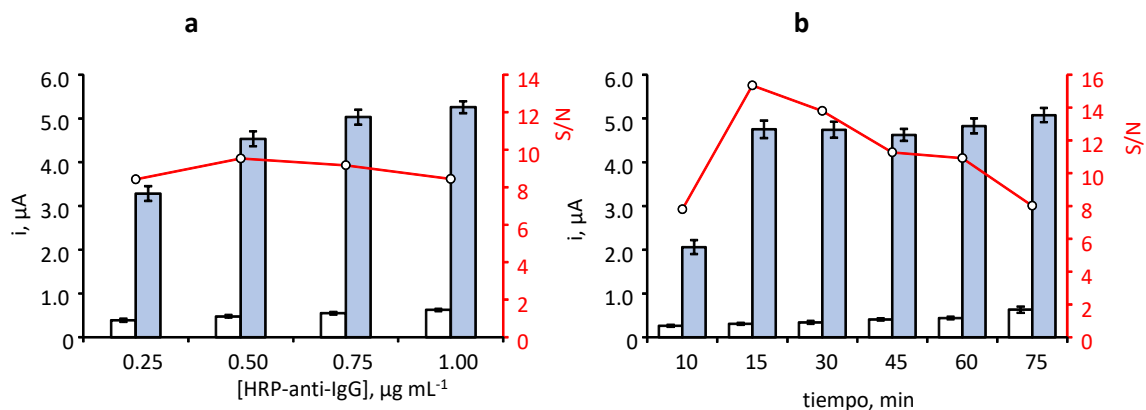


Figura 5.85. Influencia de la concentración y el tiempo de incubación de HRP-Strept sobre la respuesta amperométrica en ausencia (blanco) y presencia (azul) de 1 ng mL⁻¹ de anti-MBP inc. 60 min. 0.50 $\mu\text{g mL}^{-1}$ HRP-IgG (b) inc. 60 min (a). Otras condiciones, como en la Figura 5.81.

Influencia de la concentración y el tiempo de incubación de anti-CCL5-Biotin

Como ya se ha indicado, el empleo de neutravidina en lugar de avidina o estreptavidina para la unión de biomoléculas biotiniladas proporciona ventajas relacionadas con la elevada afinidad y una menor probabilidad de adsorciones no deseadas [Nguyen, 2012]. En este caso, se emplearon Neutr-MBs para inmovilizar el anticuerpo de captura biotinilado utilizado para la unión específica con el antígeno CCL5. Con el fin de optimizar su concentración se midió la respuesta amperométrica en ausencia y en presencia de 2 ng mL⁻¹ de la quimioquina, en el intervalo de concentraciones de anti-CCL5-Biotin comprendido entre 10 y 50 $\mu\text{g mL}^{-1}$.

Como se observa en la Figura 5.86a, es necesario utilizar al menos 10 $\mu\text{g mL}^{-1}$ de anticuerpo para obtener una buena corriente específica. Por otro lado, las respuestas inespecíficas permanecen prácticamente constantes y con una magnitud muy baja en todo el intervalo estudiado, alcanzándose la mayor relación S/N para 25 $\mu\text{g mL}^{-1}$, que fue la elegida como óptima. Una tendencia similar se encuentra al estudiar el efecto del tiempo de incubación del anticuerpo (Figura 5.86b), requiriéndose un periodo de 30 min para alcanzar la mayor relación S/N.

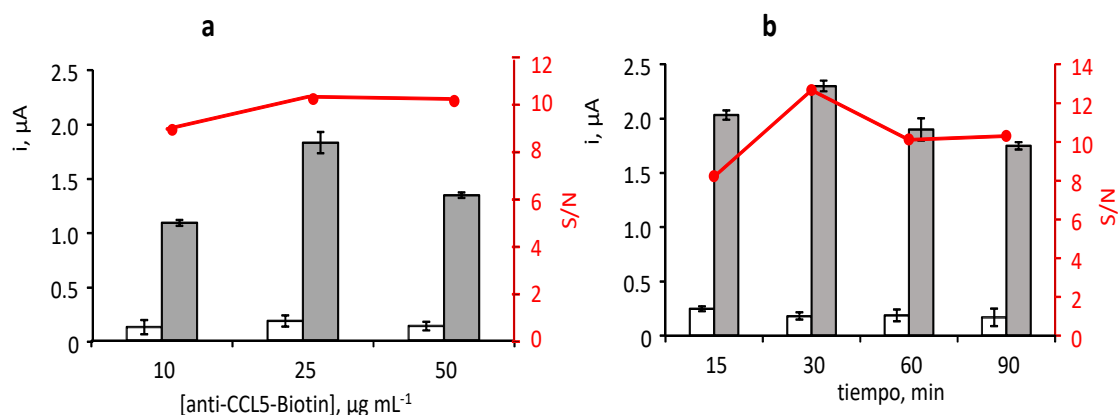


Figura 5.86. Influencia de la concentración y el tiempo de incubación de anti-CCL5-Biotin sobre la respuesta amperométrica en ausencia (blanco) y presencia (gris) de 2 ng mL⁻¹ de CCL5 inc. 60 min; 25 $\mu\text{g mL}^{-1}$ anti-CCL5-Biotin (b), inc. 30 min (a); 0.5 $\mu\text{g mL}^{-1}$ de anti-CCL5, inc. 30 min; 0.5 $\mu\text{g mL}^{-1}$ de HRP-IgG, inc. 30 min. Barras de error estimadas como el triple de la desviación estándar (n=3).

Influencia de la concentración y el tiempo de incubación de anti-CCL5

La Figura 5.87a muestra la tendencia observada en el estudio de la influencia de la concentración de anti-CCL5 sobre la respuesta amperométrica, observándose un rápido incremento de la relación S/N entre 0.25 y 0.5 $\mu\text{g mL}^{-1}$. Esta mejora es debida tanto al aumento de la señal en presencia de CCL5 como a la disminución de la corriente inespecífica. En efecto, para esta última concentración probablemente existe anticuerpo en una cantidad suficiente como para abarcar el mayor número de sitios de unión posible. Sin embargo, a concentraciones superiores, el exceso de anti-CCL5 provoca el efecto contrario, y en estas condiciones de saturación se reduce la señal específica y aumenta la inespecífica. Por ese motivo, se eligió 0.5 $\mu\text{g mL}^{-1}$ como concentración óptima para estudios posteriores. En el estudio del efecto del tiempo de incubación de anti-CCL5 en el intervalo de 15 a 90 min (Figura 5.87b) se encontraron las mejores respuestas específicas para los periodos intermedios, de 30 y 60 min, si bien el ligero aumento de las corrientes en ausencia de anti-CCL5 proporcionaron un máximo de la relación S/N a 30 min, que es el que se eligió para la preparación de los magneto-bioconjugados.

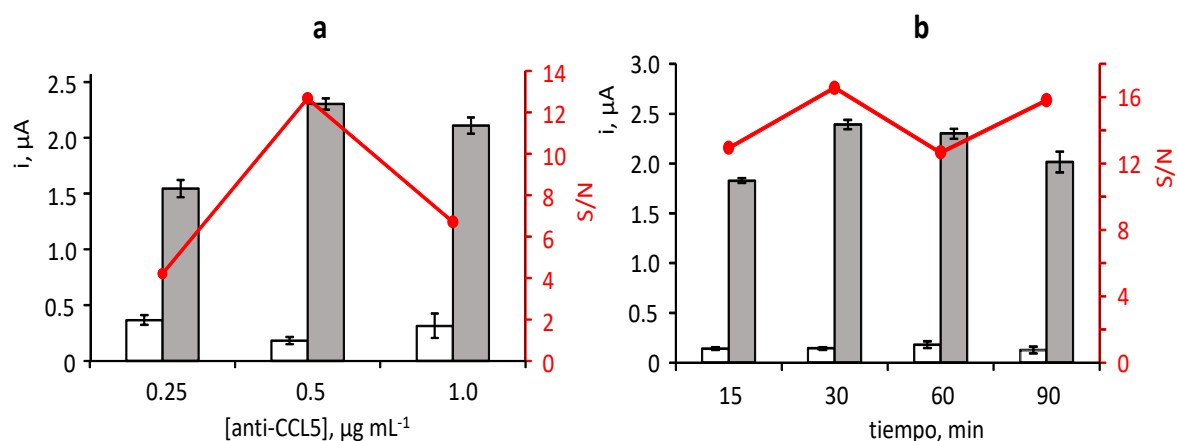


Figura 5.87. Influencia de la concentración y el tiempo de incubación de anti-CCL5 sobre la respuesta amperométrica en ausencia (blanco) y presencia (gris) de 2 ng mL⁻¹ de CCL5 inc. 60 min; 0.5 $\mu\text{g mL}^{-1}$ de anti-CCL5 (b), inc. 30 min (a). Otras condiciones como en la Figura 5.85.

Influencia de la concentración y el tiempo de incubación de HRP-IgG

Finalmente se estudiaron estas variables, encontrándose tendencias similares a las observadas en el estudio anterior. Respecto al efecto de la concentración de conjugado enzimático sobre la respuesta amperométrica, la mayor relación S/N se obtuvo para 0.5 $\mu\text{g mL}^{-1}$ de HRP-IgG (Figura 5.88a), mientras que según muestra la Figura 5.88b, un tiempo de 30 min resultó suficiente para alcanzar una elevada respuesta específica y, al igual que en el estudio anterior, un valor de S/N máximo.

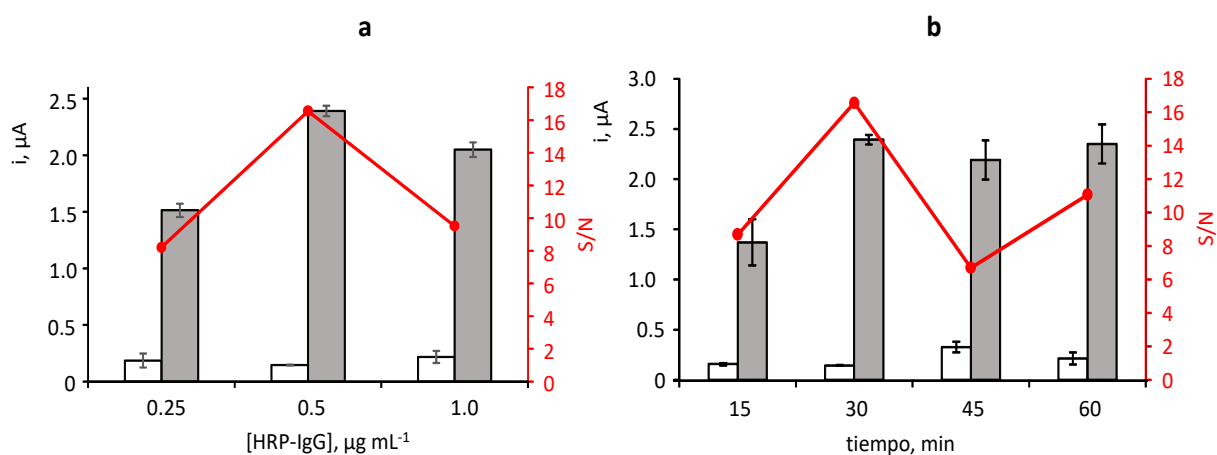


Figura 5.88. Influencia de la concentración y el tiempo de incubación de HRP-IgG sobre la respuesta amperométrica en ausencia (blanco) y presencia (gris) de 2 ng mL⁻¹ de CCL5 inc. 60 min; 0.5 $\mu\text{g mL}^{-1}$ de HRP-IgG (b), inc. 30 min (a). Otras condiciones como en la Figura 5.85.

Una vez completados los estudios de optimización de las inmunoplateformas asistidas magnéticamente para la determinación de anti-MBP y CCL5, un resumen de las condiciones experimentales elegidas se muestra en la Tabla 5.13. En este punto se ha considerado adecuado, además, destacar la baja magnitud de las respuestas inespecíficas obtenidas. En efecto, de forma similar a lo observado en otros casos, estas representan en torno a un 6% y un 8%, respetivamente, de las corrientes específicas obtenidas con los inmunoconjugados HRP-IgG-anti-MBP-MBP-MBs o HRP-IgG-anti-CCL5-CCL5-anti-CCL5-Biotin-Neutr-MBs. Esta ventaja se atribuye nuevamente a una adecuada optimización de las variables implicadas en la preparación y en el funcionamiento de las bioplateformas, así como también al uso de micropartículas magnéticas funcionalizadas como sustratos para la inmovilización de los biorreactivos.

Tabla 5.13. Optimización de las variables experimentales para la determinación de anti-MBP (azul) y de CCL5 (blanco)

Variable	Intervalo estudiado	Valor seleccionado
Concentración de MBP, $\mu\text{g mL}^{-1}$	10 - 25	20
Tiempo incubación MBP, min	30 - 75	45
Tiempo bloqueo etanolamina, min	30 - 90	60
Concentración de HRP-IgG, $\mu\text{g mL}^{-1}$	0.25 - 1.0	0.5
Tiempo incubación HRP-IgG, min	10 - 75	15
Etapas incubación anti-MBP/HRP-IgG	1 - 2	2
Concentración de anti-CCL5-Biotin, $\mu\text{g mL}^{-1}$	10 - 50	25
Tiempo incubación anti-CCL5-Biotin, min	15 - 90	30
Concentración de anti-CCL5, $\mu\text{g mL}^{-1}$	0.25 - 1	0.5
Tiempo incubación anti-CCL5, min	15 - 90	30
Concentración de HRP-IgG, $\mu\text{g mL}^{-1}$	0.25 - 1	0.5
Tiempo incubación HRP-IgG, min	15 - 60	30

5.3.3.3. Calibrados y características analíticas

Los calibrados obtenidos para la determinación de anti-MBP y de CCL5 con las bioplateformas preparadas en las condiciones experimentales previamente optimizadas se han representado, respectivamente, en las Figuras 5.89 y 5.90.

En los dos casos se observa que la respuesta amperométrica está relacionada con el logaritmo de la concentración del biomarcador. En el caso del anti-MBP, la ecuación del calibrado es: $i, \mu\text{A} = (3.19 \pm 0.07) \log ([\text{anti-MBP}], \text{ng mL}^{-1}) + (4.54 \pm 0.06)$ con un valor de $R^2 = 0.997$ extendiéndose en el intervalo de 0.05 a 50 ng mL^{-1} , mientras que para CCL5, el calibrado abarca el margen de 0.1 a 300 ng mL^{-1} ($R^2 = 0.998$), ajustándose a la ecuación $i, \mu\text{A} = (1.76 \pm 0.02) \log ([\text{CCL5}], \text{ng mL}^{-1}) + (1.95 \pm 0.03)$. Los límites de detección, calculados con el criterio habitual de $3s_b$ fueron: 16 pg mL^{-1} de anti-MBP y 40 pg mL^{-1} de CCL5.

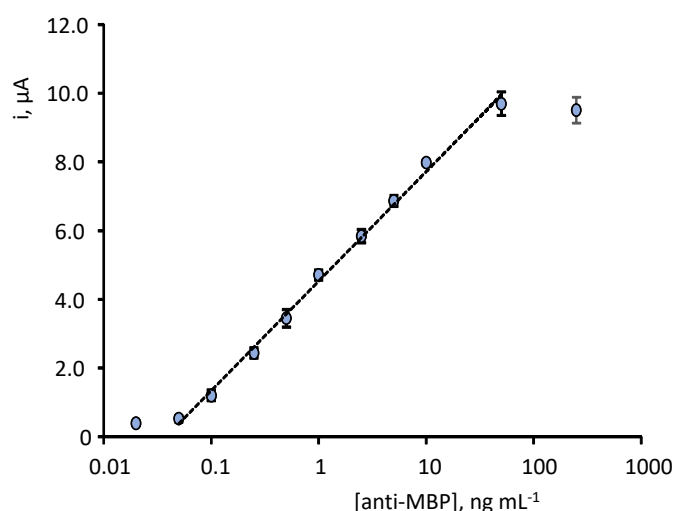


Figura 5.89. Calibrado obtenido para la determinación de anti-MBP utilizando la inmunoplateforma HRP-IgG - anti-MBP-MBP-cMBs/SPCE. Barras de error estimadas como el triple de la desviación estándar ($n=3$).

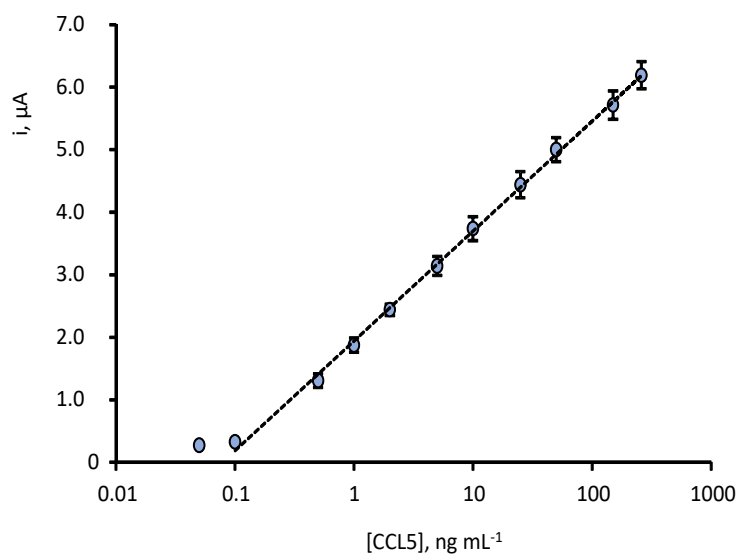


Figura 5.90. Calibrado obtenido para la determinación de CCL5 utilizando la inmunoplateforma HRP-IgG-anti-CCL5-CCL5-anti-CCL5-Biotin-Neutr-MBs/SPCE. Barras de error estimadas como el triple de la desviación estándar (n=3).

Estas características revelan que los inmunosensores desarrollados son competitivos con los kits ELISA comerciales (ver Tablas 3.5.4 y 3.5.8) en términos de sensibilidad e intervalo lineal de los calibrados. Así, por ejemplo, en el caso del anti-MBP, el valor de LOD es 62,5 veces menor que el denominado LLD ("lower limit of detection") igual a 1 ng mL⁻¹ del kit basado en los mismos inmunorreactivos [<http://www.cloud-clone.com/manual/ELISA-Kit-for-Anti-Myelin-Basic-Protein-Anti-body-Anti-MBP-E99509Hu.pdf>]. Además, el intervalo de concentraciones del calibrado es más amplio y comienza a una concentración más de sesenta veces menor (0.05–50 ng mL⁻¹ vs. 3.12–200 ng mL⁻¹). Finalmente, en relación al tiempo de ensayo hay que resaltar que la bioplateforma requiere 1 h 15 min a partir de la preparación de los conjugados MBP-cMBs frente a los 150 min necesarios para el kit ELISA. Por otro lado, cuando se considera el único biosensor descrito en la literatura hasta el momento, se aprecia que el LOD alcanzado (0.15 ng mL⁻¹) [Derkus, 2013] es un orden de magnitud superior al obtenido con la plataforma inmunosensora.

En el caso de la quimioquina CCL5, el intervalo del calibrado obtenido con el inmunosensor, entre 0.1 y 300 ng mL⁻¹, se extiende a un nivel de concentraciones superior al del kit ELISA basado en los mismos reactivos, 15.6 - 1000 pg mL⁻¹ [rndsystems.com/products/human-ccl5-rantes-duosetelisa_dy278], aunque este calibrado es no lineal y

doble logarítmico. Sin embargo, el inmunosensor desarrollado es competitivo en cuanto al tiempo de ensayo, ya que requiere solo 2h frente a las 4h 45 min del test ELISA.

La reproducibilidad de las medidas amperométricas realizadas, en ausencia y presencia de 1 ng mL^{-1} de anti-MBP se estudió preparando una batería de bioconjugados el mismo día y en días diferentes. Para los ensayos realizados el mismo día, los valores de la desviación estándar relativa en la determinación de anti-MBP fueron del 4.5% y 4.8% ($n=5$), respectivamente. Además, los valores RSD fueron del 4.6% ($n=5$) en ausencia y 5.3 % ($n=5$), y en presencia de anti-MBP para las medidas realizadas utilizando bioplataformas preparadas en días distintos. Considerando ahora la reproducibilidad del método para CCL5, los resultados obtenidos del mismo modo, en ausencia y en presencia de 2 ng mL^{-1} de CCL5 ($n=5$) fueron: RSD = 1.5 y 2.3 %, respectivamente, y del 3.7 y el 4.1 % empleando cinco bioconjugados preparados en días diferentes. Es importante señalar que estos valores son mucho menores que los que proporcionan los kits ELISA comerciales para ambos biomarcadores. Por ejemplo, para CCL5, el método ELISA proporciona valores de CV (RSD) $<12\%$ para este tipo de ensayos. Estos resultados demuestran el alto nivel de precisión alcanzado en la preparación y funcionamiento de los biosensores desarrollados. Finalmente, otra ventaja importante a señalar es el que el volumen de disolución patrón o de muestra que se requieren para la determinación es de $25 \mu\text{L}$, volumen que representa la cuarta parte del necesario en cada kit.

5.3.3.4. Estabilidad y selectividad

La estabilidad de almacenamiento de los bioconjugados preparados sobre las cMBs y las Neutr-MBs, respectivamente, para la determinación de anti-MBP o CCL5, se evaluó preparando una serie el mismo día y conservándolos a 8°C , en tubos eppendorf con $25 \mu\text{L}$ de PBS 100 mM pH 7.4 para ser posteriormente utilizados en distintos días en presencia de 1 ng mL^{-1} de anti-MBP o de 2 ng mL^{-1} de CCL5. En la Figura 5.91 se recogen los resultados obtenidos, apreciándose que las respuestas amperométricas quedan dentro de los intervalos establecidos para cada inmunosensor del modo habitual, durante al menos 15 días en el caso del anti-MBP (máximo periodo evaluado) y de 12 días para CCL5. Estos resultados ponen de manifiesto la aceptable estabilidad de los bioconjugados inmovilizados sobre las MBs y la posibilidad de utilizarlos a demanda para la preparación de los inmunosensores para el análisis clínico de rutina.

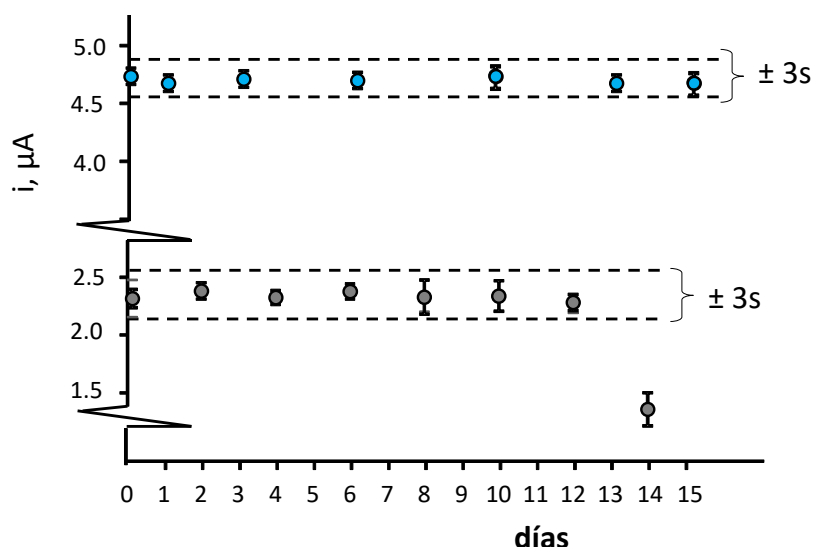


Figura 5.91. Gráfico de control para evaluar la estabilidad de los conjugados anti-MBP-MBP-cMBs (azul) y anti-CCL5-Biotin-Neutr-MBs (gris) a 8 °C en PBS. Ver el texto para más información.

Los resultados de los estudios de selectividad para ambos biosensores se han representado en las Figuras 5.92 y 5.93. Las especies ensayadas como interferentes potenciales y sus concentraciones fueron, para el inmunosensor de anti-MBP: 7 $\mu g mL^{-1}$ de ácido ascórbico (AA), 100 IU mL^{-1} de anticuerpo anti-péptido citrunilado cíclico (CCPA), 70 $pg mL^{-1}$ de endoglina (CD105), 50 $ng mL^{-1}$ de C-X-C ligando 4 (CXCL4), 50 $ng mL^{-1}$ de C-X-C ligando 7 (CXCL7), 5 $ng mL^{-1}$ de hemoglobina (HB), 1 $mg mL^{-1}$ inmunoglobulina G humana (hIgG), 5 $mg mL^{-1}$ de albúmina de suero humana (HSA), 2 $ng mL^{-1}$ de metaloproteinasa de matriz-3 (MMP-3), 50 $pg mL^{-1}$ de ligando de receptor activador para el factor nuclear κB (RANKL), 100 IU mL^{-1} de factor reumatoide (RF) y 100 $\mu g mL^{-1}$ de ácido úrico (UA).

Para el inmunosensor de CCL5 se evaluó el efecto de 5 $mg mL^{-1}$ de hemoglobina (HB), 50 $ng mL^{-1}$ de C-X-C ligando 7 (CXCL7), 50 $pg mL^{-1}$ de factor de crecimiento transformante $\beta 1$ (TGF- $\beta 1$), 50 $pg mL^{-1}$ de interleucina 1 beta (IL-1 β) 300 $pg mL^{-1}$ de interleucina 6 (IL-6), 2 $ng mL^{-1}$ de metaloproteinasa de matriz-3 (MMP-3), 100 IU mL^{-1} de factor reumatoide (RF), 70 $pg mL^{-1}$ de endoglina (CD105), 5 $mg mL^{-1}$ de albúmina de suero humana (HSA), 100 IU mL^{-1} de anticuerpo anti-péptido citrunilado cíclico (CCPA) y 1 $mg mL^{-1}$ de inmunoglobulina G humana (hIgG).

Como puede verse, la mayor parte de los compuestos ensayados coinciden en los estudios con ambas plataformas, pero en cada caso también se han añadido otros más afines a las características de cada biomarcador o sus muestras de interés. Los interferentes potenciales se ensayaron a los niveles de concentración esperables en pacientes sanos, preparando las bioplataformas en ausencia y en presencia de 1 ng mL^{-1} de anti-MBP, así como en ausencia y en presencia de 2 ng mL^{-1} de CCL5.

Los límites de control se establecieron mediante el cálculo de $\pm 2 \cdot s_b$, siendo s_b la desviación estándar correspondiente a la medida obtenida por triplicado utilizando 3 bioconjugados diferentes. Como se puede observar, no se aprecian diferencias significativas en ninguno de los casos estudiados, ni con las proteínas ensayadas ni con las sustancias electroactivas, AA y UA, las cuales no proporcionan respuesta alguna al potencial de reducción utilizado.

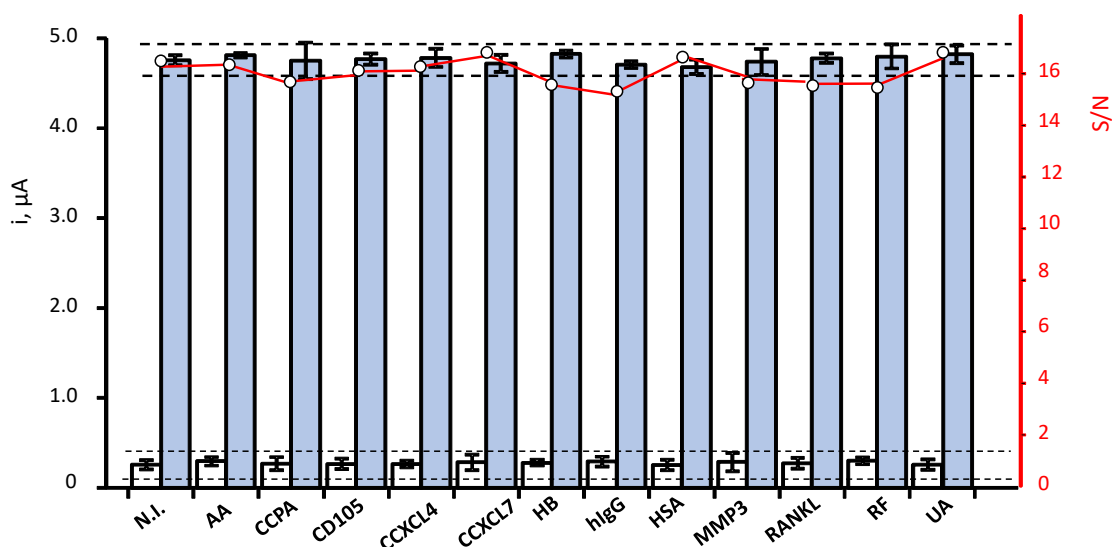


Figura 5.92. Respuestas amperométricas obtenidas con el inmunosensor en ausencia (blanco) y en presencia de 1 ng mL^{-1} de anti-MBP (azul) y en disoluciones preparadas en ausencia (N.I.) o en presencia de AA, CCPA, CD105, CXCL4, CXCL7, HB, hIgG, HSA, MMP3, RANKL, RF y UA. Barras de error estimadas como tres veces la desviación estándar de tres replicados. Ver el texto para más información.

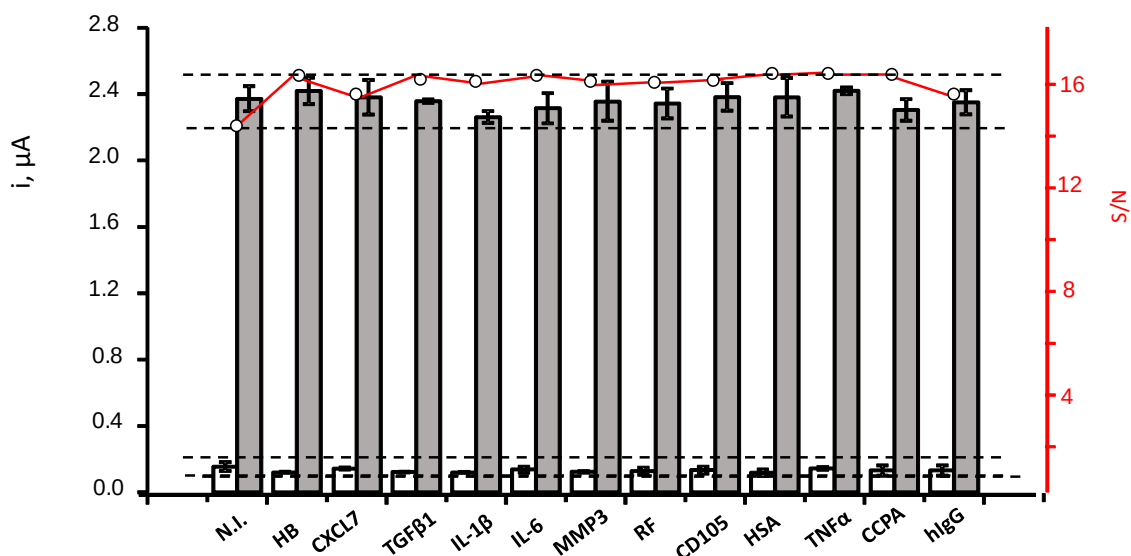


Figura 5.93. Respuestas amperométricas obtenidas con el inmunosensor en ausencia (blanco) y en presencia de 2 ng mL⁻¹ de CCL5 (gris) y en disoluciones preparadas en ausencia (N.I.) o en presencia de HB, CXCL7, TGFβ1, IL1β, IL6, MMP3, RF, CD105, HSA, TNFα, CCPA, y hIgG. Barras de error estimadas como tres veces la desviación estándar de tres replicados. Ver el texto para más información.

5.3.3.5. Determinación de anti-MBP y de CCL5 en suero

La utilidad analítica de los métodos desarrollados se evaluó por aplicación de cada bioplataforma al análisis de muestras de suero de individuos sanos y de pacientes diagnosticados con esclerosis múltiple, todas ellas proporcionadas por Central Biohub® Biospecimens. [[https://www. centralbiohub. com/](https://www.centralbiohub.com/)]. La existencia de efecto matriz en estas muestras se investigó elaborando calibrados de adiciones estándar sobre suero que no contenía ninguno de los biomarcadores. Los resultados obtenidos aplicando este procedimiento a sueros diluidos diez (anti-MBP) y cien (CCL5) veces con disolución reguladora PBS 0.1 M de pH 7.4, se han representado en la Figura 5.94.

Como puede apreciarse, los calibrados realizados sobre las muestras son prácticamente coincidentes con los de patrones. En efecto, en el caso del anti-MBP, la pendiente del calibrado obtenido por adición al suero de concentraciones crecientes de anticuerpo en el intervalo de 0.5 a 3.0 ng mL⁻¹, expresada en unidades de μA por década de concentración (Figura 5.93a), fue de 3.32 ± 0.02 , valor muy parecido a la pendiente

del calibrado de patrones, $3.19 \pm 0.07 \mu\text{A}$ por década de concentración. Como en estudios anteriores, se aplicó el test t de Student para comparar estadísticamente ambas pendientes, obteniéndose un resultado de $t_{\text{exp}}=1.808$ ($\alpha=0.05$) inferior al $t_{\text{tab}}=2.262$. Por otro lado, en el caso de la quimioquina CCL5, el calibrado de adiciones patrón se construyó en el intervalo de 0.1 a 60 ng mL^{-1} (Figura 5.93b), obteniéndose una pendiente de $1.69 \pm 0.04 \mu\text{A}$ por década de concentración, que es similar a la del calibrado de patrones: $1.76 \pm 0.02 \mu\text{A}$ por década de concentración. En este caso, el valor de $t_{\text{exp}}= 1.467$ también resultó inferior al teórico, $t_{\text{tab}}= 2.145$ ($\alpha=0.05$), lo que revela para ambos analitos la ausencia de efecto matriz.

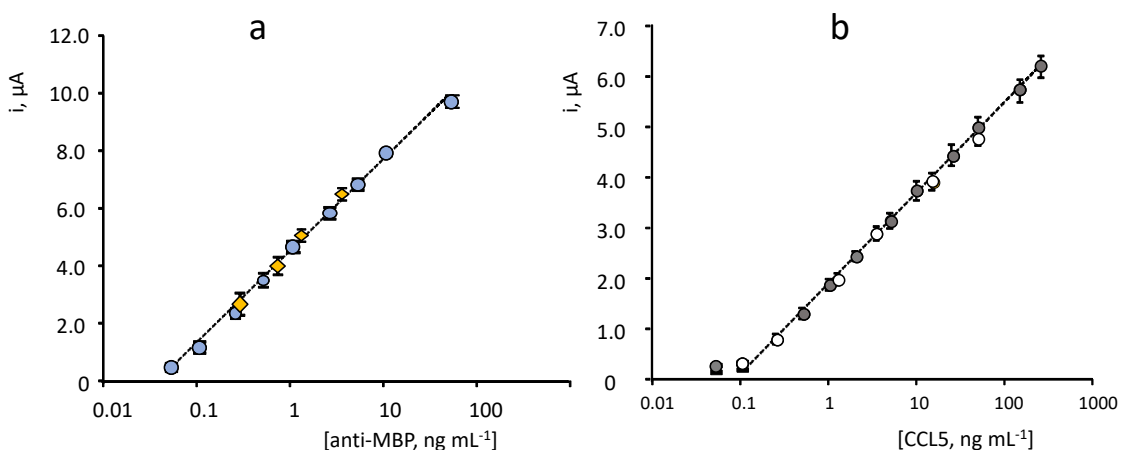


Figura 5.94. Comparación de calibrados de disoluciones patrón y de adiciones de anti-MBP (a) o de CCL5 (b) sobre muestras de suero. Ver el texto para más información.

Los resultados de las determinaciones de anti-MBP y CCL5 en las muestras reales usando las dos bioplataformas desarrolladas en estos trabajos y los kits ELISA comerciales disponibles para cada analito, se comparan en la Tabla 5.14. En primer lugar, hay que destacar la buena correlación existente entre las dos series de datos, tanto en el análisis de los sueros de individuos sanos como en el de los pacientes diagnosticados de MS. Dicha correlación se hace más evidente en la representación mostrada en la Figura 5.95.

Tabla 5.14. Determinación de anti-MBP y de CCL5 en suero de individuos sanos y pacientes con esclerosis múltiple*.

Grupo	Muestra*	anti-MBP, ng mL ⁻¹		CCL5, ng mL ⁻¹	
		Bioplateforma	ELISA	Bioplateforma	ELISA
Individuos sanos	S468087	0.50 ± 0.07	0.48 ± 0.05	15.0 ± 0.1	15.5 ± 0.5
	S468160	0.54 ± 0.04	0.51 ± 0.03	13.0 ± 0.1	13.2 ± 0.2
Pacientes con MS	EM338745	2.71 ± 0.02	2.7 ± 0.1	48.9 ± 0.3	48.6 ± 0.6
	EM337913	2.05 ± 0.06	2.0 ± 0.1	79.7 ± 0.5	80.1 ± 0.5
	EM395952	2.44 ± 0.04	2.42 ± 0.06	94.9 ± 0.5	94.5 ± 0.6
	EM354878	2.43 ± 0.05	2.38 ± 0.05	-	-
	EM398619	1.67 ± 0.04	1.7 ± 0.1	147.7 ± 0.6	148.1 ± 0.8

Valores medios ± ts/√n (n=4). * Números de referencia de la lista de productos de Central BioHub: CBH#2445 multiple_sclerosis_210621(1).xlsx; la muestra EM354878 no estaba disponible en el momento de la determinación de CCL5.

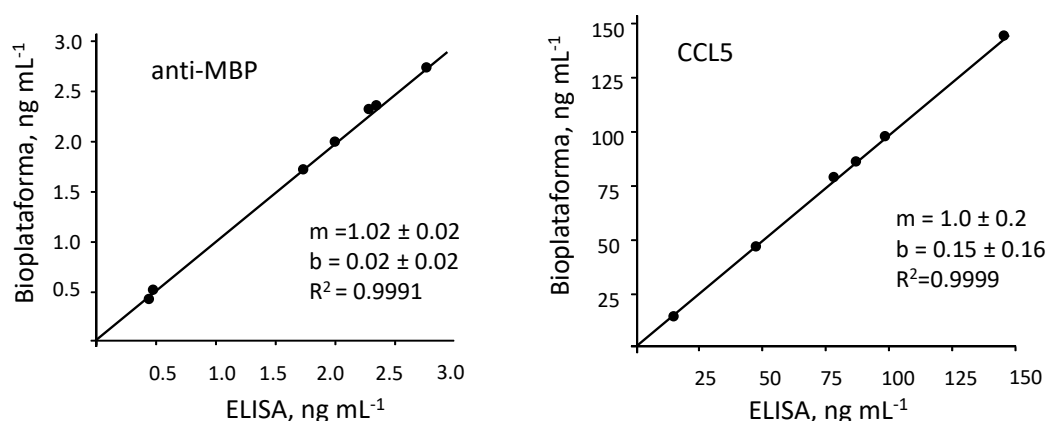


Figura 5.95. Correlación entre las concentraciones de MBP y CCL5 determinadas en suero de individuos sanos y pacientes con esclerosis múltiple con la bioplateforma dual desarrollada y con los test ELISA individuales.

Las concentraciones de anti-MBP encontradas en las muestras de individuos sanos y de pacientes diagnosticados con esclerosis múltiple de acuerdo con los niveles reportados en la bibliografía [Derkus, 2013]. En el caso de la quimioquina CCL5, se sabe que, en pacientes con esta enfermedad, el intervalo de concentraciones en que puede encontrarse está comprendido entre 65 y 120 ng mL⁻¹ [Lechner, 2021; Rentzos, 2010]. Sin embargo, estos niveles dependen de otros parámetros clínicos del paciente. Por

ejemplo, se ha observado un aumento significativo CCL5 sérico en individuos que presentaban un elevado índice de masa corporal (IMC) [Keophiphath, 2010]. Esta circunstancia justificaría la elevada concentración de CCL5 encontrado en la muestra número 5, EM398619, al corresponder a un paciente con un IMC de 32.

5.3.3.6. Conclusiones

En estos trabajos se han desarrollado las primeras bioplataformas electroquímicas para la determinación del anticuerpo anti-MBP y la quimioquina CCL5, biomarcadores de esclerosis múltiple, empleando MBs carboxiladas o funcionalizadas con neutravidina, respectivamente, para inmovilizar el anticuerpo de captura anti-MBP o el anticuerpo biotinilado Biotin-anti-CCL5 y establecer configuraciones de tipo sándwich haciendo uso del conjugado enzimático HRP-IgG para llevar a cabo la detección amperométrica por adición de peróxido de hidrógeno con hidroquinona como mediador redox. En ambos casos se alcanzó una sensibilidad elevada, obteniéndose calibrados con un amplio intervalo de concentraciones, y otras características apropiadas para aplicar el método desarrollado a muestras clínicas reales. La utilidad práctica de estas plataformas se demostró aplicándolas a muestras de individuos sanos y de pacientes con esclerosis múltiple, sin necesidad de tratamiento previo salvo una dilución 1/100 en disolución reguladora.

Conclusions

6

Throughout the chapters of this Thesis, the partial conclusions drawn from the results obtained with each configuration have been exposed, highlighting the achievements, their novelty and relevance. For this reason, this section aims to summarize what has been done during these years of research, taking into account the fulfilment of the initial objectives and the importance of what has been achieved.

In the first place, regarding the main objective embodied in the title to this Doctoral Thesis, it should be noted that four electrochemical immunosensors involving novel immobilization strategies as well as four bioplayers for implementing magnetically assisted immunoassays, two of them dual, have been developed. All these designs were applied to the determination of biomarkers of autoimmune diseases, focusing on systemic inflammatory diseases, especially rheumatoid arthritis (RA) and multiple sclerosis (MS).

In this work, two types of immunosensor configurations have been developed: integrated and prepared from platforms based on magnetic microparticles, which allows a comparison between both configurations to be concluded. Thus, in the former, the possibility of using different ways of modifying the electrode surface to implement new ways of immobilizing biomolecules as well as increasing the magnitude of the analytical response has been taken advantage of, while in the case of platforms, it has been done use of the advantages of the magnetic microparticles on which they are based, which make possible the preparation of the immunoconjugates outside the sample, therefore avoiding unspecific adsorptions, whilst reducing the number of steps involved in the working protocol and facilitating the incubation and cleaning operations.

Two immobilization schemes for immunoreagents involving the functionalization of screen-printed gold electrodes by oxidative grafting of arylamines were developed for the first time. One of the resulting immunosensors was applied to the determination of the cytokine TGF- β 1, a relevant biomarker of inflammation processes. The other, allowed demonstrating the usefulness of this strategy against the preparation of monolayers of thiol derivatives, more frequent on these electrodes, and was applied to the determination of peptide PYY, recently considered a biomarker of interest in RA patients, due to its role as regulator of energy balance in inflammatory processes.

Screen-printed electrodes modified with carbon nanotubes functionalized with azide groups linked to ethynylated immunoglobulin by Cu(I)-catalyzed azide-alkyne cycloaddition ("click") have been used as supports for antibody immobilization. The immunosensor prepared by chemical synthesis of the catalyst was applied to the determination of the chemokine CXCL7, overexpressed in the serum of patients with RA at the beginning of the disease. In addition, the advantage of the electrochemical synthesis of the catalyst ("electro-click") by applying a reduction potential in the presence of a Cu(II) salt was also demonstrated by developing an immunosensor for the determination of the pro-inflammatory interleukin IL -1 β .

With the aim of preparing multiple schemes for the simultaneous detection of various biomarkers, these providing a greater amount of clinically relevant information, two dual electrochemical immunoplatfroms involving functionalized magnetic microparticles (MBs) were developed and applied to the simultaneous determination of two auto-antibodies: factor rheumatoid (RF) and anti-cyclic citrullinated peptide (CCPA), as well as two proteins, the chemokine CXCL7 and the enzyme matrix metalloproteinase-3 (MMP-3), all of them relevant biomarkers of RA.

A similar strategy was used for the preparation of two individual immunoplatfroms for MS biomarkers: the auto-antibody anti-myelin basic protein (anti-MBP), considered an essential biomarker to monitor the progression of the degenerative disease, and the chemokine CCL5, triggering of inflammation in the central nervous system, which might be key in the disease's development, and whose remarkable increased levels are directly associated with MS activity.

The particular advantages of the methods developed for these biomarkers using the proposed immunodetection devices in comparison with other methods involving electrochemical transduction, if they exist, and with respect to ELISA immunoassay procedures, have been described throughout the Thesis.

Finally, it should be noted that both immunosensors and detection immunoplatfroms demonstrated their analytical utility through the application and validation of results on real samples of serum or saliva, being the results obtained in the analysis of sera from patients with diagnosed RA or MS especially important.



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Glosario de abreviaturas

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A

AA: ácido ascórbico

Ab: anticuerpo

Ag: antígeno

anti-CCL5: anticuerpo frente a quimioquina (C-C) ligando 5

anti-CCL5-Biotin: anticuerpo biotinilado frente a quimioquina (C-C) ligando 5

anti-CXCL7: anticuerpo frente a quimioquina (C-X-C) ligando 7

anti-IL1 β : anticuerpo frente a interleucina 1 β

anti-MBP: anticuerpo frente a proteína básica de mielina

anti-MMP3: anticuerpo frente a metaloproteinasa de matriz 3

anti-PYY: anticuerpo frente a péptido YY

anti-RF: anticuerpo frente a factor reumatoide

anti-TGF: anticuerpo frente a factor de crecimiento transformante beta 1

alkyne-IgG: inmunoglobulina G etinilizada

APN: adiponectina

AP-Strept: conjugado de fosfatasa alcalina y estreptavidina

Apt: aptámero

AU: ácido úrico

B

BB: blocking buffer (caseína comercial)

Biotin-anti-CXCL7: anticuerpo biotinilado frente a quimiocina (C-X-C) ligando 7

Biotin-anti-IL1 β : anticuerpo biotinilado frente a interleucina 1 β

Biotin-anti-MMP3: anticuerpo biotinilado frente a metaloproteinasa de matriz 3

Biotin-anti-TGF: anticuerpo biotinilado frente a factor de crecimiento transformante β 1

Biotin-PYY: péptido YY biotinilado

BR/BIL: bilirrubina

BSA: albúmina de suero bovino

C

Cad-17: cadherina 17

CCL5: quimiocina (C-C) ligando 5

CCP: péptido cíclico citrunilado

CCP-Biotin: péptido cíclico citrunilado biotinilado

CCPA: anticuerpo frente a péptido cíclico citrunilado

CD105: endoglina

CFFCP1: péptido sintético de fibrina quimérica/ filagrina

CHRL/CHL: colesterol

CIA: artritis inducida por colágeno

cMBs: partículas magnéticas carboxiladas

CNS: sistema nervioso central

CPAs: anticuerpos frente a péptidos citrunilados

CRP: proteína C reactiva

CSF: fluído cerebrospinal/ líquido cefaloraquídeo

CTAP-III: péptido III activador de tejido conector

CuAAC: cicloadición 1,3-dipolar azida-alquino catalizada por cobre (I)

CV: voltamperometría cíclica

CXCL4: quimiocina (C-X-C) ligando 4

CXCL7: quimiocina (C-X-C) ligando 7

D

DPV: voltamperometría diferencial de impulsos

E

E-cad: E-cadherina

EDC: 1-etil-3-[3-dimetilaminopropil] carbodiimida

EIS: espectroscopía de impedancia electroquímica

ELISA: Enzyme-Linked Immunosorbent Assay

ERBB2: receptor tirosina quinasa

Etinil-IgG: inmunoglobulina G etinilada

E2: estradiol

F

Fc: fracción constante del anticuerpo que determina su actividad biológica

Fc(IgG): fracción constante de una inmunoglobulina G

FGFR4: receptor del factor de crecimiento de fibroblastos 4

G

GHRL: grelina

GPES: General Purpose Electrochemical System

GSH: glutathione

H

HB: hemoglobina

hGH: hormona de crecimiento humana

hIgG: IgG humana

HQ: hidroquinona

HRP: enzima de peroxidasa de rábano

HRP-IgG: conjugado de enzima de peroxidasa de rábano e inmunoglobulina G

HRP-IgM: conjugado de enzima de peroxidasa de rábano e inmunoglobulina M

HRP-Strept: conjugado de enzima de peroxidasa de rábano y estreptavidina

HSA: albúmina de suero humano

I

IDEs: electrodos interdigitados

IFN: interferon γ

IgG: inmunoglobulina G

IgG-MWCNT: nanotubos de carbono de pared múltiple con inmunoglobulina G
etnilada

IL: familia de interleucinas

IL-1 β : interleucina 1 β

IL-6: interleucina 6

IL-8: interleucina 8

M

MBP: proteína básica de mielina

MBs: partículas magnéticas

MDD: dosis mínima detectable

MES: ácido 2-(*N*-morfolino)etanosulfónico

MHA: ácido mercaptohexanoico

MMPs: familia de metaloproteinasas

MMP3: metaloproteinasa de matriz 3

MS: esclerosis múltiple

MWCNTs: nanotubos de carbono de pared múltiple

MWCNT-PS: nanotubos de carbono de pared múltiple y poliestireno

4-MBA: ácido 4-mercaptobenzoico

N

NAP-2: péptido 2 activador de neutrófilos

Neutr-MBs: partículas magnéticas con neutravidina

NHSS: N-hidroxisulfo-succinimida

NPY: neuropéptido Y

1-NP: 1-naftol

1-NPP: 1-naftilfosfato

L

Lep: Leptina

LOD: límite de detección

LOQ: límite de cuantificación

P

***p*-ABA:** ácido *p*-aminobezoico

PB: disolución reguladora fosfato

PBS: disolución reguladora fosfato salino

PBST: disolución reguladora fosfato salino suplementada con Tween[®] 20

PEG: polietilenglicol

Poly-HRP-Strept: polímero de estravidina y peroxidase de rábano

PP: polipéptido pancreático

PYY: péptido YY

P53: proteína supresora de tumores humana P53

R

RA: artritis reumatoide

RANKL: ligando de receptor activador para el factor nuclear- κ B

Rct: resistencia a la transferencia de carga

RF: factor reumatoide

RSD: desviación estándar relativa

S

SAM: monocapas autoensambladas

SLE: lupus eritematoso sistémico

SPE: electrodo serigrafiado

SPAuE: electrodo serigrafiado de oro

SPCE: electrodo serigrafiado de carbono

SPdCE: electrodo serigrafiado de carbono dual

SSc: esclerosis sistémica

SWV: voltamperometría de onda cuadrada

SWCNT: nanotubos de carbono de pared simple

T

TGF- β 1: factor de crecimiento transformante β 1

TMB: 3,3',5,5'-tetrametilbencidina

TNF/TNF- α : factor de necrosis tumoral α

V

V: viológeno

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Publicaciones

El trabajo de investigación presentado en esta Tesis Doctoral ha dado lugar las siguientes publicaciones científicas:

1. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Screen-printed gold electrodes functionalized with grafted p-aminobenzoic acid for the construction of electrochemical immunosensors. Determination of TGF- β 1 cytokine in human plasma, *Electroanalysis* 30 (2018) 1327 – 1335.
2. S. Guerrero, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Oxidative grafting vs. monolayers self-assembling on gold surface for the preparation of electrochemical immunosensors. Application to the determination of peptide YY, *Talanta* 193 (2019) 139 – 145.
3. S. Guerrero, D. Cadano, L. Agüí, R. Barderas, S. Campuzano, P. Yáñez-Sedeño, J.M. Pingarrón, Click chemistry-assisted antibodies immobilization for immunosensing of CXCL7 chemokine in serum, *Journal of Electroanalytical Chemistry* 837 (2019) 246 – 253.
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5. S. Guerrero, E. Sánchez-Tirado, G. Martínez-García, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Electrochemical biosensor for the simultaneous determination of rheumatoid factor and anti-cyclic citrullinated peptide antibodies in human serum, *Analyst* 145 (2020) 4680 – 4687.
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Oxidative grafting vs. monolayers self-assembling on gold surface for the preparation of electrochemical immunosensors. Application to the determination of peptide YY



Oxidative grafting vs. monolayers self-assembling on gold surface for the preparation of electrochemical immunosensors. Application to the determination of peptide YY

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ABSTRACT

A comparison of the performance of two electrochemical immunosensors for the determination of the anorexigen biomarker peptide YY (PYY) is reported by using as scaffolds screen printed gold electrodes modified either by oxidative grafting of *p*-aminobenzoic acid (*p*-ABA) or by assembling of a 4-mercaptopbenzoic acid (4-MBA) SAM. Covalent immobilization of capture antibodies on the surface-confined carboxyl groups was carried out by EDC/NHSS chemistry, and competitive immunoassays between target PYY and Biotin-PYY were implemented. Upon labeling with alkaline phosphatase (AP)-streptavidin conjugate and 1-naphtyl phosphate addition, differential pulse voltammograms recorded between -0.2 and $+0.7$ V were used as analytical readout. All the steps involved in the functionalization of the electrodes and the preparation of the immunosensors were monitored by electrochemical impedance spectroscopy. The calibration plot for PYY using the AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor provided a linear current vs. $\log$ [PYY] plot extending between 10^{-6} and 10^3 ng/mL PYY with a detection limit of 3×10^{-7} ng/mL. These analytical characteristics are remarkably better than those obtained with the immunosensor prepared with 4-MBA SAM-SPAUEs. The AP-Strept-Biotin-PYY (PYY)-anti-PYY-Phe-N-SPAUE immunosensor was used to analyze human serum and saliva samples spiked with PYY at concentrations fitting with normal levels in these biological fluids.

1. Introduction

Gold electrodes have been much less explored for the preparation of electrochemical immunosensors than carbon surfaces despite gold is a very conductive and stable material and can be easily modified and characterized. Preparation of self-assembled monolayers (SAMs) by chemisorption of thiols has been the common way to modify gold electrodes for constructing electrochemical biosensors. This methodology, characterized by its simplicity and versatility, leads to densely-packed organized structures on which a high loading of different functional groups may be incorporated [1]. In contrast to these advantages, an important practical limitation concerns the stability of the gold-thiolate bond and, therefore, of the entire SAM. Clearly this is a critical issue when biosensors should operate over long times or their fabrication involves multiple coupling steps. In these cases, the oxidation of alkanethiols and the resultant loss of SAM becomes limiting. An alternative approach is gold surface modification by electrografting of aryl-derivatives. This strategy not only provides an effective way for electrode functionalization but also improves stability related to long-

term storage in air and offers a suitable environment for immobilization of bioreagents and a wider potential window for electrochemical detection [2,3].

Two procedures can be utilized to implement this methodology. On one hand, the electrochemical reduction of aryl-diazonium salts results in the formation of the corresponding aryl-diazenyl radicals. These radicals decompose rapidly by loss of nitrogen to generate aryl radicals covalently attached onto the electrode surface. This is by far the most common approach and has been employed on several conducting materials such as carbon or metals [4]. However, this strategy usually leads to disordered multilayer formation, unless special precautions are taken. On the other hand, electrochemical oxidation of arylamine-derivatives results in the formation of aminated cation radicals and, subsequently, of well-defined and covalently assembled aryl layers by nitrogen linkages on the electrode surfaces [5]. Our group reported previously electrochemical immunosensors through oxidative grafting of aryl-amines onto screen-printed carbon electrodes for the determination of estradiol hormone [6] and sulfonamide antibiotics [7]. Surprisingly, the use of oxidative electrochemical grafting on gold surfaces

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has been much less explored despite it is an excellent electrode modification alternative since a strong NH-Au covalent bond is generated [8]. Recently, we have reported an immunosensor for the determination of TGF- β 1 cytokine by implementing a sandwich-type assay upon immobilization of the specific antibody onto *p*-aminobenzoic acid (*p*-ABA) using oxidative grafting onto screen-printed gold electrodes [9].

In this paper, we perform a comparison of two electrochemical immunosensors using screen printed gold electrodes constructed either by oxidative grafting of *p*-ABA or by assembling of a 4-mercaptobenzoic acid (4-MBA) SAM. The peptide YY (PYY) was selected as the target analyte. This peptide is a potent anorexigen that belongs to the pancreatic polypeptide family and whose name derives from the tyrosine residues (Tyr or Y) at both its N- and C-termini [10]. It is produced in the gut by the L cells of the terminal ileum and colon and it is secreted to the circulation in response to food consumption [11]. Circulating PYY controls food intake acting on the arcuate nucleus in the hypothalamus [12]. Effects of PYY on satiety and body weight suggest that this peptide could mediate both the satiating effects of protein and the associated reduction in long-term adiposity [13]. This behavior has led PYY to become a therapeutic target to reduce hunger and caloric intake [11]. Concentration levels of PYY in plasma are around 40–70 pg/mL, although variability in circulating levels provides postprandial peaks of about one hundred pg/mL. In obese individuals their fasting plasma concentration is lower while in anorexia nervosa is higher than in healthy subjects with normal weight [14]. According to these roles, there is an increasing interest in the determination of PYY for the control of obesity and related diseases as well as in the follow-up of appetite disorders [15].

Despite this, available methods for the determination of PYY are scarce. ELISA commercial kits use similar immunoassay schemes involving specific PYY antibodies or biotinylated PYY binding, HRP-labeled avidin or streptavidin conjugates and colorimetric detection. These kits allow the determination of PYY at concentration ranges between 0.1–1 and 100–1000 pg/mL, with minimum detectable concentration between 0.5 and approximately 3 pg/mL. The assays take around 2–5 h although some of them require more than 20 h or overnight incubations. In a previous work, our group reported the first immunosensor for the determination of peptide YY. The method involved the preparation of an electrochemical platform consisted of a diazonium salt grafted onto a reduced graphene oxide-modified glassy carbon electrode and provided a linear range extending between 10^{-4} and 10^2 ng/mL with a LOD value of 0.01 pg/mL [16].

Using both strategies for the modification of gold surface, a covalent immobilization of anti-PYY antibody was accomplished, followed by a competitive immunoassay involving biotinylated antigen (Biotin-PYY) and streptavidin labeled with alkaline phosphatase (AP-Strept). The transduction event was monitored by differential pulse voltammetry after addition of 1-naphthyl phosphate (1-NPP) as the AP enzyme substrate.

2. Experimental

2.1. Reagents and solutions

PYY (3-36) (human) purified IgG antibodies (anti-PYY), PYY (3-36) (human) and biotinylated-PYY (Biotin-PYY) (3-36) (human) were purchased from Phoenix Pharmaceuticals, Inc. Lithium perchlorate (Aldrich, 99.9%), *p*-ABA, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysulfo-succinimide (NHSS) were purchased from Across. 4-MBA was from Sigma-Aldrich. Alkaline phosphatase-labeled streptavidin from *Streptomyces avidinii* (AP-Strept) [S9210] was from Thermo Scientific. Ethanolamine (Sigma), bovine serum albumin (BSA, Gerbu), and casein solution (Blocking buffer, Thermo Scientific) were tested as blocking agents. A 0.025 M MES solution of pH 5.0 was prepared from 2-morpholinoethane-sulfonic acid (Gerbu). 0.1 M phosphate buffer solution (PBS) of pH 7.4 was prepared from sodium di-

hydrogen phosphate and di-sodium hydrogen phosphate (Scharlau). A 100 mM tris(hydroxymethyl)amino-methane (Sigma) solution (Tris) of pH 7.2 and a 50 mM tris(hydroxymethyl)amino-methane buffer solution containing 10 mM MgCl₂ (Labkem) pH 9.6 (Trizma) were also used. All reagent solutions were prepared in 0.1 M PBS except the AP-Strept and 0.05 M 1-NPP solutions, which were prepared in Tris and Trizma buffers, respectively. Transforming growth factor β 1 (TGF- β 1, R & D Systems), human growth hormone (hGH, Sigma-Aldrich), adiponectin (APN, Abnova), hemoglobin (Hb, Sigma-Aldrich), ghrelin (GHRL, Anaspec) and leptin (Lep, BioVendor) were tested as potential interfering compounds. Deionized water was obtained from a Millipore Milli-Q purification system (18.2 M Ω cm).

2.2. Apparatus

All electrochemical measurements were carried out using a PGSTAT 101 potentiostat from Autolab, provided with a NOVA 1.8 electrochemical analysis software. Screen-printed gold electrodes (SPAUE, DRP-220BT 4 mm diameter) were from Metrohm DropSens (Oviedo, Spain). These electrodes include a silver pseudo-reference electrode and a gold counter electrode. All experiments were performed at room temperature. A P-Selecta Ultrasons ultrasonic bath, a Velp-Scientifica small samples homogenizer, a P-Selecta heater and a precision pH meter Crison model Basic 20+ were also used.

2.3. Procedures

2.3.1. Preparation of the electrochemical scaffold

SPAUEs were pre-treated electrochemically according to the supplier instructions. After deposition of 50 μ L 0.1 M sulfuric acid (Scharlau, 97%) on the electrode surface, ten successive cyclic voltammetric scans were applied between 0.00 and 1.25 V vs. Ag at a potential scan rate of 100 mV/s. Subsequently, the electrode was washed with water and sonicated for 1.5 min then allowing drying at room temperature. Thereafter, two different routes for SPAUE functionalization were followed (Fig. 1): A) oxidative electrochemical grafting with *p*-ABA or B) self-assembling of 4-mercaptobenzoic acid (4-MBA) SAM. Electrochemical grafting was performed by immersion of the electrode into a 1.5 mM *p*-ABA and 0.1 M LiClO₄ solution prepared in 10 mL of absolute ethanol [17] and applying three successive cyclic voltammetric scans between 0.0 and +0.6 V vs. Ag at a potential scan rate of 30 mV/s. Thereafter, the modified electrode was washed with water, stirred ultrasonically for 30 s and dried at room temperature. Assembling of the 4-MBA SAM was carried out by immersion of the SPAUE into a 1.5 mM 4-MBA ethanol solution at room temperature for 6 h. After a washing step with water, the electrode surface modified with thiol groups was dried at room temperature.

2.3.2. Preparation of the immunosensors

The carboxyl groups of the electrode surface were activated by dropping 20 μ L of 0.1 M EDC/NHSS solution prepared in 0.025 M MES buffer of pH 5.0 and incubation for 30 min at room temperature in a humid ambient and in darkness. After washing with water and drying at room temperature, anti-PYY was covalently immobilized by incubation with 10 μ L of a 20 μ g/mL antibody solution at 25 °C for 60 min under the conditions mentioned above. The resulting anti-PYY-Phe-N-SPAUE was rinsed with water and the remaining free sites on the electrode surface were blocked by incubation with 10 μ L of 0.2% casein (Blocking buffer) solution for 30 min to avoid unspecific adsorptions. A competitive immunoassay was designed by 5 μ L incubation of a solution containing 100 ng/mL of Biotin-PYY conjugate and the target PYY for 30 min. Then, 10 μ L of a 5 μ g/mL AP-Strept solution were added and, after 30 min incubation, the resulting AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor was washed with 0.1 M Tris buffer solution of pH 7.2 and kept in humid ambience until measurements. The determination of PYY was accomplished upon deposition of 45 μ L

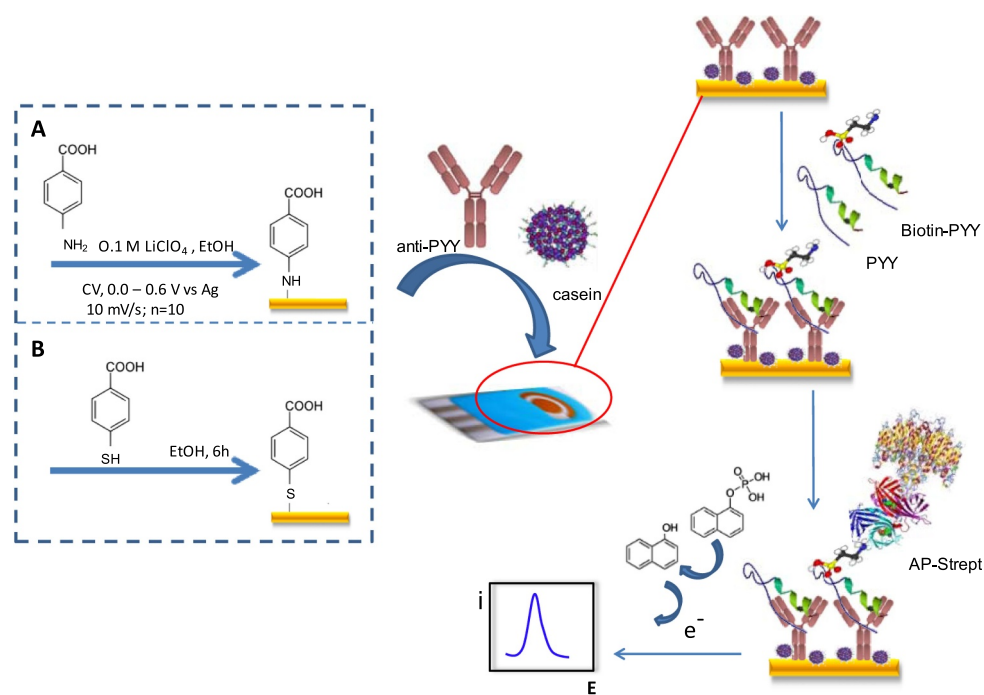


Fig. 1. Scheme of the preparation and functioning of the immunosensors constructed for PYY determination using as scaffolds SPAuEs modified by oxidative grafting of *p*-ABA (A) or 4-MBA-SAM (B).

of Trizma buffer solution of pH 9.6 followed by adding 5 μ L of 5 mM 1-NPP in the same Trizma buffer. After 5 min to let the enzyme reaction took place, differential pulse voltammograms were recorded between -0.2 and 0.7 V vs. Ag.

To perform the comparison, the same methodology for antibody immobilization and the same assay protocol was implemented on the 4-MBA SMA-modified gold screen printed electrodes.

2.3.3. Sample analysis

Spiked human serum and saliva samples were analyzed with only one-step treatment involving a 1:10 sample dilution with Biotin-PYY conjugate solution in PBS of pH 7.4.

3. Results and discussion

The different steps involved in the preparation and functioning of the AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor are schematically illustrated in Fig. 1. Upon functionalization of the SPAUE by oxidative grafting with *p*-ABA (A) or with the 4-MBA SMA (B), covalent immobilization of capture antibodies on the surface-confined carboxyl groups was carried out by EDC/NHSS chemistry. After a blocking step of the remaining activated un-reacted sites with casein blocking buffer, a competitive immunoassay between target PYY and Biotin-PYY was implemented. Upon labeling with AP-Strept conjugate and 1-NPP addition, DP voltammograms recorded between -0.2 and $+0.7$ V were used as analytical readout.

3.1. Optimization of the experimental variables

The different variables involved in the preparation of the modified SPAuEs and in the performance of the resulting AP-Strept-Biotin-PYY (PYY)-anti-PYY-Phe-N-SPAUE immunosensors were tested and optimized. The ratio values between the specific DP peak currents and the unspecific current (measured without Biotin-PYY) both measured in the absence of PYY were taken as the criterion to select each variable. The steps involved in the modification of the SPAuEs by CV electrografting were: a) range of scanned potential, b) number of cycles, c) potential

scan rate, and d) *p*-ABA concentration. The tested variables affecting the preparation of the immunosensor were: a) anti-PYY loading and time for incubation, b) type and concentration of the blocking agent, c) Biotin-PYY loading and time for incubation, and d) AP-Strept loading. In addition, the optimized variables involved in the preparation of the immunosensor using SPAUE modified with a 4-MBA SAM were: a) the thiol concentration and b) the electrode incubation time in the 4-MBA ethanol solution. Other experimental variables implied in the methodology such as the AP substrate used (1-NPP), the pH value and the time lasted for the enzymatic reaction were the same than those optimized previously [18]. The results of these studies are described in the Supplementary Information and in Figs. S1–S7. A summary of the tested ranges and the selected values for all the studied variables is included in Table 1.

3.2. Electrochemical impedance spectroscopy characterization of the immunoplatform

All the steps involved in the functionalization of the electrodes and the preparation of the immunosensors were monitored by electrochemical impedance spectroscopy (EIS) using 5 mM $\text{Fe}(\text{CN})_6^{4-/3-}$ as the redox probe in 0.1 M KCl. Fig. 2A shows that the SPAUE pretreatment with H_2SO_4 gave rise to a noticeable decrease in the electron transfer resistance, R_{CT} , from 1950 Ω (curve 1) to 212 Ω (curve 2). Grafting of *p*-ABA provoked a large increase in the R_{CT} up to 1075 Ω (curve 3) due to the electrostatic repulsion between the redox probe and the negatively charged carboxylate groups. Upon activation with EDC/NHSS, the R_{CT} value dramatically decreased ($R_{\text{CT}} = 12$ Ω , curve 4) because of neutralization of charges. Fig. 2B displays the Nyquist plots recorded for the following steps involved in the immunosensor preparation. Conjugation with anti-PYY to prepare anti-PYY-Phe-N-SPAUE produced an enhancement in the R_{CT} value (170 Ω , curve 5) due to the partially insulating barrier on the electrode surface provoked by the antibody immobilization. Further blocking of the unreacted sites gave rise to a larger R_{CT} value (1047 Ω , curve 6) in agreement with the expected decrease in the electrode conductivity at a quasi-passivated surface. Successive conjugation of Biotin-PYY and AP-Strept provoked

Table 1

Optimization of the different experimental variables involved in the preparation of the electrochemical platforms and the immunosensors developed for the determination of PYY.

Variable	Tested range/ compound	Selected value
<i>p</i> -ABA electrografting on SPAuE		
Potential range, V vs. Ag	0.0 to +0.6 to +1.0	+0.6
Number of cycles	1–10	3
Potential scan rate, mV/s	5–100	30
<i>p</i> -ABA concentration, mM	0.35–3.6	1.5
Immunosensor using <i>p</i> -ABA electrografting on SPAuE		
Anti-PYY loading, µg/mL	5–30	20
Incubation time for anti-PYY, min	15–90	60
Incubation temperature for anti-PYY, °C	4, 25, 37	25
Type of blocking agent	ethanolamine, BSA, casein (blocking buffer)	casein (blocking buffer)
Casein concentration (blocking buffer), %	0.05–1	0.2%
Incubation time for casein (blocking buffer), min	15–90	30
Biotin-PYY loading, ng/mL	50–400	100
Incubation time for Biotin-PYY, min	15–60	30
AP-Strept loading, µg/mL	2.5, 5.0, 7.5	5.0
Immunosensor using 4-MBA SAM on SPAuE		
4-MBA concentration, mM	0.5–2.5	1.5
Incubation time for 4-MBA, h	2–10	6

further increases in R_{CT} (1355 Ω for Biotin-PYY-anti-PYY-Phe-N-SPAUE, curve 7, 1725 Ω for AP-Strept-Biotin-PYY-anti-PYY-Phe-N-SPAUE, curve 8).

EIS characterization was also made for the SPAuEs modified with a 4-MBA SAM. Fig. 2 C shows that 4-MBA-SPAUE provided R_{CT} value (461 Ω) much larger than that obtained after activation with EDC-NHSS (59 Ω) for the same reasons commented above. Fig. 2D shows as the immobilization of anti-PYY (curve 3) followed by blocking (curve 4) produced sequential increases in R_{CT} , 384 Ω and 1074 Ω , respectively. However, unlike it was observed with the immunosensor prepared by oxidative grafting, the subsequent immobilization of Biotin-PYY provoked only a slight increase in R_{CT} (1155 Ω) and the incorporation of AP-Strept conjugate did not cause any significant change (R_{CT} = 1160 Ω). This behavior suggests that the amount of Biotin-PYY immobilized on the anti-PYY-Phe-S-SPAUE is smaller than that on the immunosensors prepared from grafted electrodes. In addition, no significant incorporation of the HRP-Strept conjugate is observed.

3.3. Analytical characteristics for PYY determination

Under the optimized working conditions, a calibration plot for PYY using the AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor was constructed (Fig. 3A). As expected for a competitive immunoassay, a sigmoidal curve was obtained. The current vs. analyte concentration was fitted by non-linear regression using the Sigma Plot data analysis software with the adjusted equation: $y = i_{min} + [(i_{max} - i_{min}) / 1 + (x / EC_{50})^{-h}]$. The r^2 value was 0.991 and the displayed error bars were calculated from measurements made with three different immunosensors. The maximum and minimum currents were $i_{max} = 760 \pm 22$ nA and $i_{min} = 288 \pm 13$ nA, respectively. The EC_{50} value, corresponding to the PYY concentration exhibiting fifty per cent competition, was 0.007 ng/mL, and the Hill slope, h , was -0.20 ± 0.04 . Logit transformation of the sigmoidal curve by plotting $\ln(p / 1-p)$ vs. $\ln x$, with $p = (y - i_{min}) / (i_{max} - i_{min})$, provided a linear

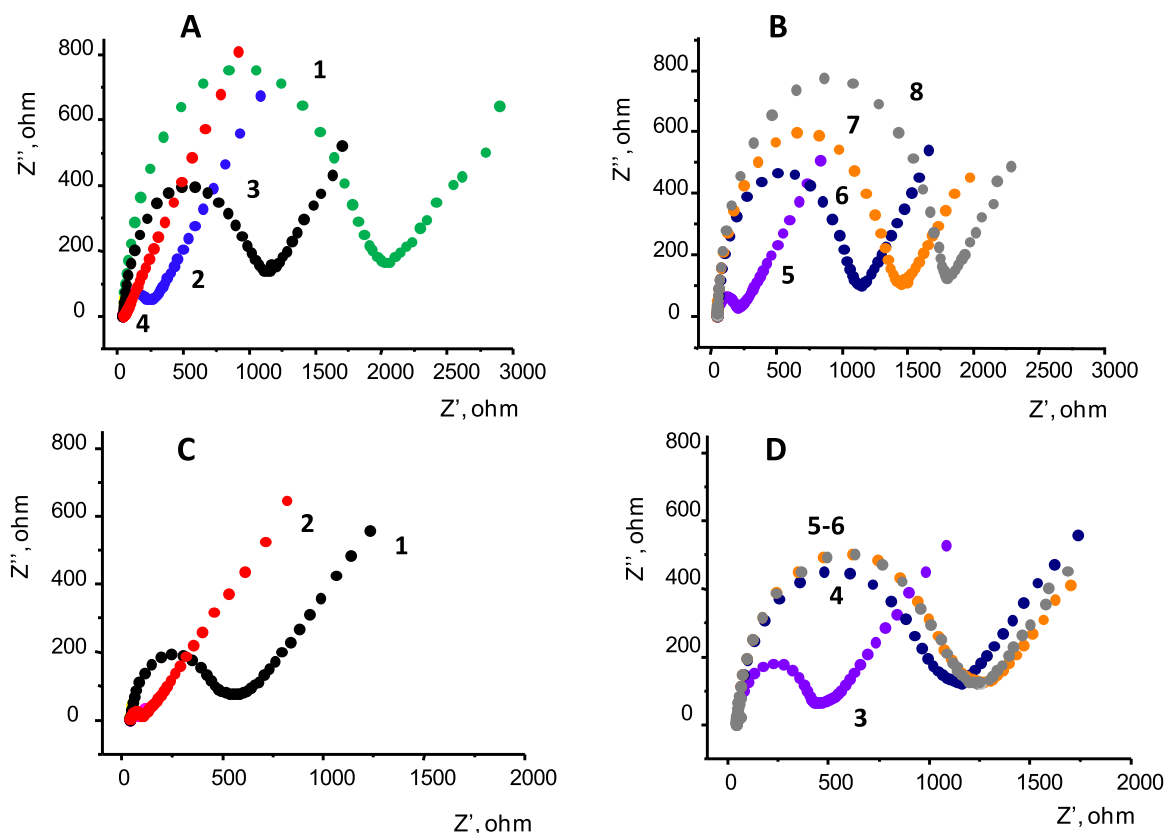


Fig. 2. Nyquist plots recorded for (A) untreated bare SPAuE (1); SPAuE treated with H_2SO_4 (2); *p*-ABA-grafted-SPAuE (3); *p*-ABA-grafted-SPAuE after activation with EDC/NHSS (4); (B) anti-PYY-Phe-N-SPAuE (5); blocked anti-PYY-Phe-N-SPAuE (6); Biotin-PYY-anti-PYY-Phe-N-SPAuE (7); AP-Strept-Biotin-PYY-anti-PYY-Phe-N-SPAuE (8); (C) 4-MBA-SPAuE (1); 4-MBA-SPAuE after activation with EDC/NHSS (2); (D) anti-PYY-Phe-S-SPAuE (3); blocked anti-PYY-Phe-S-SPAuE (4); Biotin-PYY-anti-PYY-Phe-S-SPAuE (5); AP-Strept-Biotin-PYY-anti-PYY-Phe-S-SPAuE (6).

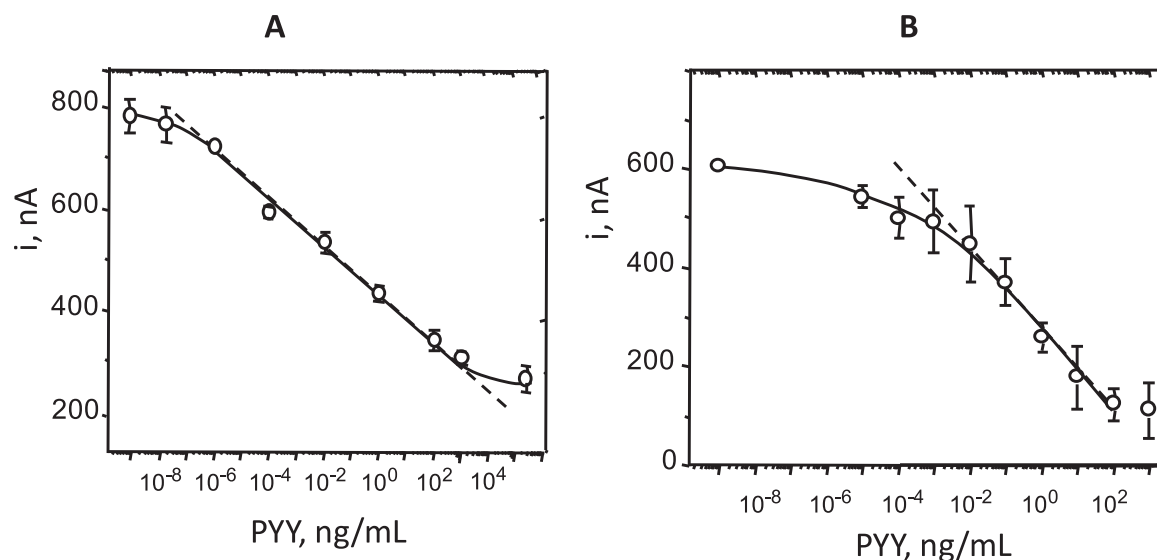


Fig. 3. Calibration plots for PYY constructed with the AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE (A) and AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-S-SPAUE (B). Error bars estimated as a triple of the standard deviation ($n = 3$).

graph ($r^2 = 0.997$) with a slope value of -0.2 in agreement, as expected, with the h value calculated above. Least square fitting of the curve provided a linear current vs. $\log$ [PYY] plot extending between 10^{-6} and 10^3 ng/mL PYY ($r^2 = 0.990$) with a slope value of -49 ± 8 nA per concentration decade.

For comparison, a calibration plot was constructed with the immunosensor prepared from 4-MBA-SPAUE (Fig. 3B). It exhibited lower currents and a shorter linear range ($r^2 = 0.999$) extending from 10^{-2} to 10^2 ng/mL PYY. However, a slightly higher slope in this linear range, -89 ± 4 nA per decade of concentration, was found. The differences observed are probably due to the smaller anti-PYY loading immobilized on the electrode in the configuration prepared with 4-MBA. As is well known, when using competitive type immunosensor configurations, a lower concentration of capture antibody produces an increase in sensitivity and a shortening of the linear range because a smaller antigen concentration is needed to reach the maximum response [19,20]. Furthermore, the 4-MBA SAM-SPAUE-based immunosensor showed a worse reproducibility. In fact, a RSD value of 17.5% was calculated from repetitive measurements of 0.1 ng/mL PYY made with five immunosensors prepared in different days. This poor RSD value was much larger than that calculated under the same experimental conditions for 0.01 ng/mL PYY (in both cases, a PYY concentration located in the central position of the corresponding linear calibration range was used) with the immunosensor prepared through *p*-ABA grafting, 4.6%. The poor precision of the measurements made with the immunosensors using 4-MBA-SPAUE can be attributed to the poor stability of this architecture under the humid environment in which the incubations of the immunoreagents on the modified electrode must be carried out.

The limit of detection was calculated as the analyte concentration for which the voltammetric signal at the AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor was reduced by 10%. The estimated LOD value was 3×10^{-7} ng/mL in good agreement with the value calculated from the equation [21]:

$$LOD = EC_{50} \{ [(i_{max} - i_{min}) / (i_{max} - i_{min} - 3s)] - 1 \}^{-1/h}$$

where s was the standard deviation of measurements from solutions prepared in the absence of PYY ($n = 10$), ± 20 nA, with $LOD = 5 \times 10^{-7}$ ng/mL PYY. According to the former criterion, a LOD value of 10^{-5} ng/mL (two hundred times higher than at the AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor) was calculated for the immunosensor constructed with 4-MBA-SPAUE.

Considering that the expected concentration of PYY in human

serum, which varies around 40–70 pg/mL to 100 pg/mL [13], and in saliva, ranging between 15 and 75 pg/mL [22], it can be deduced that both of immunosensing strategies would allow quantification of this hormone in both types of samples upon an appropriate sample dilution. However, it seems clear from the results commented above that the immunosensor prepared using *p*-ABA grafting on SPAUEs exhibits better analytical characteristics and, accordingly, this configuration was selected for further application to real samples. It is worth mention that the LOD value achieved with this immunosensor is much lower than that reported for the immunosensor prepared by immobilizing anti-PYY onto a reduced graphene oxide-modified SPCE, 0.01 pg/mL, which also showed a narrower range of linearity between 10^{-4} and 10^2 ng/mL PYY [16].

The storage stability of the anti-PYY-Phe-N-SPAUE conjugates was checked. Different bioelectrodes were prepared on the same day and stored in a freezer at 4 °C. Each bioelectrode was used to construct the corresponding immunosensor and to measure the voltammetric response for 5 mM 1-NPP in the absence of PYY. The results obtained (Fig. S8) revealed the acceptable storage stability of this configuration since the measured peak currents remained within the control limits set at $\pm 3 \times$ the standard deviation of the responses obtained on the first day, for 10 days.

3.4. Selectivity

To evaluate the selectivity of the AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor toward the target hormone, various non-target compounds that can accompany PYY in its samples were tested. Human growth hormone (hGH), leptin (Lep), ghrelin (GHR), adiponectin (APN), hemoglobin (Hb) and transforming growth factor $\beta 1$ (TGF- $\beta 1$) were assayed as potential interfering compounds. The responses obtained from solutions without PYY and containing 0.1 μ g/mL Biotin-PYY and each tested specie at a concentration level of 100 ng/mL are shown in Fig. 4. As it can be seen, no significant differences in the measured responses were apparent in the ± 3 s range of the signal obtained with no potential interfering substance, thus demonstrating the high selectivity of the proposed configuration for PYY determination.

3.5. Determination of PYY in spiked human serum and saliva

The developed AP - Strept - Biotin - PYY (PYY) - anti - PYY - Phe - N -

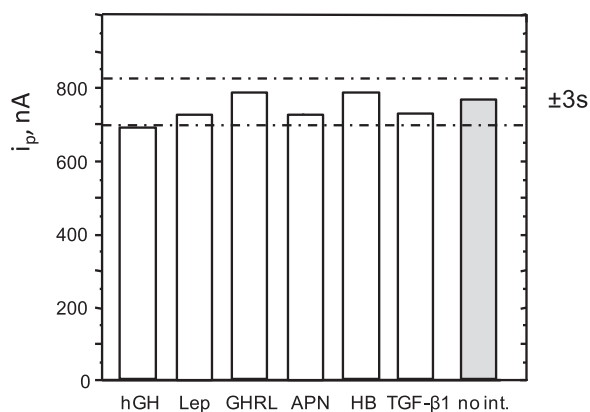


Fig. 4. Effect of the presence of 100 ng/mL hGH, Lep, GHRL, APN, HB, and TGF-β1 on the DPV responses obtained with the AP-Strept-Biotin-PYY-anti-PYY-Phe-N-SPAUE immunosensor in competition with 0.1 μg/mL Biotin-PYY.

Table 2

Determination of PYY in spiked human serum and saliva at a concentration level of 100 and 35 pg/mL, respectively, with the AP-Strept-Biotin-PYY-anti-PYY-Phe-N-SPAUE immunosensor.

Serum	Found, pg/mL ^a	Recovery, %	Saliva	Found, pg/mL ^a	Recovery, %
1	99.6/102	101 ± 3	1	36/33	100 ± 3
2	102/95.0	99 ± 5	2	36/33	99 ± 4
3	105/101	103 ± 3	3	32/35	96 ± 4
4	102/99.4	101 ± 3	4	35/36	102 ± 1
5	99.6/101	100 ± 2	5	35/33	97 ± 2
6	101/102	101 ± 2	6	35/37	102 ± 3
7	100/98.8	99 ± 2	7	34/35	99 ± 2

^a $\bar{x} \pm \frac{s}{\sqrt{n}}$ (n = 2).

SPAUE immunosensor was used to analyze human serum and saliva samples spiked with PYY at 100 and 35 pg/mL, respectively. As it was commented in the Introduction section, these concentrations fit with normal levels in these biological fluids. The working protocol was described in Section 2.3.3. It is worth to note that the methodology involved only a 1:10 sample dilution with the biotinylated antigen conjugate as treatment. The potential occurrence of matrix effects was evaluated by comparing the slope value of the calibration plot constructed with PYY standard solutions with that obtained by spiking samples in the 10^{-4} – 10^2 ng/mL concentration range. The slope values with the spiked serum and saliva were -48 ± 7 and -43 ± 6 nA per decade of concentration, respectively. The Student *t*-test provided *t*_{exp} values of 0.215 (serum) and 1.227 (saliva) which were smaller than the tabulated *t* value for n = 6, α = 0.05, *t*_{tab} = 2.228. These results suggested that no significant matrix effect was observed in the analysis of serum and saliva samples using the described methodology. Accordingly, the determination of PYY in spiked serum or saliva was accomplished by simple interpolation of the measured *i*_p values into the respective calibration plot constructed with standard solutions. The results obtained are summarized in Table 2. Recovery values ranged between 96% and 103% and no significant differences were apparent because of the different nature of biological sample, thus demonstrating the suitability of the approach for the accurate analysis of this biomarker in human serum and saliva.

4. Conclusions

From the comparative study on the preparation of electrochemical immunosensors for the biomarker peptide YY using screen printed gold electrodes constructed either by oxidative grafting of *p*-ABA or by assembling of a 4-MBA SAM, it can be concluded that the grafting approach allows achieving better analytical characteristics in terms of

wider range of linearity and reproducibility. Moreover, a remarkably shorter time required for the preparation of the electrochemical scaffold and a higher stability appear as important practical advantages for routine determinations. The suitability of the prepared AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAUE immunosensor for the analysis of real samples has been demonstrated by applying it to human serum and saliva spiked with PYY at concentrations fitting with normal levels in these biological fluids.

Acknowledgement

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2018.09.089.

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SUPPORTING INFORMATION

Oxidative grafting of carboxyphenyl groups vs. monolayers self-assembling on gold surface for the preparation of electrochemical immunosensors. Application to the determination of peptide YY

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1. Optimization of variables for *p*-ABA electrografting on SPAuE

1.1 Effect of the potential range scanned and the number of cycles

The electrochemical oxidation of *p*-ABA generates a cationic radical through the one-electron oxidation of the amino-group [1] which is grafted onto the SPAuE by covalent binding of nitrogen to the gold surface. Figure S1a shows as repetitive CV scans in a 1.5 mM *p*-ABA and 0.1 M LiClO₄ absolute ethanol solution yielded voltammograms with decreased current as a consequence of the electrode surface coating with a non-conductive material. No significant differences were apparent from the third cycle.

The range of scanned potential for the CV electrografting was studied. Figure S1b shows the results obtained with different immunosensors prepared by electrografting in a 1.5 mM *p*-ABA solution and applying potential scan ranges from 0.0 V to different maximum potential values between +0.6 and +1.0 V vs. Ag. The specific responses of the immunosensor in the absence of PYY and in the presence of 100 ng/mL Biotin-PYY (black points) slightly decreased whereas the unspecific currents in the absence of Biotin-PYY (white points) increased with the positive limit potential. Accordingly, a better S/N ratio was observed for a maximum potential value of +0.6 V which was selected for further work.

Regarding the number of cycles, this variable was checked by preparing different immunosensors with SPAuEs modified by CV electrografting with application of successive scans at 30 mV/s. Figure S1c shows as the best S/N ratio was reached for three potential cycles, in agreement with the behavior shown in Figure S1a. This behavior can be attributed to that a small number of scans provides a low loading of phenyl-derived radicals on the electrode surface and, subsequently, few carboxylic groups are available for antibody binding. However, a large number of potential cycles may produce many binding sites which, in turn, gives rise to steric effects and a loose of effectiveness in the antibody immobilization process.

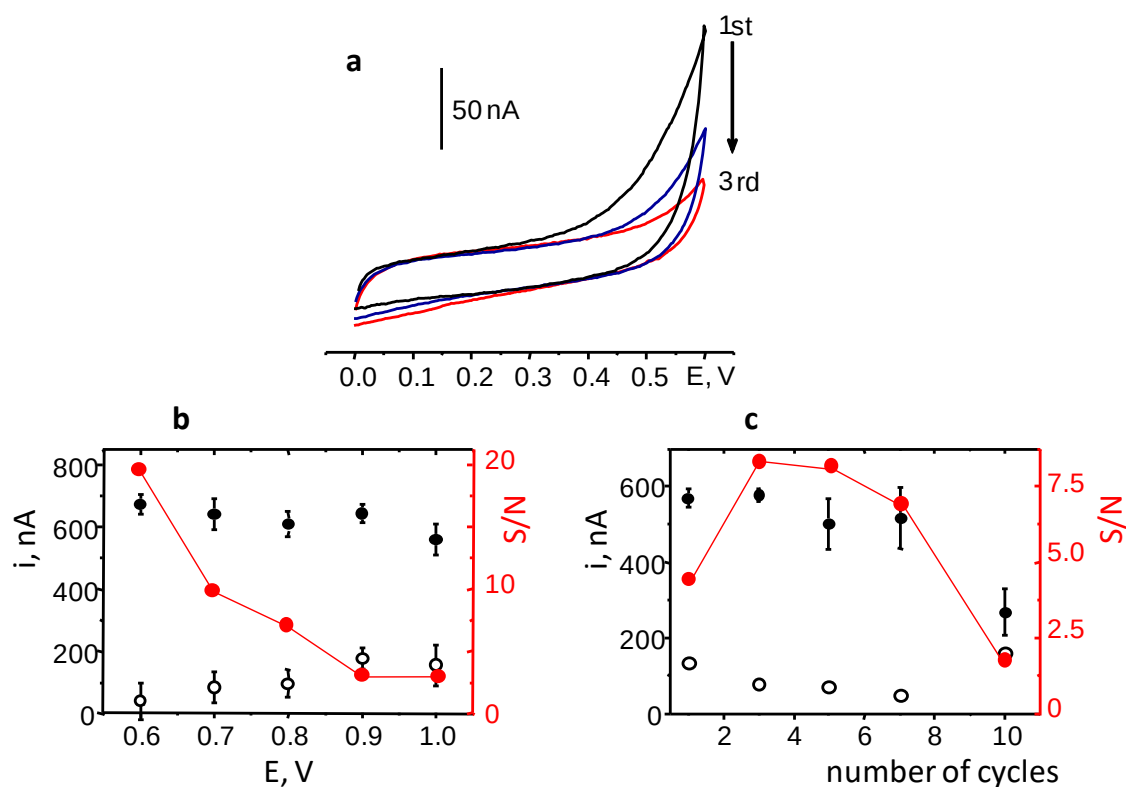


Figure S1. (a) Successive CV scans in 1.5 mM *p*-ABA 0.1 M LiClO₄ in absolute ethanol at SPAuE; effect of (b) the extreme positive potential value in the scanned range and (c) the number of potential cycles on the DPV responses measured with AP-Strept-Biotin-PYY(PYY)-anti-PYY-Phe-N-SPAuE immunosensors. Other used variables were: 0.1M EDC/NHSS, 30 min; 20 µg/mL anti-PYY, 60 min; 0.2 % casein (Blocking buffer), 60 min; 100 ng/mL (●) or 0 ng/mL (○) Biotin-PYY, 30 min; 5 µg/mL AP-Strept, 30 min. CV electrografting: (b) 0.0 - (+0.6- +1.0) V vs. Ag, *n*=3; (c) 0.0 - +0.6 V vs. Ag, *n*=1-7; *v* = 30 mV/s. Detection: 5 mM 1-NPP, 5 min; DPV: -0.2 - +0.7 V vs. Ag; ΔE = 50 mV; *v* = 10 mV/s. Error bars estimated as a triple of the standard deviation (*n* = 3).

1.2 Effect of the potential scan rate and *p*-ABA concentration

The potential scan rate applied for CV electrografting of SPAuE was tested over the 5 - 100 mV/s range and using the previous selected conditions. Figure S2a shows that the specific responses of the immunosensor increased with the scan rate up to 30mV/s and then levelled off. Furthermore, the unspecific currents remained practically constant over the whole range of scan rates. The observed behavior can be explained considering that high scan rates may generate radicals not homogeneously and orderly distributed leading to some of the -COOH groups are unsuitable for binding with antibodies. However, very slow potential scan rates probably do not induce an efficient grafting of the radicals to the electrode surface. In addition, the time needed for electrode

functionalization is longer. Accordingly, 30 mV/s was selected to be used in the grafting procedure.

The concentration of *p*-ABA affects dramatically the immunosensor response, since it regulates the number of biomolecules immobilized on the electrode. This variable was tested using the above mentioned experimental conditions over a *p*-ABA concentration range between 0.35 and 3.6 mM. Figure S2b shows as a larger S/N ratio was measured for a 1.5 mM *p*-ABA solution. Probably, this concentration allows the immobilization of a larger anti-PYY capture antibody loading thus implying larger analytical responses and a better specific-to-non-specific current ratio. Using a smaller amount of *p*-ABA provoked that not sufficient radicals were formed while very large *p*-ABA concentrations leads to electrode saturation.

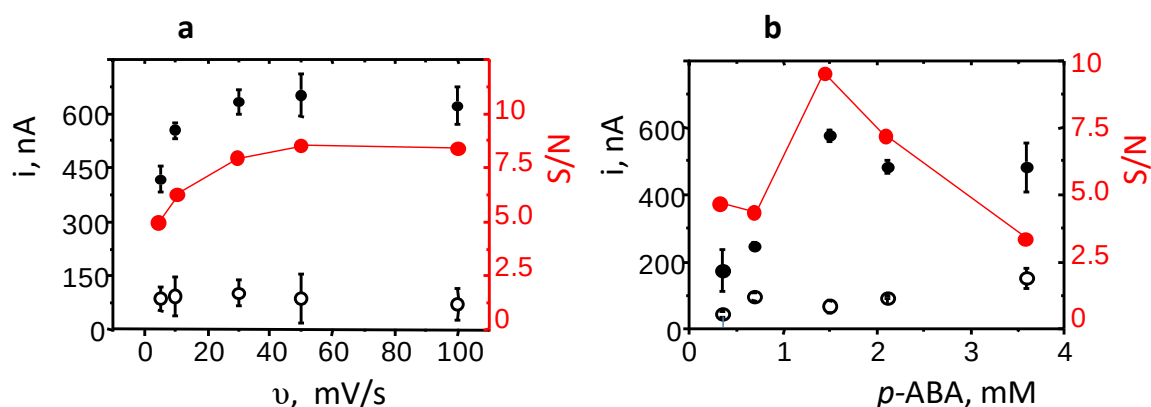


Figure S2. Effect of the potential scan rate (a) and the *p*-ABA concentration (b) used in the grafting process on the DPV responses of the immunosensors. Working conditions: 0.1 M EDC/NHSS, 30 min; 20 μ g/mL anti-PYY, 60 min; 0.2 % casein (Blocking buffer), 60 min; 100 ng/mL (●) or 0 ng/mL (○) Biotin-PYY, 30 min; 5 μ g/mL AP-Strept, 30 min; CV electrografting: 1.5 mM *p*-ABA 0.1 M LiClO₄ in ethanol, 0.0 - +0.6 V vs. Ag, *n*=3, *v* = 5 -100 mV/s (a); 0.37 - 3.6 mM *p*-ABA (b) 0.1 M LiClO₄ in ethanol, 0.0 - +0.6 V vs. Ag; *n*=3; *v* = 30 mV/s. Other conditions as in Figure S1.

2. Optimization of variables for the preparation of the immunosensor using *p*-ABA electrografting on SPAuE

2.1 Effect of anti-PYY loading and the time and temperature for antibody incubation

Optimization of anti-PYY loading and the time and temperature for its incubation gave the results shown in Figure S3. Using the previously optimized conditions and an incubation time of 60 min, different immunosensors were prepared with antibody concentrations ranging between 5 and 30 μ g/mL. Figure S3a shows as the current for the

specific responses increased with the anti-PYY loading due to the larger number of Biotin-PYY binding sites. However, the unspecific currents also increased. As a compromise, a loading of 20 $\mu\text{g/mL}$ was selected. Optimization of the incubation time (Figure S3b) and temperature (Figure S3c) led to select 60 min and 25 $^{\circ}\text{C}$, respectively.

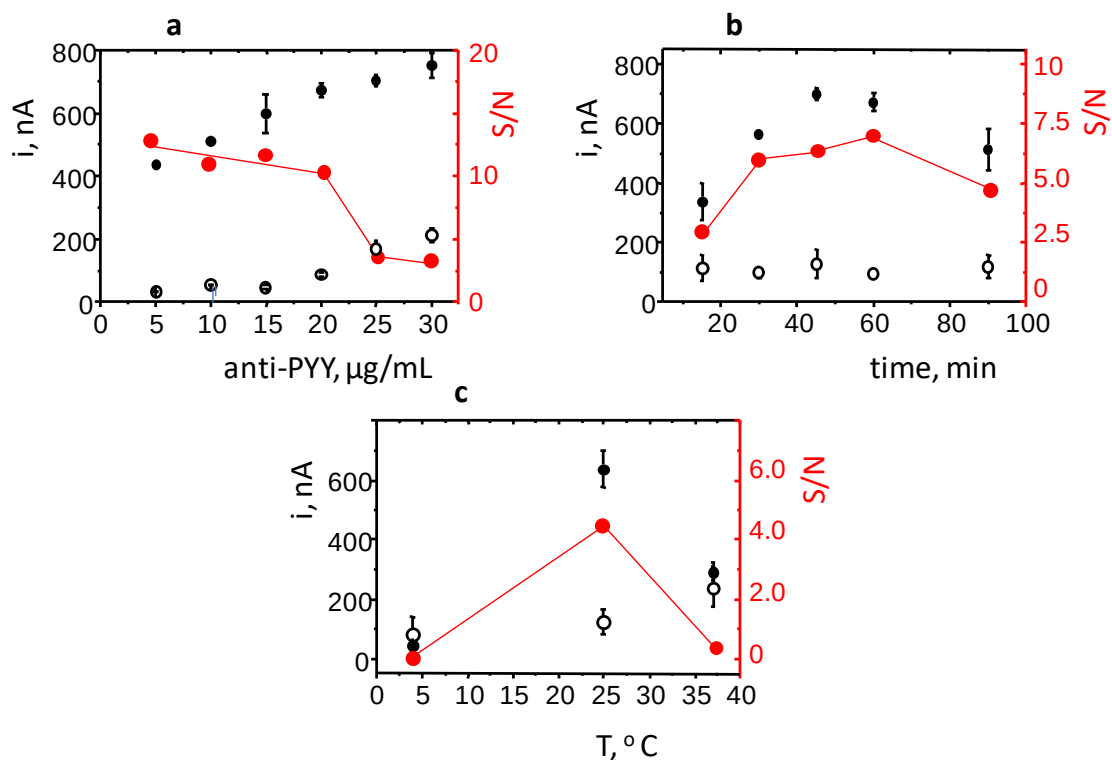


Figure S3. Effect of the anti-PYY loading (a), the time (b) and temperature (c) for incubation of anti-PYY on the DPV responses of the immunosensors. Working conditions: 0.1 M EDC/NHSS, 30 min; 5 - 20 $\mu\text{g/mL}$ anti-PYY (a), 60 min, 25 $^{\circ}\text{C}$; 20 $\mu\text{g/mL}$ anti-PYY, 15 - 90 min, 25 $^{\circ}\text{C}$ (b); 20 $\mu\text{g/mL}$ anti-PYY, 60 min, 4 - 37.5 $^{\circ}\text{C}$ (c); 0.2 % casein (Blocking buffer), 60 min; 100 ng/mL ($\bullet$) or 0 ng/mL ($\circ$) Biotin-PYY, 30 min; 5 $\mu\text{g/mL}$ AP-Strept, 30 min. Other conditions as in Figure S1.

2.2 Blocking step optimization

In order to minimize unspecific adsorptions of immunoreagents on the electrode surface, various blocking strategies were evaluated. Ethanolamine, BSA, and commercial casein solution (Blocking buffer) at a 1% concentration, were tested. The protocol consisted of adding 20 μL of each blocking solution onto the anti-PYY-SPAUE immunosensor allowing incubation for 60 min. The results shown in Figure S4a revealed that both specific and unspecific responses were larger when ethanolamine was used, probably due to the instability of the electrode coating with this compound. A better specific-to-unspecific current ratio was obtained using casein (Blocking buffer) and,

therefore, this blocking agent was selected for further work. Its concentration and the time for incubation were also checked. Figures S4b and S4c led us to select a 0.2% casein concentration and 30 min incubation for further work.

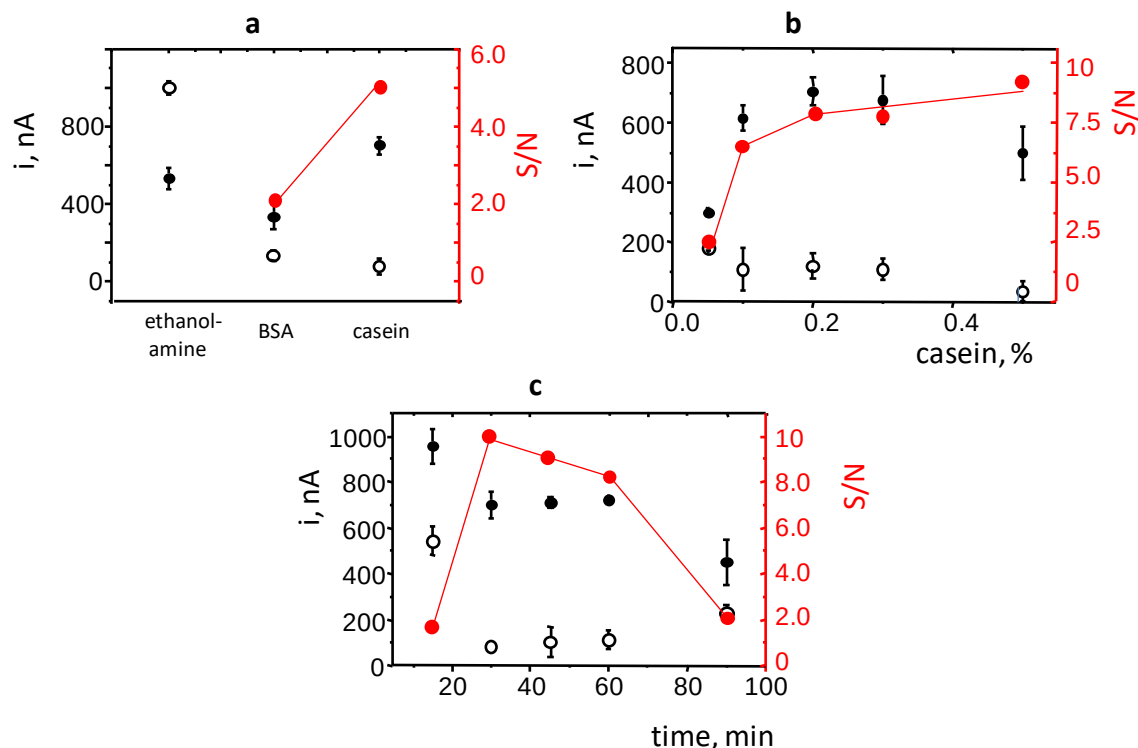


Figure S4. Effect of the type of blocking agent (a), concentration (b) and incubation time (c) of casein (Blocking buffer) on the DPV responses of the immunosensors. Working conditions: 20 μ g/mL anti-PYY, 60 min; (a) blocking agents, 60 min, (b) 0.1 - 0.5 % casein (Blocking buffer). 60 min; (c) 0.2 % casein (Blocking buffer) 15-90 min; 100 ng/mL (●) or 0 ng/mL (○) Biotin-PYY, 30 min; 5 μ g/mL AP-Strept, 30 min; 5 mM 1-NPP, 5 min. Other conditions as in Figure S1.

2.3 Effect of the Biotin-PYY loading and the time for incubation

The Biotin-PYY loading was optimized in the 50-400 ng/mL range. Figure S5a shows that the specific responses of the immunosensor sharply increased with Biotin-PYY concentration up to 75 ng/mL remaining practically constant for larger loadings. Dues to the better reproducibility of the measurements, 100 ng/mL was chosen. In addition, an incubation time for the Biotin-PYY conjugate of 30 min was selected (Figure S5b).

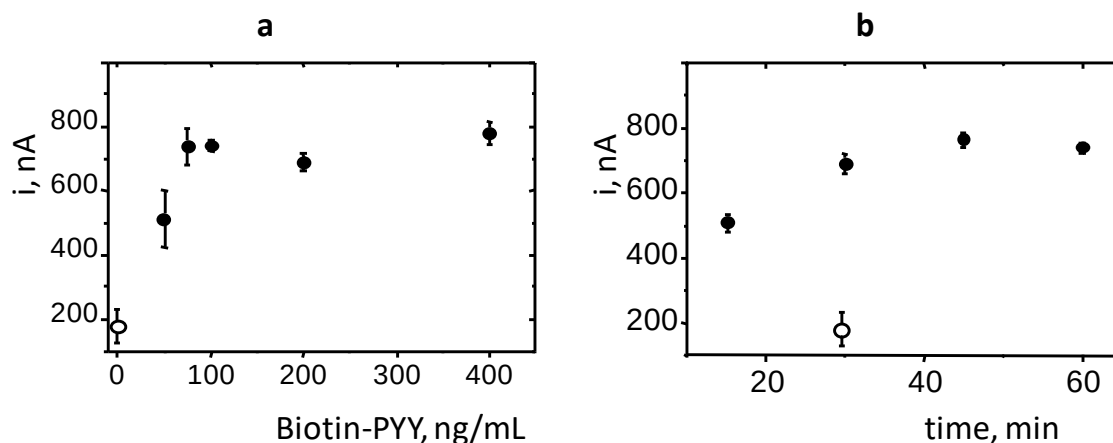


Figure S5. Effect of the Biotin-PYY concentration (a) and the time for incubation (b) on the DPV responses of the immunosensors. Working conditions: 20 $\mu\text{g/mL}$ anti-PYY, 60 min; 0.2 % casein (Blocking buffer) 30min; (a) 50 - 400 ng/mL (●) or 0 ng/mL (○) Biotin-PYY, 30 min; (b) 100 ng/mL Biotin-PYY (●), 15 - 60 min; 5 $\mu\text{g/mL}$ AP-Strept, 30 min. Other conditions as in Figure S1.

2.4 Effect of the AP-Strept loading

The effect of the AP-Strept concentration on the immunosensor response was evaluated by constructing various calibration plots for PYY in the 10^{-9} - 10^2 ng/mL range using 2.5, 5.0 and 7.7 $\mu\text{g/mL}$ AP-Strept and an incubation time of 30 min. As can be seen in Figure S6, a wider linear i_p vs. $\log [\text{PYY}]$ range and a higher slope value was obtained using a 5.0 $\mu\text{g/mL}$ concentration of the labeled conjugate. Therefore, this value was chosen for the preparation of the immunosensor.

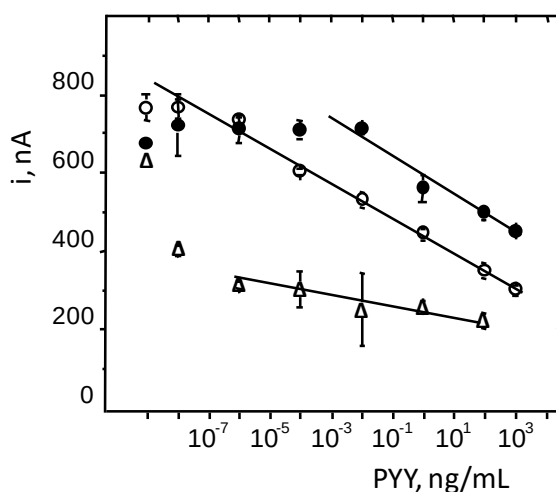


Figure S6. Calibration plots for PYY obtained for 7.5 (●); 5.0 (○) and 2.5 (Δ) $\mu\text{g/mL}$ AP-Strept, 30 min; 20 $\mu\text{g/mL}$ anti-PYY, 60 min; 0.2 % casein (Blocking buffer), 30 min; 100 ng/mL Biotin-PYY, 30 min. Other conditions as in Figure S1.

3. Optimization of variables for the preparation of the immunosensor using a 4-MBA SAM on SPAuE

3.1 Effect of the 4-MBA concentration and the time for incubation

The variables affecting the preparation of the immunosensor using a 4-MBA SAM on SPAuE were also evaluated. Figure S7a shows the results obtained when varying the 4-MBA concentration used for the assembling of the prepared SAM onto the SPAuE. The DPV responses of the immunosensors prepared using the conditions previously optimized were accordingly measured. The specific current (black points) increased in the whole range of tested concentrations due to the increase of the number of functional groups suitable for the covalent immobilization of Biotin-PYY. Conversely, the unspecific currents (in the absence of Biotin-PYY, white points) slightly decreased at low 4-MBA concentrations and remained practically constant for larger concentrations. Regarding the time of incubation, 6 h was selected.

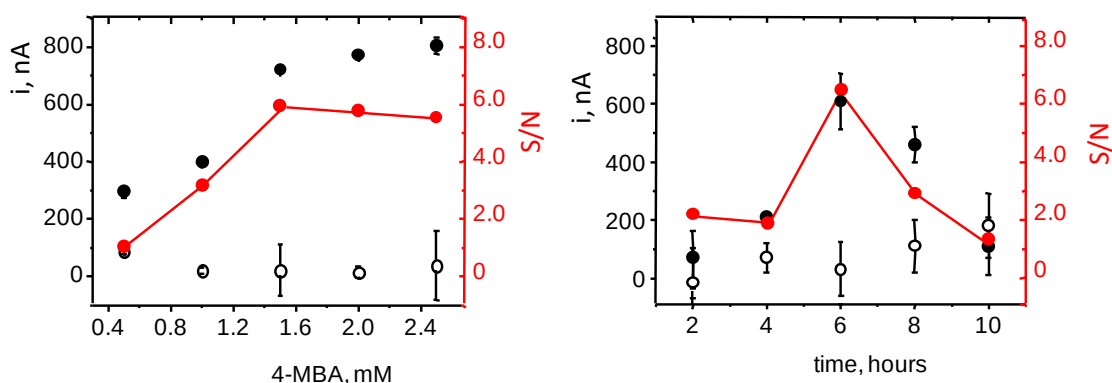


Figure S7. Effect of the 4-MBA concentration (a) and the time for incubation (b) on the DPV responses of the immunosensors. Working conditions: 0.1 M EDC/NHSS, 30 min; 20 μ g/mL anti-PYY, 60 min, 25 $^{\circ}$ C; 0.2 % casein (Blocking buffer), 30 min; 100 ng/mL (●) or 0 mg/mL (○) Biotin-PYY, 30 min; 5 μ g/mL AP-Strept, 30 min. Detection: 5 mM 1-NPP, 5 min; DPV: -0.2 - +0.7 V vs. Ag; ΔE = 50 mV; v = 10 mV/s. Error bars estimated as a triple of the standard deviation (n = 3).

4. Storage stability of anti-PYY-Phe-N-SPAUE conjugates

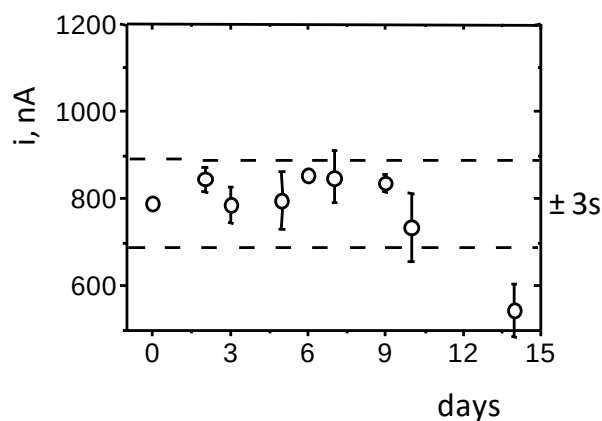
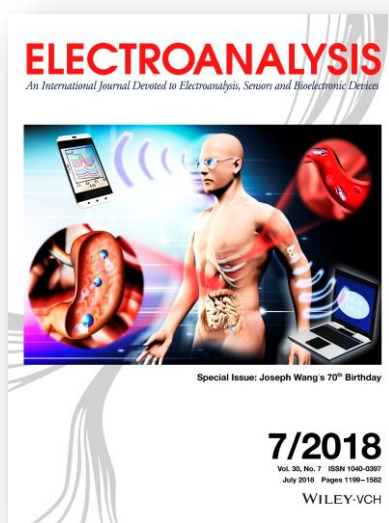


Figure S8. Control chart constructed to check the storage stability of anti-PYY-Phe-N-SPAUE conjugates at 4 °C. The central value was set as the average amperometric current for ten measurements of different solutions in the absence of PYY. Upper and lower limits of control were set as three times the standard deviation of the central value.

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***Electroanalysis* 30 (2018) 1327 – 1335**

Screen-printed gold electrodes functionalized with grafted p-aminobenzoic acid for the construction of electrochemical immunosensors. Determination of TGF- β 1 cytokine in human plasma

Screen-printed Gold Electrodes Functionalized with Grafted p-Aminobenzoic Acid for the Construction of Electrochemical Immunosensors. Determination of TGF- β 1 Cytokine in Human Plasma

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Abstract: Screen-printed gold electrodes were modified with grafted p-aminobenzoic acid to provide scaffolds with high loading of surface confined carboxyl groups that can be used to construct electrochemical immunosensors. As a specific application, an immunosensor for the determination of the cytokine transforming growth factor beta 1 (TGF- β 1) is reported. The capture antibody (anti-TGF) was covalently immobilized on the surface of the prepared disposable scaffold and a sandwich-type immunoassay was implemented with biotinylated detector antibody (Biotin-anti-TGF) and streptavidin labeled with alkaline phosphatase (AP-Strept). The transduction event was monitored by differential pulse voltammetry after addition of 1-naphthyl phosphate (1-NPP) as the enzyme substrate. The

different variables affecting the preparation and the analytical behavior of the AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAUE immunosensor were optimized, and the functionalized gold electrodes and the prepared immunosensor were characterized using several techniques. A linear range extending between 0.05 and 3.0 ng/mL TGF- β 1, adequate for the determination of the cytokine in human plasma, and a LOD of 10 pg/mL, better than that claimed for commercial ELISA kits, were achieved. The immunosensor exhibits also a great selectivity against other proteins and an excellent storage ability. The usefulness of the immunosensor was demonstrated by analyzing human plasma spiked with TGF- β 1.

Keywords: Screen-printed gold electrodes • cytokines • transforming growth factor beta 1 • electrochemical immunosensor

1 Introduction

Gold electrodes constitute useful scaffolds for construction of electrochemical biosensors due to their relative chemical inertia and large potential window. However, gold electrodes have been scarcely employed for the development of electrochemical immunosensors in comparison with carbon electrodes. A common well-known method to modify gold surfaces for biomolecules immobilization is the formation of self-assembled monolayers (SAMs) through binding of thiol (SH) or disulfide (–S–S–) functional groups to Au [1]. This method is characterized by the simplicity and efficiency [2,3] and permits to modify the surface with diverse functional groups which can be used for further covalent immobilization of the bio-reagents. However, apart from the long time required for the spontaneous formation of the monolayer, another important limitation is the low stability of the S–Au link and, consequently, the lack of stability. In fact, the application of reductive potentials, high temperatures or durable assays can cause desorption of the monolayer, thus making it difficult the commercialization of the immunosensors using this methodology. To face these problems, other alternatives should be considered. Electrochemical grafting is a simple and efficient method for electrodes modification providing compact and stable monolayers with specific functionalities [4,5].

Two procedures can be utilized to implement this methodology. On the one hand, the electrochemical reduction of diazonium salts of aryl compounds resulting in covalent attachment of aryl radicals. This strategy has been used, for instance, to prepare mixed monolayers for the investigation of protein electrochemistry [6], the bonding of gold nanoparticles [7] and the fabrication of electrochemical genosensors [8] and immunosensors [9–12]. On the other hand, electrochemical oxidation of aminated compounds results in the formation of amine cation radicals and, subsequently, nitrogen linkages with the electrode material. This strategy has been used to prepare electrodes modified with β -alanine [13], or glycosylated enzymes [14], and also for the preparation of immunosensors [15].

Grafting methods possess a high versatility, hence allowing the attachment of a broad spectrum of substances. The main problem that can arise is the formation of a multilayer coating that passivates the electrode and

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prevents the charge transfer. However, this drawback can be avoided through a deep control of the surface coverage by optimization of the chemical and electrochemical variables involved [16]. Interestingly, these methods have been applied mainly to the modification of carbon electrodes but, despite the advantages of grafting relative to SAM preparation, only a few methods involving gold electrodes have been reported in the literature [17].

In this work, we have constructed an electrochemical scaffold using screen printed gold electrodes modified with grafted p-aminobenzoic acid (ABA-g-SPAuE). The resulting surface, with a high loading of carboxyl groups, was successfully employed for the development of an immunosensor applied to the determination of the cytokine transforming growth factor beta 1 TGF- β 1. Cytokines are low molecular weight bioactive proteins strongly associated with the immune system [18,19]. There is an enormous clinical interest in the determination of cytokines since elevated concentrations are associated with inflammation or disease progression. Therefore, various types of cytokines are widely used as biomarkers to characterize the immune function, predict diseases and monitor their evolution and treatments. Since the expected levels of cytokines in the extracellular milieu are at pM concentration range, their determination requires the availability of highly sensitive analytical methods.

TGF- β 1 is a 25 kDa peptide belonging to the TGF- β family, a collection of structurally related multi-functional cytokines which regulates a wide range of physiological and pathological processes [20]. Three isoforms (TGF- β 1, - β 2, and - β 3) are present in mammals with some differences in biological activities and also in their potencies. TGF- β 1 is involved in immune and inflammatory responses showing a hundred times more potent behavior as growth inhibitor of hematopoietic stem cells than the others. This cytokine has been considered as a good biomarker of liver fibrosis [21] or bladder carcinoma [22]. Increasing evidence also links TGF- β 1 with the progression of renal fibrosis and scarring associated with diabetic nephropathy or hypertensive nephro-sclerosis [23] and glomerulonephritis [24]. TGF- β 1 concentration levels from below 0.1 ng/mL to more than 25 ng/mL have been reported in plasma from healthy individuals [20]. Circulating levels of TGF- β 1 increase in patients suffering various types of cancer in addition to the aforementioned kidney diseases, and are severely depressed in advanced atherosclerosis [25].

Table 1 summarizes bioanalytical methods for the determination of TGF- β 1. Immunoassay strategies using sandwich-type configurations with peroxidase-labeled or biotinylated anti-TGF- β 1 as detection antibodies are employed in commercial ELISA colorimetric kits. Some of these kits are provided by R&D Systems (https://www.rndsystems.com/products/human-tgf-beta-1-quantikine-elisa-kit_db100b), Abcam (<http://www.abcam.com/human-tgf-beta-1-elisa-kit-ab100647.html>) or Abnova (http://www.abnova.com/products/productsdetail.asp?catalog_id=KA0151) and claim to be valid for determining

TGF- β 1 in the range from several tens to thousands of pg/mL with minimum detectable concentrations that can drop to a few units of pg/mL. Regarding biosensors for TGF- β 1 determination, few configurations have been found in the literature. An aptasensor involving aptamer-modified Au electrodes integrated with microfluidics was reported. Thiolated aptamers labeled with methylene blue were self-assembled on gold surfaces. The linear range covered up to 250 ng/mL with a detection limit of 1 ng/mL. This sensor was used to monitor TGF- β 1 release from hepatic cells [25]. An impedimetric immunosensor was developed for the determination of TGF- β 1 in human serum. A polyethyleneglycol (PEG) SAM prepared onto interdigitated electrodes was used for the covalent immobilization of the antibodies. A linear impedance vs log TGF- β 1 concentration range between 1 and 1000 ng/mL was found with a detection limit of 0.570 ng/mL [26]. Very recently, an electrochemical immunosensor for the determination of TGF- β 1 in urine using magnetic microbeads for antibodies immobilization was also reported [27]. Moreover, another immunosensor configuration was implemented and applied to serum samples using multi-walled carbon nanotubes-modified screen-printed carbon electrodes (MWCNTs/SPCE) functionalized by means of copper(I) catalyzed azide-alkyne cycloaddition ("click" chemistry) for the covalent immobilization of immunoreagents [28].

In this work, the immunosensors prepared at the grafted ABA-g-SPAuE involved covalent binding of the capture antibody (anti-TGF) and implementation of a sandwich-type immunoassay with biotinylated detector antibody (Biotin-anti-TGF) and streptavidin labeled with alkaline phosphatase (AP-Strept). The transduction event was monitored voltammetrically after addition of 1-naphthyl phosphate (1-NPP) as the enzyme substrate.

2 Experimental

2.1 Reagents and Solutions

Lithium perchlorate (Aldrich, 99.9%), p-aminobenzoic acid (p-ABA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysulfo-succinimide (NHSS) were purchased from Across. Human monoclonal anti-TGF- β 1 antibody from mouse [#840116] (anti-TGF), human TGF- β 1 [#840118], and biotinylated human monoclonal anti-TGF- β 1 from chicken [#840117] (Biotin-anti-TGF) were all from R&D Systems. The immunoreagents were reconstituted in Reagent Diluent pH 7.2–7.4 from R&D Systems. Alkaline phosphatase labeled-streptavidin from *Streptomyces avidinni* (AP-Strept) [S9210] and 1% casein solution in phosphate buffer were from Thermo Scientific. AP-Strept solutions were prepared in 0.1 M Tris buffer solution of pH 7.2. Solutions of 1-naphthylphosphate (1-NPP) (Across) used as the enzyme substrate were prepared in 0.05 M Trizma buffer of pH 9.6.

Buffer solutions used were: 0.025 M MES of pH 5.0, prepared from 2-morpholinoethane-sulfonic acid (Gerbu,

Table 1. Methods for the determination of TGF- β 1.

Method/ Technique	Procedure	LOD, pg/mL	Dynamic range, pg/mL	Sample volume, μ L	Assay time	Precision, RSD (%)	Sample	Reference
Colorimetric ELISA	50 μ L assay diluent + 50 μ L standard + 100 μ L TGF- β 1 + 100 μ L substrate soln.	15.4*	31.2–2000	40	4 h 30 min	Intra/Inter-assay = 2.9/9.1 (serum, plasma); 3.4/8.4 (cell culture, urine) Intra/Inter-assay <10/12	cell culture, serum, plasma, urine	R&D Systems (DB100B) Human TGF- β 1 Quantikine ELISA Kit
Colorimetric ELISA	100 μ L standard or sample + 100 μ L Biotin-TGF- β 1 detection Ab + 100 μ L HRP-Strept + 100 μ L TMB	80*	82–6000	100	4 h 45 min		cell culture, serum, plasma	Abcam (ab100647) TGF- β 1 Human ELISA Kit
Colorimetric ELISA	100 μ L assay buffer + 100 μ L Biotin-conjugate + 100 μ L Strept-HRP + 100 μ L TMB	8.6*	31–2000	40	5 h 45 min	Intra/Inter-assay = 3.2/4.9	serum, plasma	Abnova (KA0151) TGF- β 1 ELISA Kit
Colorimetric ELISA	capture Ab + 350 μ L FAF-BSA + 100 μ L sample + 20 μ L detection Ab + Ab against rabbit IgG + chromogenic substrate	50	(2–500) $\cdot 10^2$	100	17 h 30 min	Intra/Inter-assay = 7.4/13.2	serum	[24]
Aptasensor	stellate cells + mitogenic soln. + blocking soln. + Ab + anti-rabbit IgG + DAPI	1000	(1–250) $\cdot 10^3$	–	2d	–	Hepatic stellate cells	[25]
Impedimetric immunosensor	(4,6-diamidino-phenylindole) 1:3 v/v, H ₂ O ₂ /H ₂ SO ₄ soln. + 200 μ L COOH-PEG-SH soln. + Ab and EDC/NHS soln	586	(1–1000) $\cdot 10^3$	–	> 1 h 15 min	–	serum	[27]
Amperometric immunosensor	HOOC-MBs + anti-TGF + Biotin-anti-TGF + poly-HRP-Strept	10	15–3000	25	3 h 20 min	Intra/Inter-assay = 3.9/4.2	urine	[28]
Amperometric immunosensor	azide-MWCNTs + alkyne-IgG + anti-TGF + TGF- β 1 + Biotin-anti-TGF	1.3	5–200	50	1 h 20 min	Intra/Inter-assay = 1.9/1.5	serum	[29]
Voltammetric immunosensor	HOOC-Phe/SPAuE + anti-TGF + Biotin-anti-TGF + AP-Strept + 1-NPP	10	50–3000	5	3 h	Intra/Inter-assay = 3.7/3.2	plasma	This paper

* Minimum detectable concentration

100 %); phosphate buffer saline solution (PBS) of pH 7.4 containing 1.37 mM NaCl (Labkem, 99 %), 2.7 mM KCl (Scharlau, 99 %), 1.4 mM NaH_2PO_4 (Scharlau, 98 %) and 4.3 mM Na_2HPO_4 (Alfa Aesar, 99 %); Wash Buffer diluent pH 7.2–7.4 prepared by 1/24 dilution of commercial buffer from R&D Systems, (catalog n° WA 126) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 and 0.05 % Tween® 20; Reagent Diluent pH 7.2–7.4 prepared by addition of 1.4 mL of commercial Reagent Diluent Concentrate 1 solution (R&D Systems, catalog n° DY997) to 98.6 mL of Wash Buffer pH 7.2–7.4; 0.1 M Tris buffer of pH 7.2 prepared from tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl, Scharlau, 99 %), and Trizma buffer of pH 9.6, containing 0.05 M Tris-HCl and 0.01 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Panreac, 99–102 %).

2.2 Apparatus

All electrochemical measurements were carried out using a PGSTAT 101 potentiostat-galvanostat from Autolab provided with a NOVA 1.8 electrochemical analysis software. Screen-printed gold electrodes (SPAuE, DRP-220BT 4 mm diameter) were from DropSens (Oviedo, Spain). These electrodes include a silver pseudo-reference electrode and a gold counter electrode. All experiments were performed at room temperature. A P-Selecta Ultrasons ultrasonic bath, a Velp-Scientifica small samples homogenizer, a P-Selecta heater, and a precision pH-meter Crison model Basic 20+ were also used.

2.3 Procedures

2.3.1 Preparation of the Electrochemical Scaffold

The electrode surface was pre-treated electrochemically according with the manufacturer instructions. The pretreatment consisted in deposition of 50 μL 0.1 M sulfuric acid (Scharlau, 97 %) on the electrode and application of ten successive cyclic voltammetric scans over the 0.00 to 1.25 V vs Ag potential range at a scan rate of 100 mV/s. Subsequently, the electrode was washed with Milli-Q water by shaking in the ultrasonic bath for 1.5 min and allowed drying at room temperature. Then, the electrode surface was functionalized by electrochemical grafting by immersion into a 1.5 mM p-ABA and 0.1 M LiClO_4 solution prepared in 10 mL of absolute ethanol [4] and applying three successive cyclic voltammetric scans between 0.0 and 0.6 V vs Ag at a potential scan rate of 30 mV/s. Thereafter, the modified electrode was washed with water, stirred ultrasonically for 30 s and dried at room temperature.

2.3.2 Preparation of the Immunosensor

The carboxyl groups on the electrode surface were activated by dropping 20 μL of 0.1 M EDC/NHSS solution prepared in 0.025 M MES buffer of pH 5.0 and incubation for 30 min at room temperature in a humid ambient and in the dark. After washing with water and drying, anti-TGF antibody was

covalently immobilized by incubation with 5 μL of 10 $\mu\text{g/mL}$ antibody solution at 25 °C for 30 min under the same conditions. Further, the resulting anti-TGF-SPAuE was rinsed with water and blocking of the remaining free sites was accomplished to avoid unspecific adsorptions by incubation with 5 μL of 1 % casein solution for 30 min. A sandwich-type immunoassay configuration was employed by deposition of 5 μL of standard TGF- β 1 solution or the sample on the anti-TGF-SPAuE and allowing it to stand for 1 h. Thereafter, 5 μL of 2 $\mu\text{g/mL}$ Biotin-anti-TGF were dropped onto the electrode surface and incubated for 1 h. The Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAuE immunosensor was washed with water and 0.1 M Tris buffer solution of pH 7.2 to remove possible phosphate residues on the electrode surface that could interact with the enzyme AP. The immunosensor was incubated with 5 $\mu\text{g/mL}$ AP-Strept for 30 min, and the determination of TGF- β 1 was accomplished upon deposition of 45 μL of Trizma buffer solution of pH 9.6 followed by addition of 5 μL of 5 mM 1-NPP in Trizma buffer solution of pH 9.6. After 5 min for the enzyme reaction to take place, differential pulse voltammetry between –0.2 and 0.7 V vs Ag was recorded.

2.3.3 Sample Analysis

Plasma (Cat 8BFP; Lot 11/03-8BFP; HyTest Ltd) spiked with 25 ng/mL TGF- β 1 was 1/100 diluted with Reagent Diluent pH 7.2–7.4 and analyzed by duplicate by applying the procedure described above. Determination of cytokine was performed by interpolation of the measured peak current into the calibration plot constructed with TGF- β 1 standard solutions.

3 Results and Discussion

The rationale of the design and functioning of the developed immunosensor is illustrated in Figure 1. In a first step, SPAuEs were modified with p-aminobenzoic acid (p-ABA) by performing three successive cyclic voltammetric scans between 0.0 and 0.6 V at a potential scan rate of 30 mV/s in a p-ABA ethanolic solution containing lithium perchlorate, this leading to the generation of the corresponding amine cation radical and the grafting to the SPAuE surface. Thereafter, ABA-g-SPAuE was activated with EDC/NHSS, the anti-TGF antibody immobilized covalently on the electrode surface and the remaining active free sites on the electrode surface blocked with casein (step 2). Then, a sandwich-type immunoassay was implemented by the capture of the target TGF- β 1 (step 3), subsequent incubation with a biotinylated detector antibody (Biotin-anti-TGF, step 4) and further labeling with streptavidin-labeled alkaline phosphatase (AP-Strept, step 5). Monitoring of the affinity reaction was accomplished by measuring the differential pulse voltammetric peak current corresponding to the oxidation of 1-naphthyl phosphate (1-NP) generated enzymatically after addition of 1-naphthyl phosphate (1-NPP) as the enzyme substrate (step 6).

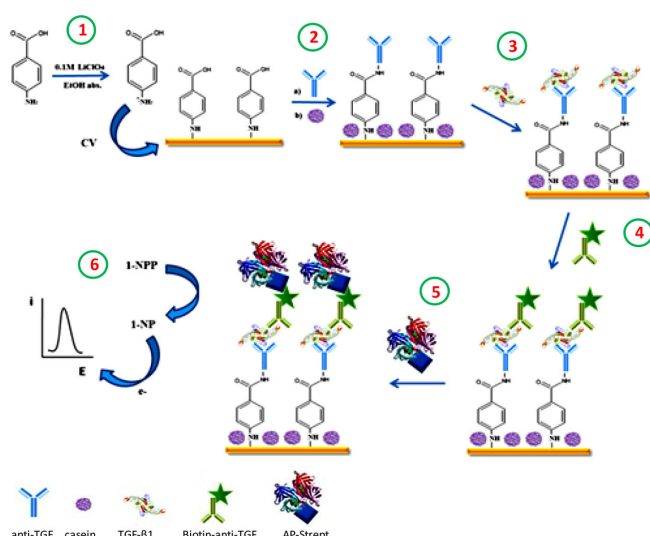


Fig. 1. Schematic display of the different steps involved in the construction of the voltammetric immunosensor for TGF- β 1 by grafting of p-ABA on SPAuEs and covalent immobilization of anti-TGF.

3.1 Optimization of the Experimental Variables

The different variables affecting the preparation and the analytical behavior of the AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAUE immunosensor were optimized. The largest ratio between the currents measured with the immunosensor in the presence (S) and in absence (N) of TGF- β 1 was taken as the selection criterion for each tested variable. These were: a) the number of potential cycles and the potential scan rate for grafting of p-ABA on SPAuEs; b) the p-ABA concentration; c) the anti-TGF loading and the incubation time for immobilization; d) the type and concentration of the blocking agent and the incubation time for this step; e) the Biotin-anti-TGF loading and the corresponding incubation time; f) the AP-Strept loading and the corresponding incubation time. Details on these optimization studies are provided in

Supplementary Material and Figs. S1–S6 and the results are summarized in Table 2.

3.2 Characterization of the Functionalized Gold Electrodes and the Prepared Immunosensor

Cyclic voltammetry using 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl as the redox probe was employed to monitor the steps involved in the electrode modification. As it is shown in Figure 2a, the pretreatment of SPAuEs with 0.1 M H_2SO_4 gave rise, as expected, to a better electrochemical behavior of the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox probe, with values for the oxidation peak current of $i_{\text{pa}} = 92 \mu\text{A}$, and peak potential difference, $\Delta E_p = 124 \text{ mV}$ (50 μA and 286 mV, respectively, for the untreated bare SPAuE). The subsequent grafting of p-ABA on the SPAuE resulted in a well-defined cyclic voltammogram with a lower oxidation peak current, $i_{\text{pa}} = 62.5 \mu\text{A}$, as a consequence of the electrostatic repulsion between the redox probe and the negatively charged surface confined carboxylate groups. Electrochemical impedance spectroscopy (EIS) was also employed to characterize the steps involved in the preparation of the immunosensor.

The corresponding spectra together with the equivalent circuit used to fit the experimental data are displayed in Figure 2b and c. Nyquist plots recorded at the bare SPAuEs (curves 1 and 2) exhibited notably lower charge transfer resistance after the treatment of the electrode surface with sulfuric acid (37 Ω vs. 796 Ω), in agreement with the behavior observed by CV. Modification of the SPAuE with p-ABA (curve 3) gave rise to a significant increase in the electron transfer resistance ($\text{RCT} = 266 \Omega$) which is mainly due to the effect of electrostatic repulsion commented above. Activation of surface confined carboxylate groups with EDC/NHSS produced a dramatic decrease in the RCT value (39 Ω , curve 4) due to the neutralization of the negative electrode surface charge. As expected, antibodies immobilization forming anti-TGF-SPAUE resulted in an increase of RCT (119 Ω), owing to the lower conductivity of the resulting biosurface (curve 5). Finally, the charge transfer resistance measured

Table 2. Optimization of the experimental variables affecting the preparation and functioning of the AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAUE immunosensor.

Variable	Tested range	Selected Value
number of cycles	1–7	3
potential scan rate, mV/s	5–100	30
p-ABA concentration, mM	0.73–3.6	1.5
anti-TGF, $\mu\text{g/mL}$	2.5–15	10
Incubation time for anti-TGF, min	15–60	30
Blocking agent type	ethanolamine, BSA, casein	
Casein, % (w/v) in PBS	0.2–1	1
Incubation time for blocking, min	15–60	30
Biotin-anti-TGF, $\mu\text{g/mL}$	0.5–5	2
Incubation time for Biotin-anti TGF, min	15–90	60
AP-Strept, $\mu\text{g/mL}$	1–10	5
Incubation time for AP-Strept, min	15–60	30

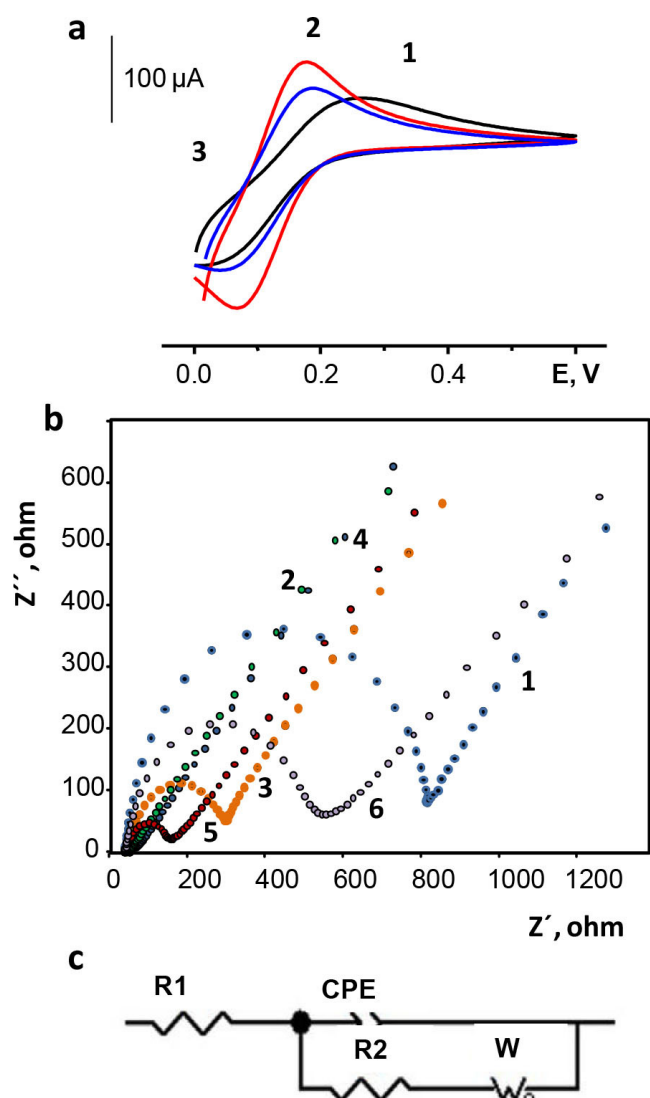


Fig. 2. Cyclic voltammograms (a) and Nyquist plots (b) recorded in 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl solution at bare SPAuE (1), SPAuE after pretreatment with 0.1 M H_2SO_4 (2), pABA-modified SPAuE (3), pABA-modified SPAuE activated with EDC/NHSS (4), anti-TGF-SPAuE (5) and Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAuE (6). (c) Equivalent circuit that optimally fits with results obtained by EIS spectra.

for the Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAuE immunosensor was $R_{CT} = 502 \text{ ohm}$ (curve 6). These results confirmed the suitability of the immuno-reagents immobilization procedure on pABA-grafted SPAuE electrodes. The best fit of the experimental results was achieved using the Randle's equivalent circuit $R_1(Q_1)[R_2 W]$, where R_1 is the solution resistance, CPE is the constant phase element attributed to the capacity of the double electric layer, R_2 refers to current flow produced by redox reactions at the interface, and W is the finite length Warburg element which results from the impedance of current due to diffusion from the bulk solution to the interface [29,30].

3.3 Analytical Characteristics of the Immunosensor

Figure 3 shows the calibration plot for TGF- β 1 constructed with the developed immunosensor under the optimized working conditions. Error bars were calculated from measurements carried out with three different immunosensors in each case. The DPV peak current vs. TGF- β 1 concentration followed the adjusted equation: $I, \mu\text{A} = (0.184 \pm 0.004) [\text{TGF-}\beta 1, \text{ng/mL}] + (0.047 \pm 0.005) \mu\text{A}$ ($R^2 = 0.992$) with a linear range extending from 0.05 and 3.0 ng/mL TGF- β 1. This range is adequate for the determination of the cytokine in real samples since the expected concentrations in plasma are at 0.1–25 ng/mL level [24]. The limit of detection, 10 pg/mL, was calculated by applying the $3s_b$ criterion, where s_b was estimated as the standard deviation in concentration units ($n=10$) of blank current measurements (0 ng/mL TGF- β 1). The analytical characteristics achieved with the proposed immunosensor are notably improved with respect to those claimed for the commercial ELISA kits (see Table 1). For example, Abcam (ab100647) TGF- β 1 Human ELISA Kit claims (https://www.rndsystems.com/products/human-tgf-beta-1-antikine-elisa-kit_db100b) for a minimal detectable dose (analyte concentration resulting in an absorbance equal to 2 s higher than that of the blank) of 80 pg/mL, which is eight times higher than that achieved in this work. The immunosensor exhibits also a remarkably higher sensitivity than those reported for the aptasensors (LOD of 1000 pg/mL) [25] and the impedimetric immunosensor (LOD = 570 pg/mL) [27]. The reproducibility of the voltammetric responses for 1 ng/mL TGF- β 1 was tested with different immuno-sensors prepared on the same day and on different days using a new anti-TGF-SPAuE immunoconjugate in each case. The relative standard deviation (RSD) ($n=6$) values were 3.2% for the assays performed on the same day, and 3.7% and 2.2% ($n=8$) for the measurements made on different

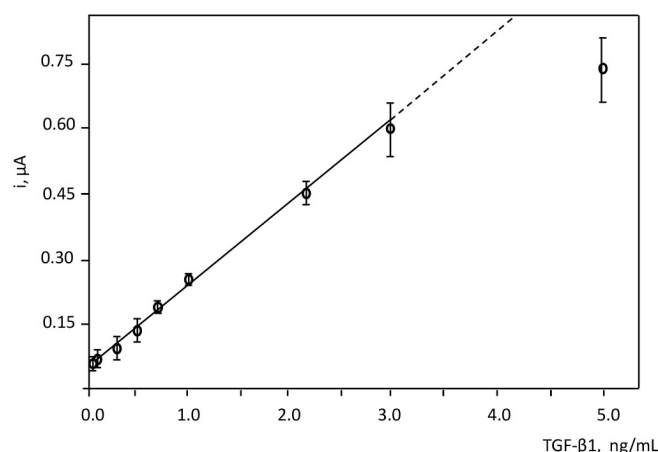


Fig. 3. Calibration plot constructed for TGF- β 1 using differential pulse voltammetry at the AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAuE immunosensor. See text and Table 2 for the experimental conditions.

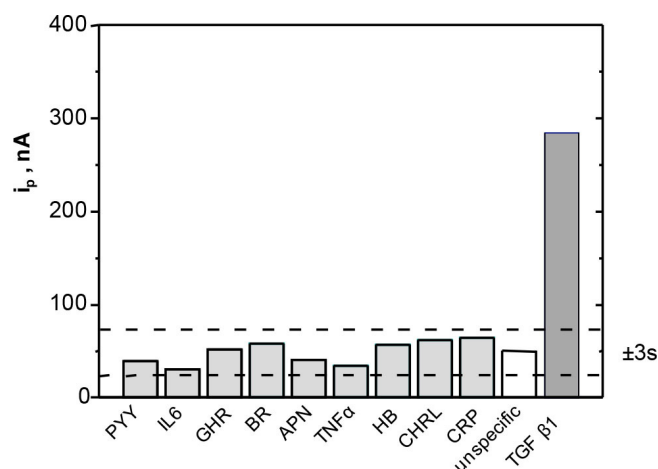


Fig. 5. Measurements carried out with the AP- Strept-Biotin-anti-TGF-TGF- β 1- anti-TGF-SPAUE immunosensor in the presence of 1 ng/mL: peptide YY (PYY), interleukin 6 (IL-6), ghrelin (GHR), bilirubin (BR), adiponectin (APN), tumor necrosis factor alpha (TNF α), hemoglobin (HB), cholesterol (CHRL) and C reactive protein (CRP); (light grey bars), 0 ng/mL TGF- β 1 (white bar); 1 ng/mL TGF- β 1 (dark grey). See text and Table 2 for the experimental conditions.

days in the absence and in the presence of the cytokine. These results revealed the good precision achieved in the preparation process and functioning of the immunosensing platform.

The storage stability of anti-TGF-SPAUEs was also tested. Different anti-TGF-SPAUEs were prepared on the same day, stored under humidity conditions at 4°C, and employed to prepare different AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAUE immunosensors to measure 1 ng/mL TGF- β 1 on different days. The results shown in Figure 4 indicated that the immunosensor responses

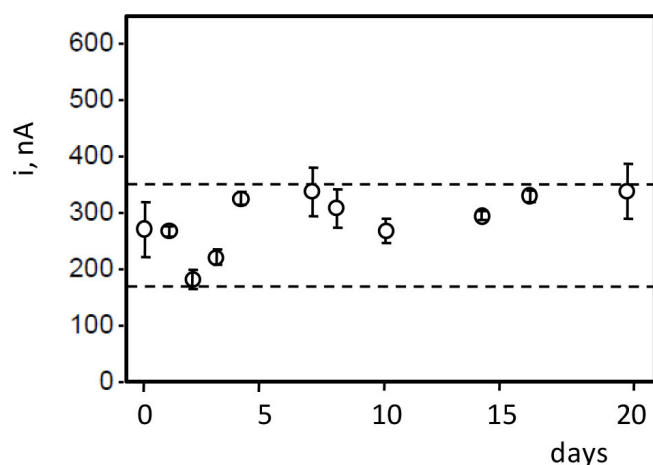


Fig. 4. Control chart constructed to evaluate the storage stability of anti-TGF-SPAUE conjugates. Each point corresponds to the mean value of three successive measurements for 1 ng/mL TGF- β 1. See text and Table 2 for the experimental conditions.

remained within the control limits, set at $\pm 3s$ where s was the standard deviation of the measurements ($n=10$) carried out on the first working day, for at least 20 days (no longer storage times were tested) demonstrating the good stability of the anti-TGF-SPAUE conjugates.

The developed method for TGF- β 1 using the AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAUE immunosensor also exhibited a great selectivity against other proteins. Figure 5 displays the immunosensor responses in the absence of TGF- β 1 and in the presence of 1 ng/mL of the potential interfering substances: peptide YY (PYY), interleukin 6 (IL-6), ghrelin (GHR), bilirubin (BR), adiponectin (ADP), tumor necrosis factor alpha (TNF), hemoglobin (HB), cholesterol (CHRL), and C reactive protein (CRP). As it is clearly seen, no significant interference was apparent in any case, with their signals being within the limits of error for the target cytokine determination. This high selectivity can be attributed to the practical specificity of the antibody used for the preparation of the immunosensor.

3.4 Determination of TGF- β 1 in Human Plasma

The usefulness of the immunosensor for the analysis of real samples was demonstrated by analyzing human plasma spiked with TGF- β 1 at a 25.0 ng/mL concentration level. A calibration graph was constructed in plasma by performing a 1/100 dilution with Reagent Diluent pH 7.2–7.4 and applying the procedure described in the Experimental section.

Figure 6 shows as similar calibration plots were obtained in plasma and with TGF- β 1 standard solutions. The slope and intercept values of the calibration in plasma were $0.192 \pm 0.003 \mu\text{A ng}^{-1}\text{mL}$ and $0.030 \pm 0.007 \mu\text{A}$, respectively. The statistical comparison of the slope value

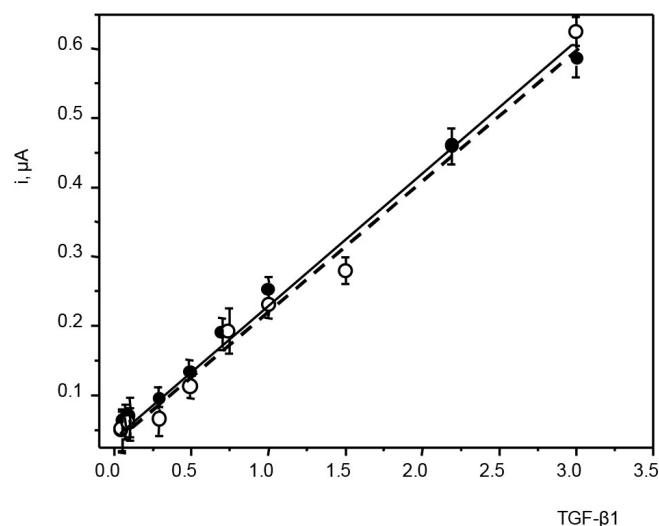


Fig. 6. Calibration plots constructed with standard TGF- β 1 solutions (black points) and in plasma spiked with TGF- β 1 (white points) using DPV at the AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAUE immunosensor. See text and Table 2 for the experimental conditions.

Table 3. Determination of TGF- β 1 in plasma with the AP-Strept-Biotin-anti-TGF-TGF- β 1-anti-TGF-SPAuE immunosensor.

Sample	TGF- β 1 added, ng/mL	TGF- β 1 found, ng/mL	Mean $\pm$ s	Recovery, %
1	0.250	0.230; 0.220	0.225 $\pm$ 0.007	90 $\pm$ 3
2		0.255; 0.246	0.250 $\pm$ 0.006	100 $\pm$ 2
3		0.243; 0.234	0.239 $\pm$ 0.006	95 $\pm$ 3
4		0.253; 0.252	0.252 $\pm$ 0.001	101 $\pm$ 1
5		0.253; 0.252	0.252 $\pm$ 0.001	101 $\pm$ 1
6		0.236; 0.244	0.240 $\pm$ 0.006	96 $\pm$ 2
7		0.230; 0.220	0.225 $\pm$ 0.007	90 $\pm$ 3
8		0.238; 0.229	0.234 $\pm$ 0.006	93 $\pm$ 2

with that of the calibration graph constructed with standards ($0.182 \pm 0.005 \mu\text{A ng}^{-1} \text{mL}$) was performed by applying the Student's *t* test. The t_{exp} value was 1.76, which is lower than the tabulated one, 2.46, indicating that no statistically significant differences existed between the slopes values for both calibration plots. Therefore, the determination of TGF- β 1 could be carried out by interpolation of the current values measured for the plasma samples into the calibration plot constructed with the standard solutions.

Table 3 summarizes the results obtained by triplicate analysis. As it can be seen, the mean TGF- β 1 concentration found in the duplicate analysis of eight different samples of spiked plasma was $0.24 \pm 0.01 \text{ ng/mL}$. Recoveries ranged between 90 and 101 %, with a mean recovery of 95 ± 4 %. These results indicated the reliability of the method to determine low TGF- β 1 concentrations in plasma following a simple working protocol.

4 Conclusions

An electrochemical immunosensor for the determination of the cytokine TGF- β 1 has been developed using as scaffold for the immobilization of the capture antibody screen-printed gold electrodes where grafting with p-aminobenzoic acid was employed to generate surface confined carboxyl groups. The methodology to prepare the electrochemical platforms is simple and efficient and shows important practical advantages with respect to conventional gold electrode modification strategies using SAMs. In particular, it is worth mention the much shorter time needed to accomplish an appropriate electrode modification (few minutes against several hours) as well as the improved stability of the modified electrodes upon storage. In the specific case of the immunosensor for TGF- β 1, the resulting immunosensor exhibits an improved analytical performance against that claimed for commercial ELISA kits and other reported electrochemical immunosensors. The usefulness of the methodology was demonstrated by analyzing human plasma spiked with TGF- β 1 through a very simple protocol, involving only a dilution step which avoids the occurrence of matrix effect and allows the determination of the cytokine to be carried out by simple interpolation of the measured peak current into the calibration plot constructed with TGF- β 1 standard solutions.

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Click chemistry-assisted antibodies immobilization for immunosensing of
CXCL7 chemokine in serum



Click chemistry-assisted antibodies immobilization for immunosensing of CXCL7 chemokine in serum

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ABSTRACT

The first electrochemical immunosensor for the determination of CXCL7 (chemokine (C-X-C motif) ligand 7) autoimmune biomarker is reported in this work. Click chemistry-assisted antibodies immobilization was performed by reaction of azide functionalized-multi-walled carbon nanotubes (MWCNTs) and ethynyl-IgG onto screen-printed carbon electrodes. The capture antibodies were further immobilized onto IgG-MWCNTs conjugates. After a blocking step with casein, a sandwich immunoassay was implemented involving biotinylated detector antibodies and alkaline phosphatase (AP)-streptavidin conjugate. Differential pulse voltammetry upon addition of 1-naphthylphosphate was used as the analytical readout. A linear calibration plot between 0.5 and 600 pg mL⁻¹ CXCL7 and a LOD value of 0.1 pg mL⁻¹ were obtained. The usefulness of the immunosensor was demonstrated by the successful analysis of serum samples from patients with rheumatoid arthritis.

1. Introduction

Chemokines are regulatory proteins related to physiologic or pathologic processes involving immune system responses. In particular, the 9.3 kDa peptide CXCL7, also known as NAP-2, is a platelet-derived growth factor belonging to the CXC chemokine subfamily. It is a potent chemoattractant and activator of neutrophils, also stimulating a great variety of cellular processes such as glycolysis, prostaglandin E2 secretion, or hyaluronic acid synthesis. CXCL7 acts also as an antimicrobial agent with bactericidal and antifungal activity. In addition, CXCL7 plays an important role in tumors growth and rheumatoid processes [1]. In particular, we are interested in the determination of CXCL7 due to its relationship with these autoimmune disorders.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint swelling, joint tenderness, and destruction of synovial joints, leading to severe disability and premature mortality [2]. It is considered as an autoimmune disease since the presence of auto-antibodies such as rheumatoid factor or anti-citrullinated protein antibodies can be detected preceding the clinical manifestation of RA [3,4]. Although structural changes can be visualized by conventional radiography allowing differentiation of RA from other arthritic disorders, joint damage is rarely apparent in the very early stages of the disease. CXCL7 chemokine is highly expressed in serum, synovial fluid

and synovial tissue of patients developing RA during the first 12 weeks. However, it is expressed at lower levels in RA with longer duration, this variation being useful to reflect local pathological changes [5]. Moreover, it has been suggested that chemokines can not only aggravate synovial inflammation but also promote its chronicity by attracting monocytes to the inflamed tissue and activating them following recruitment to the synovium. In addition, CXCL7 is reported as a potential biomarker for early diagnosis of lung cancer showing significantly higher CXCL7 levels in plasma of NSCLC (Non-small-cell lung carcinoma) patients than in controls [6].

This important involvement of CXCL7 in various relevant diseases makes its determination an interesting challenge in clinical analysis. Common methods used for this purpose are based on enzyme-linked immunosorbent assays (ELISAs) in which sandwich formats using biotinylated antibodies conjugated to HRP-Streptavidin are predominantly used. For instance, the CXCL7 Human ELISA Kit (ab100613) from Abcam [7] is claimed to provide an absorbance vs log [CXCL7] calibration plot with a dynamic range between 4.1 and 1000 pg mL⁻¹ and a minimum detectable dose of 8.5 pg mL⁻¹. The assay time takes more than 4 h. However, despite its importance, no immunosensor has been developed so far to our knowledge for the determination of this relevant chemokine.

Copper (I) catalyzed azide-alkyne cycloaddition (click chemistry),

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introduced by Sharpless and co-workers in 2001 as a tool in organic synthesis [8], has become one of the most useful techniques for building architectures and incorporate functionalities in different types of materials [9]. The high interest of this environmentally friendly reaction derives from its high yields, no by-products, selectivity, and moderate conditions suitable to be performed in aqueous medium and under physiological conditions. The methodology, involving azide and alkyne complementary functions, is tolerant to a wide range of pHs, solvents and temperatures. Click reaction also constitutes an efficient strategy for immobilization of enzymes, antibodies, aptamers, or DNA sequences that can be coupled to different substrates without altering their configurations and preserving their biological activity [10]. The catalyst incorporation can be carried out through the direct addition of copper (I) salts (chloride, iodide, acetate) to the reaction medium, or the in situ production by reaction of copper (II) salts (sulfate or acetate) with a reducing agent such as hydroquinone or ascorbic acid [11,12]. Moreover, Cu(I) catalyst can also be electrochemically generated [13].

Click chemistry has demonstrated to be a versatile tool for the construction of functionalized platforms allowing the efficient immobilization of biomolecules on solid surfaces at specific sites. In the particular case of immunosensors, click chemistry is an excellent alternative for achieving stable and oriented immobilization of immunoreagents onto electrode surfaces. However, only a few electrochemical immunosensors has been reported so far involving the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction. Qi et al. coupled alkyne-IgG on single-walled carbon nanotubes (SWCNTs) functionalized with azide groups and the resulting bionanoconjugate was casted onto a glassy carbon electrode surface to implement a competitive electrochemical immunoassay for the determination of anti-IgG as the target compound with HRP as an enzyme label [14]. The same group reported a double electrochemical covalent coupling involving click and diazonium chemistries for the fabrication of an amperometric sandwich-type immunosensor for IgG using alkyne-functionalized human IgG as the capture antibody and HRP-labeled rabbit anti-goat IgG as signal antibody. The immunosensor was prepared by electrochemically grafting of a phenylazide on a GCE and coupling the alkyne-functionalized human IgG with the phenylazide group through an electro-click chemistry in the presence of Cu(II) which was electrochemically reduced at -0.4 V vs Ag/AgCl [15].

An electrochemical immunosensor using a gold nanoparticle-modified porous paper working electrode (Au-PWE) functionalized with 1-azido undecan-11-thiol was developed for the detection of microcystin-LR. An alkyne end-terminated capture antibody was prepared with 4-pentynoic acid and coupled to azido-Au-PWE by click reaction. Azide-functionalized sphere-like peroxidase magnetic silica ($\text{Fe}_3\text{O}_4@\text{SiO}_2$) nanoparticles were prepared to couple alkynylated peroxidase and secondary antibodies as detection label tags in the presence of hydrogen peroxide and thionine [16]. The same group used also click chemistry to prepare $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles/graphene oxide (GO) composites conjugated to the secondary antibody as signal labels in the construction of a disposable electrochemical immunosensor for the detection of cancer antigen 153 (CA 153). Anti-CA 153 capture antibody was immobilized on a screen-printed electrode modified with GO [17]. An electrochemical immunosensor for the determination of transforming growth factor $\beta 1$ (TGF- $\beta 1$) cytokine was previously reported by our group using azide-functionalized MWCNTs-modified screen-printed carbon electrodes for the covalent immobilization of the capture antibody. A sandwich-type configuration using biotinylated detection antibody and signal amplification with poly-HRP-Strept conjugates was employed [18]. Very recently, a sandwich-type electrochemical immunosensor was reported for the detection of carbohydrate antigen 24-2 (CA 242) involving immobilization of the capture antibody onto a poly(ethyleneimine)(PEI)-GO modified GCE using propionic acid as the blocking agent. The secondary antibody was immobilized onto pH responsive Cu^{2+} /polydopamine particles from which Cu(II) can be released and reduced in the presence of the antigen, thus allowing further

immobilization of an azide-functionalized double-stranded DNA by click chemistry that hinders the electrochemical reaction resulting in a current decrease proportional to the CA 242 concentration [19].

We report in this paper the first immunosensor for CXCL7. To do that, we prepared integrated disposable immunoplatfroms using screen-printed carbon electrodes modified with azide-modified-multiwalled carbon nanotubes which allowed the Cu(I)-catalyzed click reaction with alkynylated IgG for the efficient immobilization of anti-CXCL7 antibodies. A sandwich-type immunoassay was implemented involving the electrochemical transduction by differential pulse voltammetry (DPV) through alkaline phosphatase (AP) and 1-naphthyl phosphate (1-NPP) as the enzyme substrate. It is important to remark that this approach requires only the addition of the enzyme substrate and not a redox mediator. An important advantage of this transduction strategy is that it does not require H_2O_2 , avoiding the interference of hemoglobin (with intrinsic peroxidase activity [20]) in serum samples. The immunosensor was successfully employed to quantify CXCL7 in human sera from patients with RA with no need for sample treatment except dilution.

2. Experimental

2.1. Apparatus and electrodes

A PGSTAT 101 potentiostat from Autolab provided with the NOVA 1.8 electrochemical analysis software was used for the voltammetric measurements. Electrochemical impedance spectroscopy (EIS) was performed with a PGSTAT 101 potentiostat from Autolab provided with the FRA analysis software. Screen-printed carbon electrodes (SPCEs, 110 DRP, ϕ 4 mm) were from Metrohm-DropSens and comprised a silver pseudo-reference electrode and a carbon counter electrode. Incubation steps were carried out at 25°C using an Optic Ivymen System constant temperature incubator shaker (Comecta S.A.). A Crison Basic 20+ pH meter, a P-Selecta Ultrasons, an ultrasonic bath (Elma) and a Vortex homogenizator from Velp Scientifica were also employed.

2.2. Reagents and solutions

An anti-mouse IgG (Fc specific) from Sigma (SAB3700848) was used as a primary antibody. The capture antibody was a mouse anti-CXCL7 from R&D Systems [Ref. 840319] reconstituted in 0.1 M phosphate buffer solution of pH 7.4 (PBS). A goat biotinylated antibody (Biotin-anti-CXCL7) [Ref. 840320] and human CXCL7 antigen [Ref. 840321] both from R&D Systems were reconstituted in commercial Reagent Diluent (R&D Systems). Multi-walled carbon nanotubes (MWCNTs, PD30L5-20) were from Nanolab. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfo-succinimide (NHSS) and 11-azide-3,6,9-trioxundecan-1-amine were from Sigma-Aldrich. Ethynyl hydrazide (Chem Space), 0.6% (w/v) casein solution (Blocker™ Casein, Thermo Fisher) in 0.1 M PBS of pH 7, ascorbic acid (Fluka) and copper (II) sulfate (Sigma Aldrich) were also used.

AP-labeled streptavidin (AP-Strept) (Thermo Fisher Scientific) solutions were prepared in 0.1 M Tris buffer of pH 7.2; and 0.05 M 1-naphthyl-phosphate (1-NPP) from (Acros) solutions were prepared in Trizma buffer of pH 9.6. Bovine serum albumin (BSA) (Gerbu), human immunoglobulin G (human IgG) (Sigma Aldrich), hemoglobin (HB) (Sigma Aldrich), transforming growth factor $\beta 1$ (TGF- $\beta 1$) (R&D Systems), interleukin 6 (IL-6) (Abcam), interleukin 1 β (IL-1 β) (R&D Systems), E-cadherin (E-cad) (R&D Systems), recombinant human ErbB2 protein (ERBB2) (Sino Biological Inc.), human p53 (p53) (Sigma Aldrich), cadherin-17 (Cad-17) (R&D Systems) and fibroblast growth factor receptor 4 (FGFR4) (R&D Systems) were used for selectivity studies. Buffer solutions used were: 10 mM phosphate buffer of pH 7.0 (PB); 0.1 M phosphate buffer and 0.15 M NaCl of pH 7.2; 0.1 M phosphate buffer and 1.37 mM NaCl (Labkem, 99%), 2.7 mM KCl (Scharlau, 99%) of pH 7.4 (PBS); 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) (Gerbu) buffer of pH 5.0; wash buffer concentrate from R&D

Systems, WA126, containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 and 0.05% Tween®20; Tris base (Sigma, 99%) 1 mM magnesium chloride (Panreac, 99%) buffer of pH 9.6. All other reagents and solvents were of analytical grade. De-ionized water was obtained from a Millipore Milli-Q purification system (18.2 M Ω cm). The analyzed samples were serum samples from RA patients provided by the Hospital Clínico San Carlos in Madrid.

2.3. Procedures

2.3.1. Preparation of azide-functionalized MWCNTs

Carbon nanotubes were firstly oxidized by addition of 16 mL 65% w/w nitric acid to 85 mL of a MWCNTs (25 mg) aqueous suspension keeping the mixture under reflux for 1 h. Once cooled at room temperature, the mixture was centrifuged at 4000g for 10 min and the supernatant was removed. The resulting carboxylated MWCNTs (cMWCNTs) were washed with deionized water until washing liquids reached pH 7 and, then, dried in an oven at 60 °C. Thereafter, they were stored at room temperature in the dark. Functionalization with azide groups was accomplished by suspending 25 mg of cMWCNTs in 3.5 mL of 25 mM MES buffer, pH 5.0, containing 26.8 mg EDC and 29.8 mg NHSS, and stirring magnetically for 2 h in the dark. After addition of 4.5 μL 11-azide-3,6,9-trioxadecan-1-amine, the mixture was magnetically stirred again for 48 h in the dark. Then, the product was centrifuged at 4000g for 10 min, sequentially washed with 10 mM PBS of pH 7.0 and deionized water and dried at 37 °C. The as prepared azide-functionalized carbon nanotubes were characterized by IR spectroscopy in our previous work [18].

2.3.2. Functionalization of IgG antibody

Alkynylation of IgG antibody was made by incubation of a 2 mg mL⁻¹ of the anti-mouse IgG solution (30 μL) with 1.88 μL of 20 mM NaIO_4 in 0.1 M sodium acetate of pH 5.5. Incubation was carried out in an ice bath under stirring in the dark for 30 min. Thereafter, the mixture was centrifuged at 14,000 rpm in an Amicon Ultra 10 filter at 4 °C during 15 min. After removal of the reagents excess by triplicate washing with 500 μL of a 0.1 M phosphate buffer and 0.15 M NaCl solution of pH 7.2, the functionalized antibody was introduced into a vial and centrifuged at 1000 rpm and 4 °C for 2 min. Then, 19 μL of 1.0 M ethynyl hydrazine (prepared in the same phosphate buffer of pH 7.2) were added and the mixture was incubated for 2 h at 25 °C. After filtering, the modified antibody was washed (five successive washings as mentioned above) followed by a last washing with 10 mM phosphate buffer of pH 7.0. The modified antibody was introduced into a vial, centrifuged at 4 °C and 1000 rpm for 2 min, and diluted to 30 μL with the same buffer (10 mM phosphate buffer of pH 7.0).

2.3.3. Preparation of IgG-alkyne-azide-MWCNTs conjugates

A 34 μL aliquot of the prepared alkyne-functionalized-IgG solution, previously diluted 100 times with 10 mM phosphate buffer of pH 7.0, was mixed with 22 μL of 5 mM ascorbic acid and 23 μL of 5 mM Cu_2SO_4 . Then, 79 μL of the azide-functionalized-MWCNTs dispersion were added and magnetically stirred at 4 °C overnight.

2.3.4. Preparation of the immunosensor

A 5 μL -aliquot of the IgG-alkyne-azide-MWCNT conjugate dispersion was dropped onto the SPCE surface and allowed drying at room temperature. Then, a blocking step was performed by adding 5 μL of a 0.6% (w/v) casein solution prepared in 0.1 M PBS of pH 7.4 and allowing incubation for 30 min at 25 °C in humid ambient in the dark.

After a washing with deionized water, 5 μL of a 2 $\mu\text{g mL}^{-1}$ anti-CXCL7 solution were added onto the IgG-MWCNTs/SPCE allowing incubation for 1 h at 25 °C in the dark and in humid environment. Thereafter, 5 μL of CXCL7 standard solution (or the sample) were added and incubated under the same conditions. The resulting CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE was washed as described above and 5 μL of

a 100 ng mL⁻¹ Biotin-anti-CXCL7 solution were added and incubated under the same conditions for 30 min. Upon washings with deionized water and Tris buffer of pH 7.2, 5 μL of a 5 $\mu\text{g mL}^{-1}$ Strept-AP solution were incubated onto the Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE in the dark and in a humid environment for 30 min at 25 °C.

2.3.5. Determination of CXCL7

Quantification of CXCL7 was accomplished by dropping 45 μL of a 5 mM 1-NPP solution in Trizma buffer of pH 9.6 onto the surface of the AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE allowing reaction for 5 min. The analytical signal was measured by differential pulse voltammetry (DPV) between -0.2 and +0.7 V vs Ag pseudo-reference electrode.

2.3.6. Samples analyzed

Serum samples were collected from patients affected by RA and provided by Hospital Clínico San Carlos (Madrid, Spain) with informed consent approved by the patients and Ethics Committee of the Institution. The samples were analyzed using AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE immunosensors after a 1/2500 dilution in Reagent Diluent (R&D Systems). Validation of the method was carried out by comparing the results with those obtained using an ELISA kit using the same immunoreagents.

3. Results and discussion

Fig. 1 shows a scheme of the steps involved in the preparation of AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE immunosensors as well as the electrochemistry used to obtain the analytical readout. IgG-alkyne-azide-MWCNTs conjugates prepared as described in Sections 2.3.1 to 2.3.3 were casted on the SPCE surface (step 1). In brief, the dispersion of these conjugates was prepared by mixing and overnight stirring, in the presence of ascorbic acid and Cu_2SO_4 , of the azide-functionalized MWCNTs, prepared by covalent modification of cMWCNTs with 11-azide-3,6,9-trioxadecan-1-amine using the EDC/NHSS chemistry, and the alkynylated anti-mouse IgG antibody, prepared by oxidation with NaIO_4 and further incubation with ethynyl hydrazine. After blocking of the remaining free active surface sites with casein, anti-CXCL7 capture antibodies were immobilized through their Fc region onto the anti-mouse IgG of the conjugates (step 2). A sandwich type immunoassay was implemented involving CXCL7, Biotin-anti-CXCL7 (step 3) and AP-Strept (step 4) and 1-NPP was added as the enzyme substrate. The affinity reaction was monitored by measurement of the peak oxidation current of 1-naphthyl phenol (1-NP) by DPV (step 5).

3.1. Optimization of the experimental variables involved in the preparation of the immunosensor

The influence of the following working variables on the immunosensor response was tested: a) concentration of blocking agent and time for incubation onto IgG-MWCNTs/SPCE; b) anti-CXCL7 antibody loading onto blocked IgG-MWCNTs/SPCE and incubation time; c) Biotin-anti-CXCL7 loading on CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE and incubation time; d) AP-Strept concentration and time for incubation onto Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE. The largest ratio between the peak current measured by DPV in the presence (S) and in the absence (N) of the target compound was taken as the selection criterion for each tested variable. Other variables involved in the immunosensor construction such as those used for the preparation of IgG-alkyne-azide-MWCNTs conjugates were the same reported in a previous work [18]. In addition, the conditions employed for the differential pulse voltammetric measurements of 1-NP were those specified in [21]. The obtained results are shown in Fig. 2 and summarized in Table 1.

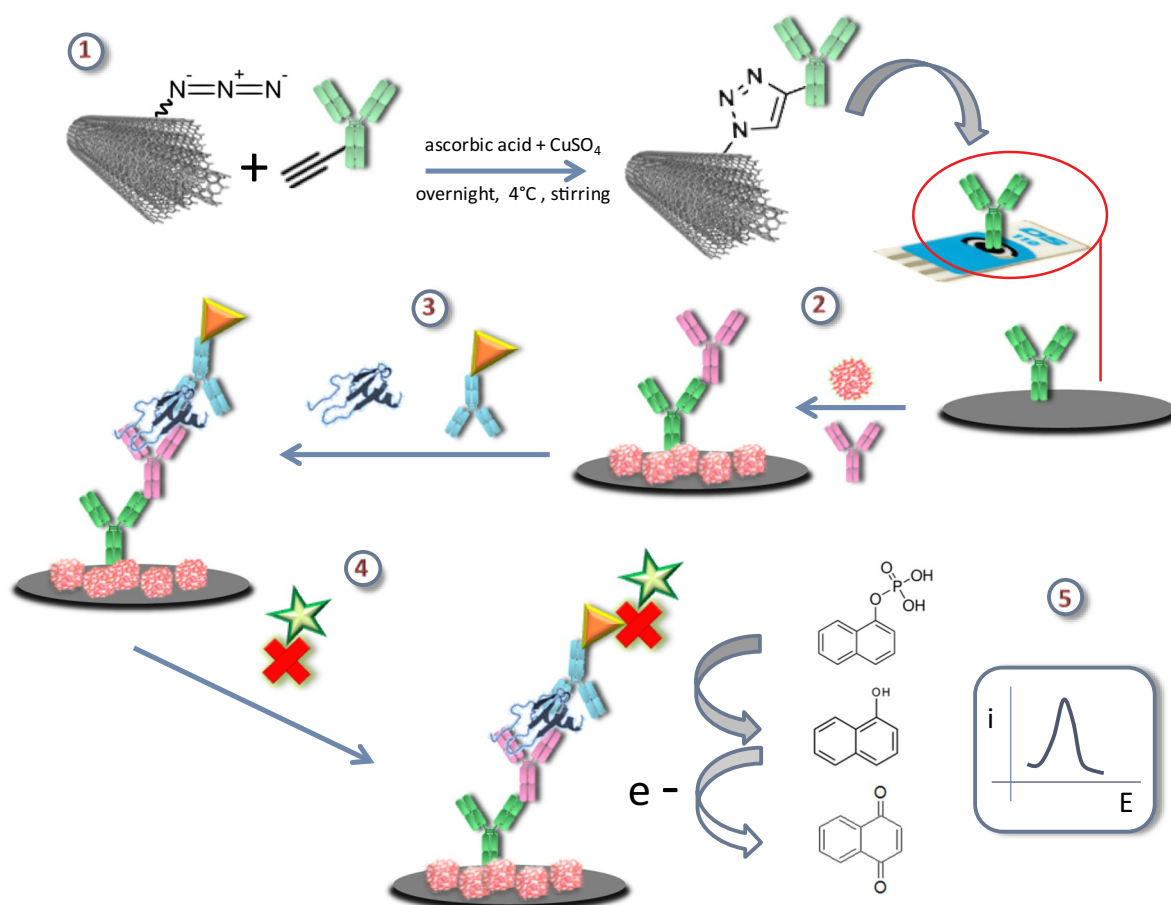


Fig. 1. Schematic display of the different steps involved in the preparation of the immunosensor for the determination of CXCL7: click chemistry-assisted synthesis of IgG-alkyne-azide-MWCNTs and modification of SPCE (1); blocking step with casein and immobilization of anti-CXCL7 (2); sandwich assay with CXCL7 and Biotin-anti-CXCL7 (3); incubation with AP-Strept (4); addition of 1-NPP and DP voltammetric monitoring of the 1-NP oxidation (5).

Fig. 2a and b show the effect of the casein blocking agent concentration and the time for incubation onto the IgG-MWCNTs/SPCE on the immunosensor response, respectively. The largest S/N current ratio was found obtained with 0.6% (w/v) casein solution and 30 min incubation. Regarding the capture antibody concentration, results shown in Fig. 2c led us to choose a $2 \mu\text{g mL}^{-1}$ anti-CXCL7 concentration for further work because the specific current decreased for larger loadings due to the steric hindrance of the antigen recognition that is produced when large amounts of capture antibodies are immobilized [22]. The corresponding incubation time (Fig. 2d) showed better S/N ratios for 60 min. Fig. 2e displays as the non-specific current provided by the immunosensor increased with the concentration of biotinylated antibody. A concentration of 100 ng mL^{-1} allowed obtaining a sufficiently large specific current, this loading being chosen for further studies as a compromise between good specific and relatively low non-specific measured currents. Fig. 2f shows that both the specific and non-specific responses increased with the incubation time, with larger S/N ratio for 30 min. The effect of the AP-Strept concentration and the time for incubation displayed in Fig. 2g and h led us to select $5 \mu\text{g mL}^{-1}$ and 30 min to get an effective binding with the biotinylated secondary antibody.

3.2. Electrochemical characterization

Once the experimental variables were optimized, the different steps involved in the preparation of the immunosensors were monitored by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) using $5 \text{ mM Fe(CN)}_6^{4-/3-}$ as the redox probe in 5 mM KCl

(Fig. 3). Fig. 3a displays the Nyquist plots recorded by EIS for the SPCE upon the successive modification steps. As expected, bare SPCE (curve 1) exhibited a higher electron transfer resistance ($R_{CT} = 868 \Omega$) than that observed at the electrode modified with azide-MWCNTs (curve 2, $R_{CT} = 155 \Omega$) as a consequence of the higher conductivity of the modified electrode. Increased R_{CT} was observed at IgG-MWCNTs/SPCE (curve 3), with $R_{CT} = 285 \Omega$, due to the presence of the insulating biomolecules on the electrode surface. Incubation of anti-CXCL7 produced an increase in the R_{CT} value to 865Ω due to the partially insulating barrier built on the electrode surface. It is important to mention that two different semicircles and a linear domain appeared in this spectrum (curve 4) corresponding to the presence of two layers on the electrode surface [23]. This behavior has been reported previously for other immunosensors involving casein as the blocking reagent [24]. Accordingly, the electrode surfaces yielding curves 1 to 3 can be examined with an equivalent circuit different from that of the curve 4. In this latter case, the $R1(R2Q1)(Q2[R3W1])$ equivalent circuit was the one that fits best to an electrode surface with two differentiated layers [25].

Cyclic voltammograms (Fig. 3b), registered at a 100 mV s^{-1} scan rate in the -0.4 to $+0.6$ potential range, show as the SPCE modification with azide-MWCNTs provoked a noticeable increase in the oxidation and reduction peaks of the redox probe with values of $i_{pa} = 164 \text{ nA}$ and $i_{pc} = 168 \text{ nA}$ suggesting a quasi-reversible behavior of the electroactive system at the modified electrode. In addition, the peak potentials values were 224 mV (E_{pa}) and -16 mV (E_{pc}), with a ΔE of 240 mV remarkably smaller than that observed at the bare SPCE (537 mV). The cyclic voltammogram obtained at the IgG-MWCNTs/SPCE showed slightly

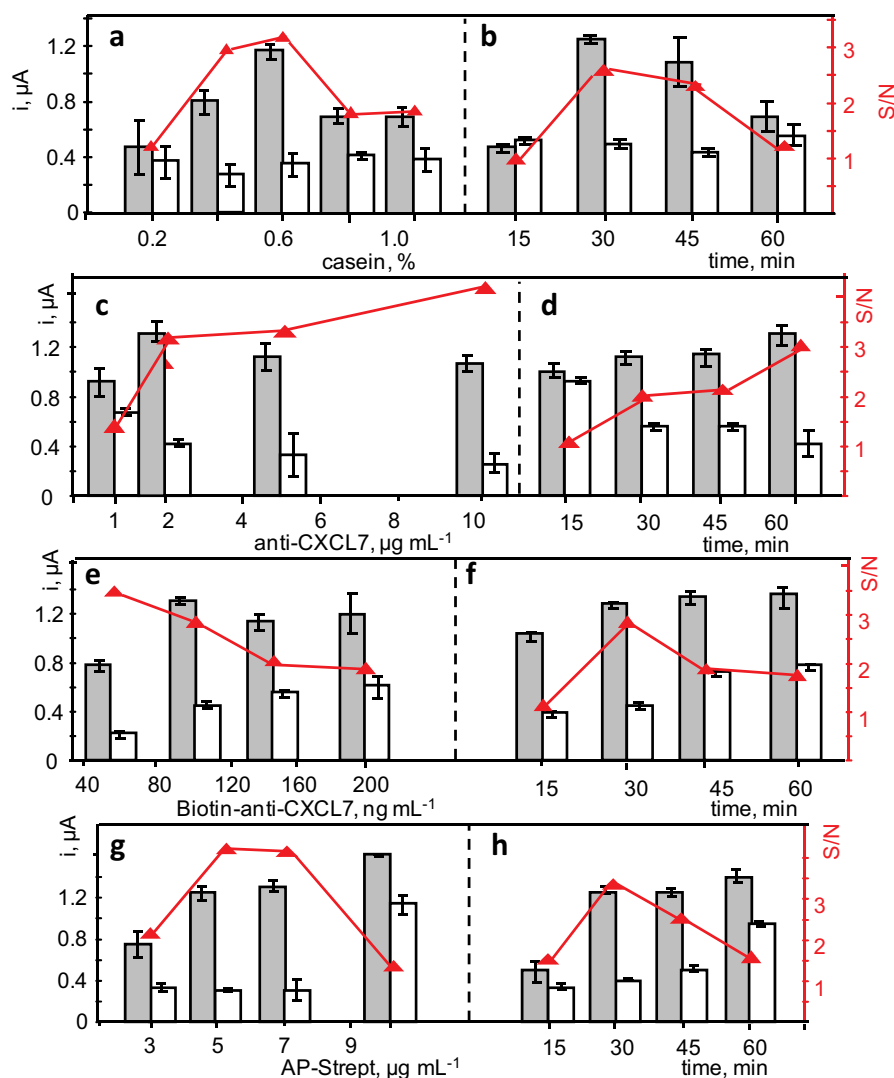


Fig. 2. Effect of the concentration and incubation time of: casein (a,b); anti-CXCL7 (c,d); Biotin-anti-CXCL7 (e,f) and AP-Strept (g,h) on the immunosensor DPV response for 0 (white) and 500 (grey) pg mL^{-1} CXCL7. 5 μL of IgG-MWCNT, 60 min. (a) 0.2–1% casein, 30 min; (b) 0.6% casein, 15–60 min; (c) 0.6% casein, 30 min; 1–10 $\mu\text{g mL}^{-1}$ anti-CXCL7, 60 min; (d) 2 $\mu\text{g mL}^{-1}$ anti-CXCL7, 15–60 min; 100 ng mL^{-1} Biotin-anti-CXCL7, 30 min; 5 $\mu\text{g mL}^{-1}$ Strept-AP, 30 min; (e) 0.6% casein, 30 min; 2 $\mu\text{g mL}^{-1}$ anti-CXCL7, 60 min; 40–200 ng mL^{-1} Biotin-anti-CXCL7, 30 min; (f) 100 ng mL^{-1} Biotin-anti-CXCL7, 15–60 min; 5 $\mu\text{g mL}^{-1}$ Strept-AP, 30 min; (g) 0.6% casein, 30 min; 2 $\mu\text{g mL}^{-1}$ anti-CXCL7, 60 min; 100 ng mL^{-1} Biotin-anti-CXCL7, 30 min; 3–10 $\mu\text{g mL}^{-1}$ Strept-AP, 30 min; (h) 5 $\mu\text{g mL}^{-1}$ Strept-AP, 15–60 min. Error bars estimated as triple of the standard deviation ($n = 3$).

Table 1

Optimization of the experimental variables affecting the DP voltammetric response of AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE immunosensors for the determination of CXCL7.

Variable	Tested range	Selected
Casein concentration, % (w/v)	0.2–1.0	0.6
Incubation time with casein, min	15–60	30
Anti-CXCL7 loading, $\mu\text{g mL}^{-1}$	1–10	2
Incubation time with anti-CXCL7, min	15–60	60
Biotin-anti-CXCL7 loading, $\mu\text{g mL}^{-1}$	50–200	100
Incubation time with Biotin-anti-CXCL7, min	15–60	30
AP-Strept concentration, $\mu\text{g mL}^{-1}$	2.5–10	5
Incubation time with AP-Strept, min	15–60	30

smaller peak currents ($i_{\text{pa}} = 136 \text{ nA}$ and $i_{\text{pc}} = 132 \text{ nA}$) and a similar ΔE (259 mV) due to the lower conductivity of the electrode surface. As expected, the subsequent immobilization of casein and anti-CXCL7 gave rise to an important decrease in the peak current values ($i_{\text{pa}} = 86 \text{ nA}$ and $i_{\text{pc}} = 91 \text{ nA}$). Therefore, the results obtained by CV were fully consistent with those found with EIS and demonstrated the successful incorporation of the immunoreagents on the electrode surface including the use of click chemistry to prepare suitable electrochemical platforms.

3.3. Analytical characteristics of the immunosensor

Fig. 4 displays the calibration plot for CXCL7 standards constructed with the developed immunosensor under the optimized working conditions. Error bars were calculated from the oxidation peak currents measured from differential pulse voltammograms recorded with three different immunosensors. The current vs. CXCL7 concentration followed the adjusted equation $\Delta i_{\text{p}}, \mu\text{A} = (2.49 \pm 0.06) [\text{CXCL7}], \text{ng mL}^{-1} + (0.02 \pm 0.02)$ ($R^2 = 0.994$), where Δi_{p} is the current obtained by subtracting the background current measured in the absence of antigen from the voltammetric response of the immunosensor [26]. The range of linearity extended between 0.5 and 600 pg mL^{-1} , covering more than two orders of magnitude, and is adequate for the determination of CXCL7 in serum according to the expected concentrations, at the ng mL^{-1} level. The LOD, 0.1 pg mL^{-1} , was calculated according to the $3s_b$ criterion, where s_b was estimated as the standard deviation, expressed in concentration units, calculated from ten DPV responses obtained with the immunosensor in the absence of CXCL7. It is worth to mention that these analytical characteristics improve notably those claimed for commercial ELISA kits. As an example, the CXCL7 Human ELISA Kit (ab100613) from Abcam [https://www.abcam.com/human-cxcl7-elisa-kit-ab100613.html] provides a non-linear dynamic range between 4.1 and 1000 pg mL^{-1} and a minimum detectable dose of 8.5 pg mL^{-1} , more than one order of magnitude higher than that obtained with the developed immunosensor.

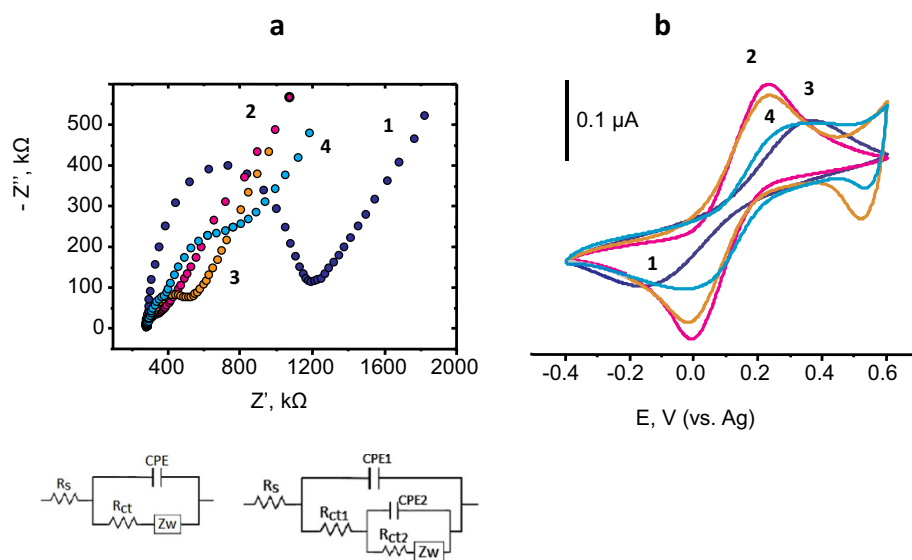


Fig. 3. Nyquist plots and Randles circuits (a) and cyclic voltammograms (b) recorded at bare SPCE (1), azide-MWCNT/SPCE (2), IgG-MWCNT/SPCE (3), and anti-CXCL7-IgG-alkyne-azide-MWCNT/SPCE (4), using 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 5 mM KCl as the redox probe in 0.1 M PBS of pH 7.4. Number of frequencies, 50; frequency range, $10^5 - 0.04$ Hz; amplitude, 0.01 V (a); $\nu = 100 \text{ mV s}^{-1}$; potential range: $-0.4 - +0.6 \text{ V}$ (b).

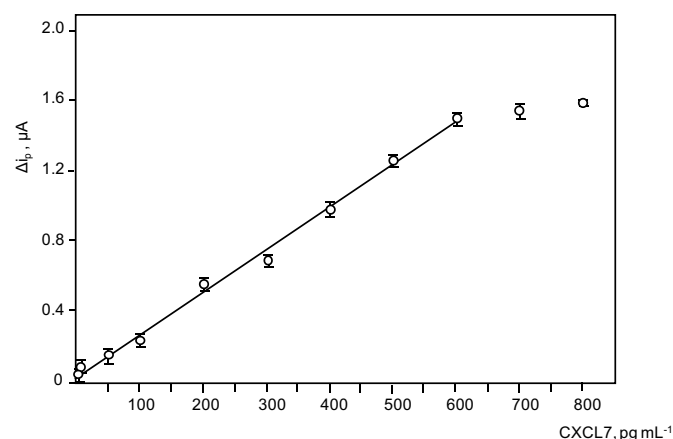


Fig. 4. Calibration plot for CXCL7 standards at the AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE immunosensors. See the text for more information. Error bars estimated as triple of the standard deviation ($n = 3$).

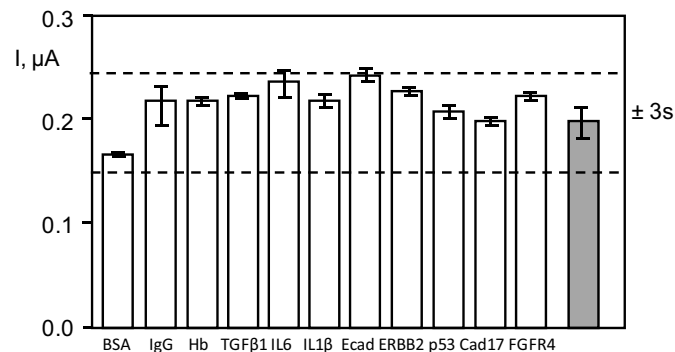


Fig. 5. Peak current responses measured with the AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE immunosensor for 0 pg mL^{-1} CXCL7 in the absence (grey) and in the presence (white) of 5 μg mL^{-1} BSA; 0.9 μg mL^{-1} IgG; 200 ng mL^{-1} Hb; 180 ng mL^{-1} TGF- β 1; 300 pg mL^{-1} IL-6; 55 ng mL^{-1} IL-1 β ; 55 ng mL^{-1} E-cad; 500 μg mL^{-1} ERBB2; 200 ng mL^{-1} p53; 100 μg mL^{-1} Cad-17; 90 ng mL^{-1} FGFR4.

Moreover, the ELISA assay time takes more than 4 h, whereas the immunosensor requires only approximately 2 h, in both cases counting from the immobilization of the capture antibody.

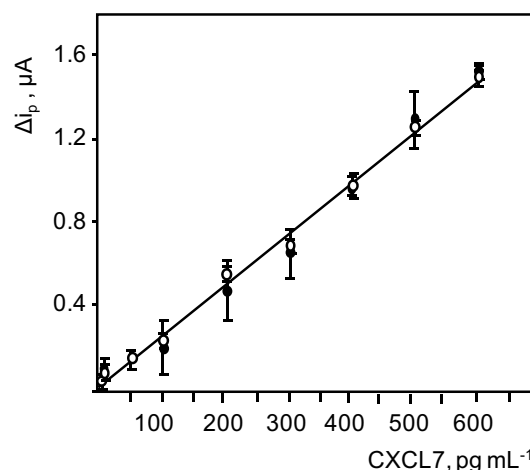


Fig. 6. Comparison of the calibration plots constructed with the AP-Strept-Biotin-anti-CXCL7-CXCL7-anti-CXCL7-IgG-MWCNTs/SPCE immunosensor for CXCL7 standards in buffered solutions (empty circles) and in 1/2500 diluted human serum (full circles). Error bars estimated as triple of the standard deviation ($n = 3$).

Table 2

Determination of CXCL7 in RA patient serum samples.

Sample	Immunosensor, ng mL^{-1} ($n = 5$)	Elisa, ng mL^{-1} ($n = 3$)	t_{exp}
1	1204 ± 57	1240 ± 90	0.84
2	1195 ± 95	1132 ± 67	1.2
3	1488 ± 52	1478 ± 96	1.4

The voltammetric responses for 300 pg mL^{-1} CXCL7 measured with ten different immunosensors prepared on the same day yielded a relative standard deviation (RSD) value of 3.7%. Moreover, measurements for solutions prepared in absence and in the presence of 500 pg mL^{-1} CXCL7 with ten different immunosensors constructed on different days gave RSD values of 1.5% and 4.4%, respectively, indicating that the protocols used for the immunosensor preparation and the electrochemical transduction were suitable to provide measurements with good reproducibility.

The storage stability of anti-CXCL7-IgG-MWCNTs/SPCE bioelectrodes was also tested. Different anti-CXCL7-IgG-MWCNTs/SPCEs were

prepared on the same day, stored under wet conditions at 4 °C, and employed each control day to measure 500 pg mL⁻¹ CXCL7 standards. The results obtained (not shown) indicated that the DPV immunosensor responses remained within the control limits, set at ± 3 times the standard deviation of the mean current value provided by 10 different immunosensors prepared the first day, for at least one week (no longer storage times were tested).

3.4. Selectivity

The effect of the presence of other protein biomarkers and non-target compounds, potentially co-existing with CXCL7 in human serum, on the immunosensor voltammetric response was tested. The voltammetric responses measured in absence of CXCL7 were measured in the presence of: BSA, IgG, hemoglobin (HB), transforming growth factor β 1 (TGF- β 1), interleukin 6 (IL-6), interleukin 1 β (IL-1 β), E-cadherin (E-cad), recombinant human ErbB2 protein (ERBB2), human p53, cadherin 17R (Cad-17R), and fibroblast growth factor receptor 4 (FGFR4), at the concentrations established as their normal levels in human serum. Fig. 5 shows that no significant differences were apparent between the peak currents measured in the absence or in the presence of the tested potential interfering compounds, thus demonstrating the high selectivity of the immunosensor for the determination of CXCL7.

3.5. Determination of CXCL7 in human serum

The immunosensor was applied to the determination of CXCL7 in human serum collected from patients suffering RA. Firstly, the possible existence of a matrix effect in serum was addressed. Successive sample dilutions with Reagent Diluent (R&D Systems) were made and calibration plots for CXCL7 were constructed with the immunosensor and compared with that prepared using CXCL7 standard solutions. Fig. 6 displays the results obtained for successive additions of CXCL7 aliquots to 1/2500-diluted serum. The slope value of this calibration plot, $2.8 \pm 0.5 \mu\text{A mL ng}^{-1}$, was not statistically different from the slope corresponding to the calibration with CXCL7 standards ($2.49 \pm 0.06 \mu\text{A mL ng}^{-1}$). The Student *t*-test provided a t_{exp} value of 0.62 which is lower than the tabulated value, $t_{\text{tab}} = 2.14$. Therefore, the determination of CXCL7 in serum can be performed by interpolation of the DPV peak current measured with the immunosensor from an aliquot of the diluted sample into the calibration plot constructed with CXCL7 standard solutions. The concentrations found in the analysis of the serum samples were compared with those provided by a commercial ELISA kit (Table 2).

As it can be seen, there was an excellent agreement between the results obtained by both methods, with t_{exp} values lower than $t_{\text{tab}} = 2.447$ at a significance level of 95% for all the samples. Furthermore, the concentrations of CXCL7 found in the three samples were significantly higher than that expected in healthy individuals (approximately 850 ng mL⁻¹) [6].

4. Conclusions

The first immunosensor for the determination of chemokine CXCL7 is reported in this work. The immunosensor preparation involved the synthesis of IgG-alkyne-azide-MWCNTs by copper (I) catalyzed cycloaddition from azide-MWCNTs electrode scaffolds and alkyne-IgG. This strategy allowed oriented immobilization of the capture antibody and the implementation of a sandwich configuration using ALP-labeling which combined to the differential pulse voltammetric transduction using the 1-NPP/1-NP system, provided a high sensitivity and an excellent precision. The analytical characteristics achieved with the electrochemical immunosensor improve notable those claimed for commercial ELISA kits with the important advantage that the assay time with the immunosensor is at least 2 h shorter. The immunosensor exhibits an excellent behavior for the accurate determination of the

target chemokine in serum samples collected from RA patients with a minimal sample treatment, just a 1/2500 dilution, and by simple interpolation in a CXCL7 standard calibration plot.

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Design of electrochemical immunosensors using electro-click chemistry. Application to the detection of IL-1b cytokine in saliva



Design of electrochemical immunosensors using electro-click chemistry. Application to the detection of IL-1 β cytokine in saliva

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ABSTRACT

Electro-click methodology was employed to prepare an electrochemical immunosensor for the cytokine interleukin 1 β (IL-1 β). The strategy involved binding of ethynylated IgG to azide-MWCNTs modified electrodes by Cu(I) catalyzed-cycloaddition reaction where the catalyst was electrochemically synthesized. This electro-click protocol is significantly faster and greener than the methods for catalyst generation through chemical reduction. The oriented immobilization of the capture antibody onto IgG-MWCNTs conjugates allowed the preparation of a sandwich-type immunosensor using biotinylated anti-IL-1 β as detector antibody labeled with alkaline phosphatase-streptavidin (AP-strept). Differential pulse voltammetric transduction through the 1-naphthylphosphate/1-naphthol system was carried out. The analytical characteristics achieved with the electrochemical immunosensor showed a calibration curve exhibiting two linear ranges between 10 and 200 pg mL⁻¹ ($r^2 = 0.998$), and from 200 to 1200 pg mL⁻¹ ($r^2 = 0.998$), and a LOD value of 5.2 pg mL⁻¹, an improvement compared with those claimed for commercial ELISA kits. In addition, the assay time was at least one hour shorter. Excellent performance was observed in the determination of IL-1 β in saliva with no need for sample treatment, and by simple interpolation using a calibration plot constructed with standard solutions of the target cytokine.

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1. Introduction

Among the different types of the so-called “click chemistry”, probably the most representative is the cycloaddition reaction between an azide group and an alkyne catalyzed by copper (I) (CuAAC) [1–3]. Although initially click chemistry was designed as a tool for organic synthesis [4], this particular process has become one of the most useful methods for incorporating functionalities into different materials due to the high yields, practical absence of by-products, moderate conditions, use of readily available reagents, and possibility of using aqueous or physiological media [5,6]. In addition, this reaction is compatible with a wide range of pHs, solvents and temperatures, and has been used successfully for the construction of electrochemical scaffolds by means of different strategies such as grafting of 4-azidophenyl diazonium [7], self-assembling of thiol monolayers functionalized with azide groups [8], and incorporation of alkynylated redox mediators such as ethynylferrocene [9], specific ethynyl-derived antibodies [10] or enzymes [11].

On the other hand, the rise of nanomaterials combined with CuAAC [12] has provided a broader space for the development of bio-analytical chemistry, whereas the application of nanostructured surfaces in electrochemical platforms has allowed the efficient immobilization of biological molecules for recognition, signal detection and amplification strategies improving the stability and sensitivity of the resulting electrochemical biosensors. In particular, the preparation of electrochemical immunosensors is one of the most benefited fields of using nanomaterials [13]. Carbon nanotubes, graphene and graphene derivatives, metal nanoparticles (gold, silver), metal oxides or metal complexes, demonstrated to be appropriate to achieve the requirements for realistic clinical diagnosis. In this work, multi-walled carbon nanotubes (MWCNTs) functionalized with azide groups (aMWCNTs) were used to modify the active surface of screen-printed carbon electrodes (SPCEs). The use of disposable SPCEs allows an ease surface modification and the use of small volumes making applications more versatile and converting the resulting devices in portable and disposable bioanalytical tools.

The applications of click chemistry in the development of electrochemical sensors were reviewed by Cernat [5] and Nie [14]. More recently, the use of CuAAC as a tool for the functionalization of nanomaterials and the preparation of electrochemical (bio)sensors were also reviewed [1]. Several methodologies involving click

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chemistry for the functionalization of different electrode materials have been reported. A frequent strategy implies electrografting and subsequent modification by CuAAC [15]. Formation of alkanethiol SAMs on gold surfaces has also been used as a previous step to the CuAAC cycloaddition reaction involving azide-terminated SAMs [16]. Regarding carbon nanomaterials, the use of CuAAC for CNT functionalization was reviewed by Campidelli [12]. In the particular case of graphene, click chemistry has been employed for the covalent linking of different compounds via complementary azide or alkyne groups. For instance, Cernat et al. [17] modified GO with azide groups generating a new material with active sites available for click chemistry reactions.

The design of enzyme biosensors involving click chemistry relies on the controlled immobilization of catalytic compounds on a specific center onto the electrode platform and the inertness of partners involved in the CuAAC reaction to biomolecules [5]. Among the different enzymes, horseradish peroxidase (HRP) is the most widely used due to the great stability and easy functionalization with both azide and alkyne groups. The tagged enzyme immobilization can be performed at room temperature on various conductive materials while maintaining its bioactivity [11] and the electrochemical detection can be performed indirectly through redox probes, or directly by means of the electron transfer between the enzyme active center and the electrode surface. Furthermore, CuAAC has been applied for surface functionalization and immobilization of oligonucleotides in the development of DNA biosensors taking advantage of mild reaction conditions and excellent chemoselectivity and orthogonality to provide a wide variety of functional groups. As example, the simple and fast electrochemical detection of sequence-specific DNA via click chemistry mediated labeling of hairpin DNA probes with ethynylferrocene was performed [9]. In addition, Tang et al. [18] reported the hairpin DNA-dependent click conjugation of oligonucleotides for electrochemical monitoring of copper (II).

Regarding immunosensors, the CuAAC reaction has been involved in the preparation of a few electrochemical platforms in which nanomaterials were used either as electrode modifiers for the immobilization of immunoreagents and in the design of carrier tags for sensitive detection. For instance, an immunosensor for microcystin-LR was constructed by coupling alkynylated-antibody and HRP by click reaction to azide-functionalized nanoparticles of silica-coated ferroferric oxide ($\text{Fe}_3\text{O}_4/\text{SiO}_2$) [19]. A disposable electrochemical immunosensor for CA 153, a breast cancer-related biomarker, was also prepared using CuAAC to synthesize graphene oxide-magnetic silica nanoparticle-secondary antibody conjugates as detection labels [20]. Our group prepared an electrochemical immunosensor for the determination of the cytokine TGF- β 1 (transforming growth factor β 1) using MWCNTs functionalized by CuAAC reaction for the covalent immobilization of capture antibodies [13]. More recently, a similar strategy was applied to prepare a sandwich-type immunosensor for CXCL7 (chemokine (C-X-C motif) ligand 7, after immobilization of the capture antibodies onto IgG-MWCNTs conjugates [21].

In all these methods, the catalyst, Cu (I), was chemically synthesized from cupric ion using reducing agents such as ascorbic acid. However, the catalyst can also be electrochemically generated [22] by applying a methodology named “electro-click” recently used for the selective functionalization of electrode surfaces [23]. In 2006, Devaraj et al. [24] reported the possibility of forming cuprous ions from copper (II) complexes by applying a potential of -300 mV vs Ag/AgCl to form 1,2,3-triazole between organic azides and terminal acetylenes. This approach was postulated as an ideal method for the modification of multielectrode surfaces without the risk of contamination of the adjacent electrodes. The use of the electro-click methodology, by application of a constant reduction potential during a short time allows the acceleration of the immobilization

process without leaving residues of the chemical reducing agent. Despite these advantages, the applications of this methodology to the development of electrochemical biosensors are very scarce [1]. Only one immunosensor involving human IgG as the target compound was described where cupric ions from Cu (II)-bis (bathophenanthroline) disulfonic acid salt were electrochemically reduced at a glassy carbon electrode by stepping the potential from open circuit to -0.4 V vs Ag/AgCl value during 30 min [25]. However, electroclick chemistry was also used for the preparation of an aptasensor for the detection of VEGF 165 biomarker [26]. In this case, the alkyne-modified aptamer was immobilized onto an azide-functionalized electrode prepared with a mixed azide/alkanethiol monolayer and the click reaction was performed by Cu(II) reduction at -0.5 V vs Ag/AgCl in DMSO/ H_2O medium.

In this work we report the use of electro-click chemistry in the preparation of an electrochemical immunosensor for the detection of interleukin 1 beta (IL-1 β), with Cu (I) being electrochemically synthesized from CuSO_4 by applying a constant potential of -0.4 V vs Ag pseudo reference electrode during a brief period of 300 s. The protein encoded by IL-1 β is a member of the interleukin 1 cytokine family in humans. It is also known as IL-1 or IL1F2 and it is mostly expressed in bone marrow or appendix, and 12 other tissues. This cytokine, with a 17.5 kDa molecular weight, is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis. Similarly to other cytokines, the determination of IL-1 β is relevant for monitoring diseases related to inflammation, immune processes, atherosclerosis and cancer [27]. However, some challenges derived from their low concentration in biological samples [28] make it difficult its reliable and accurate quantification.

Few immunoassays for the determination of IL-1 β have been described so far. Commercially available ELISA kits provide adequate sensitivity but need for a long assay time. For example, the colorimetric ab46052 IL-1 beta Human ELISA Kit from Abcam (<https://www.abcam.com/human-il-1-beta-elisa-kit-ab46052.html>), involving a sandwich-type configuration with a biotinylated secondary antibody and peroxidase-streptavidin (HRP-strept) conjugate, provides a logarithmic calibration with a linear range between 15.6 and 500 pg mL $^{-1}$ and a minimum detectable dose (determined by adding $2 \times \text{SD}$ to the mean response, $n = 6$, from the zero standard) of 6.5 pg mL $^{-1}$ IL-1 β . The assay time taken from the immobilization of the capture antibody extends up to 3 h 45 min. Regarding immunosensors, our group developed an electrochemical biosensor for the simultaneous determination of IL-1 β and TNF- α (tumor necrosis factor alpha) using dual SPCEs modified with functionalized double-walled carbon nanotubes (DWCNTs) and signal amplification [29]. The LOD value achieved for IL-1 β was 0.38 pg mL $^{-1}$, and the linear range extended between 0.5 and 100 pg mL $^{-1}$.

As stated above, in this work we have prepared an electrochemical immunosensor for IL-1 β using electro-click chemistry for bioreagents immobilization. Therefore, the novelty of this paper includes: a) the introduction of the electro-click methodology for the preparation of an electrochemical immunosensor for a relevant biomarker, and b) the development of the first electrochemical immunosensor for the individual determination of IL-1 β . Integrated disposable immunoplatfroms using SPCEs modified with azide-modified-MWCNTs (aMWCNTs/SPCE) were prepared which allowed the CuAAC electro-click reaction with ethynylated IgG for the efficient immobilization of anti-IL-1 β capture antibodies (cAbs). The electro-click step consisted of the application of a constant potential of -0.4 V vs Ag to the aMWCNTs/SPCE and deposition of a mixture solution of ethynylated IgG and CuSO_4 during 300 s. Importantly, this procedure is significantly faster and greener than that usually employed to generate the catalyst via

chemical reduction which proceeds overnight [13,21] and leaves reagent residues. A sandwich-type immunoassay was implemented involving cAb, biotinylated anti-IL-1 β detector antibody (biotin-dAb) and alkaline phosphatase-streptavidin (AP-strept) conjugates. The electrochemical transduction was performed by differential pulse voltammetry (DPV) using 1-naphthylphosphate (1-NPP) as enzyme substrate. This approach requires only the addition of the enzyme substrate and not a redox mediator. A calibration graph with two linear ranges, between 10 and 200 pg mL⁻¹ ($r^2 = 0.998$) and from 200 to 1200 pg mL⁻¹ ($r^2 = 0.998$), and a LOD value of 5.2 pg mL⁻¹ were obtained. The assay time counted from the immobilization of the capture antibody was 2 h 30 min. The immunosensor was successfully employed to quantify IL-1 β in human saliva with no need for sample treatment.

2. Experimental

2.1. Apparatus and electrodes

An Autolab type III potentiostat controlled by the FRA2 software (EcoChemie B.V.) was used to record the cyclic voltammograms and make impedimetric measurements by electrochemical impedance spectroscopy (EIS). Amperometric measurements were made with an InBea potentiostat provided by the IbGraph software. A p-Selecta Ultrasons ultrasonic bath, a Vortex homogenizator (Velp Scientifica), and a Crison Basis 20+ pH-meter were also used. All electrochemical experiments were performed at room temperature. SPCEs, 110 DRP (DropSens-Metrohm) consisting of a 4 mm $\varnothing$ -working electrode, a silver pseudo-reference electrode and a carbon counter electrode, were used as electrochemical platforms for the preparation of the immunosensor.

2.2. Reagents and solutions

MWCNTs, PD30L5-20, were from Nanolab. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS) and 11-azide-3,6,9-trioxaundecan-1-amine were from Sigma-Aldrich. 1% (w/v) casein solution (Blocker™ Casein, Thermo Fisher) in 0.1 M PBS of pH 7, and CuSO₄ (Sigma-Aldrich) were also used. An anti-mouse IgG (Fc specific) from Sigma (SAB3700848) was used as primary antibody. A monoclonal mouse IL-1 β capture antibody [Ref. 840168] (cAb), a goat biotinylated detection antibody (Biotin-dAb) [Ref. 840169], and human IL-1 β [Ref. 840170] antigen were all from R&D Systems. The immunoreagents solutions were prepared in 0.1 M PBS of pH 7.2. AP-labeled streptavidin (AP-strept) [Ref. S9210] from Thermo Fisher Scientific was reconstituted in 0.1 M Tris buffer solution of pH 7.2. A 0.05 M 1-naphthylphosphate (1-NPP, Across) solution was prepared in Trizma buffer of pH 9.6. Bovine serum albumin (BSA, Gerbu), hemoglobin (HB, Sigma-Aldrich), transforming growth factor β 1 (TGF, R&D Systems), interleukin 6 (IL-6, Abcam), interleukin-8 (IL-8, Abcam), ghrelin (GHRL, AnaSpec), tumour necrosis factor α (TNF, R&D Systems), leptin (Lep, BioVendor), bilirubin (BIL, Sigma-Aldrich), and cholesterol (CHL, Sigma-Aldrich), were evaluated as potential interfering compounds.

Buffer solutions used were: 10 mM phosphate buffer of pH 7.0 (PB); 0.1 M phosphate buffer saline solution (PBS) of pH 7.2 containing 0.15 M NaCl; 10 mM 2-(N-morpholino) ethane-sulfonic acid (MES, Gerbu, 100%) buffer of pH 7.0; 0.1 M tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl, Scharlau, 99%) of pH 7.2, and 50 mM Trizma of pH 9.6 prepared from Tris-HCl and MgCl₂·6H₂O (Panreac, 99%). All other reagents and solvents were of analytical grade. De-ionized water was obtained from a Millipore Milli-Q purification system (18.2 M Ω cm).

2.3. Procedures

2.3.1. Preparation of azide-functionalized MWCNTs

The preparation of aMWCNTs was accomplished by applying the procedure described previously [13] with slight modifications. Briefly, 25 mg of carbon nanotubes were suspended in 83.2 mL of deionized water and ultrasonically stirred during 1 h. Next, an oxidation treatment to obtain carboxylated MWCNTs (cMWCNTs) was carried out by adding 66.6 mL 65% (w/w) nitric acid and keeping the mixture under reflux overnight. Once cooled at room temperature, the mixture was centrifuged at 4000 rpm for 15 min and the supernatant was removed. The resulting cMWCNTs were washed with deionized water until washing liquids reached pH 6–7 and then dried in an oven at 60 °C. Thereafter, they were stored at room temperature in darkness.

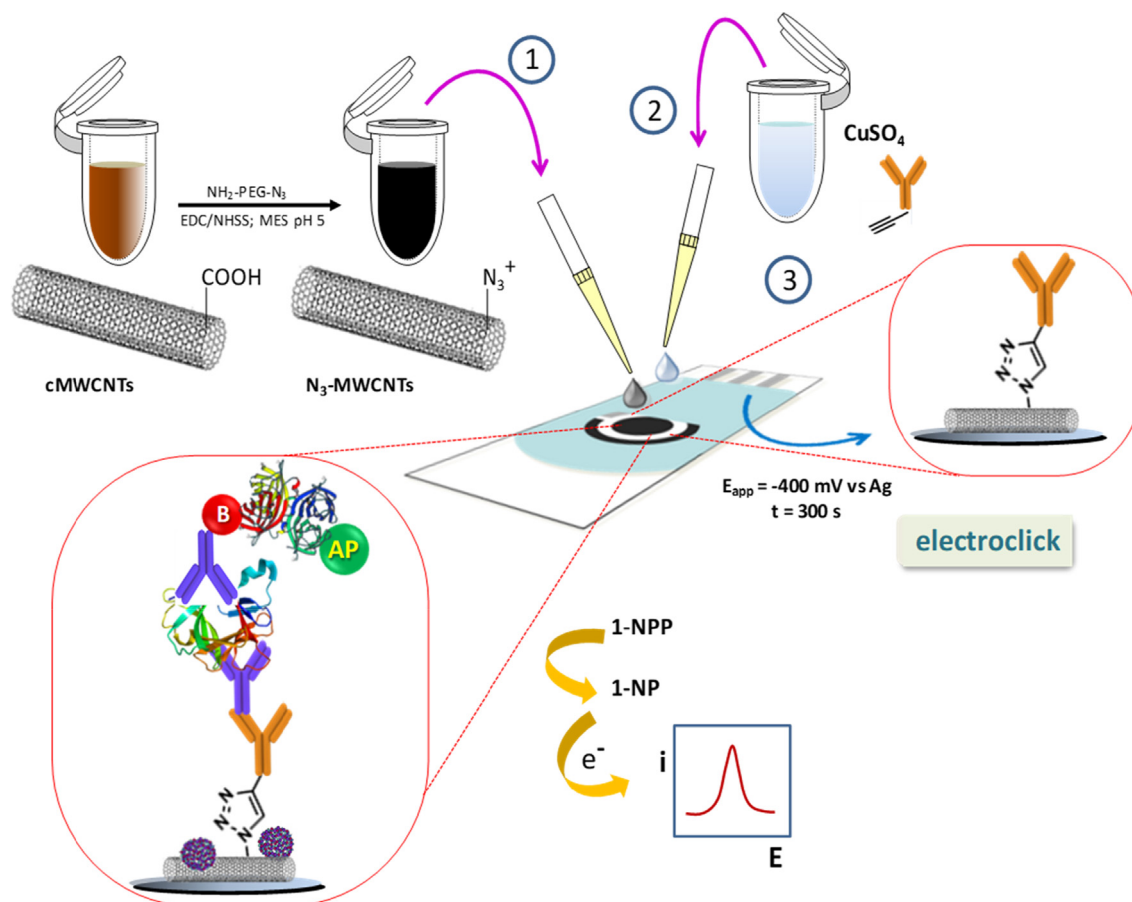
Functionalization with azide groups was accomplished by suspending 25 mg of the as prepared cMWCNTs in 3.5 mL of 25 mM MES buffer of pH 6.5 containing 26.8 mg EDC and 29.8 mg sulfo-NHS, and stirring for 2 h at room temperature in the dark. Subsequently, 4.6 μ L of 11-azide-3,6,9-trioxaundecan-1-amine were added and the mixture was stirred again for 48 h under the same conditions. Then, a medium change was carried out by centrifuging the resulting suspension at 4000 rpm, 10 min at 8 °C. After removing the supernatant, 800 μ L of 10 mM PB solution of pH 7.0 were added, centrifuged again under the above mentioned conditions, and the supernatant was removed. Then, the same process was made by replacing the buffer medium by deionized water. Finally, the solid obtained (aMWCNTs) was dried in an oven at 60 °C and stored at room temperature.

2.3.2. Functionalization of IgG antibody

Ethynylation of IgG antibody was performed by applying the method described by Le et al. [30] with slight modifications. Briefly, 1.9 μ L of a 20 mM sodium peryodate solution prepared in 0.1 M sodium acetate buffer (pH 5.5) were mixed with 30 μ L of a 2 mg mL⁻¹ anti-rabbit-IgG solution and stirred in an ice bath and in the dark for 30 min. Then, the mixture was filtered through an Amicon® Ultra 10 K centrifuge filter by centrifugation at 14,000 rpm at 4 °C for 15 min. Three washing steps were then made using each time 500 μ L of 0.1 M PB and 0.15 M NaCl solution of pH 7.2 under the same experimental conditions, then turning around the filter and centrifuging 2 min at 1000 rpm. The resulting product was incubated for 2 h at room temperature with 18.7 μ L of 1.05 M ethynyl hydrazide in the same buffer solution. After filtering, five successive washings, under the same conditions mentioned above, were made followed by a last washing step with 10 mM PB of pH 7.0. The modified antibody was introduced into a vial, centrifuged at 4 °C for 2 min at 1000 rpm, and then diluted to 30 μ L with the same buffer.

2.3.3. Preparation of IgG-alkyne-aMWCNTs conjugates by electro-click chemistry

A 5- μ L aliquot of a 0.5 mg mL⁻¹ aMWCNTs solution was deposited onto the SPCE surface, letting it dry at room temperature. Separately, 18.8 μ L of a 1/100 diluted ethynylated IgG solution in 10 mM PB of pH 7.0 were mixed with 13.5 μ L of a 5.0×10^{-3} mM CuSO₄ solution and 13.5 μ L of 10 mM MES of pH 7.0. Then, 40 μ L of the resulting solution were dropped onto the aMWCNTs/SPCE surface and a constant potential of -0.4 V vs Ag pseudo-reference electrode was applied during 300 s at room temperature in the dark. Thereafter, the electrode was washed thoroughly by immersion in deionized water and kept in a humid environment until use.



Schematic 1. Schematic display of the different steps involved in the preparation of the AP-strept-biotin-dAb-IL-1 β -cAb-IgG-MWCNTs/SPCE immunosensor by electro-click chemistry (See the text for more information).

2.3.4. Preparation of the immunosensor for the determination of IL-1 β

Schematic 1 shows the steps involved in the preparation and functioning of the immunosensor. Once the IgG-alkyne-aMWCNTs/SPCE (hereinafter IgG-MWCNTs/SPCE) was prepared, a blocking step was performed by dropping 10 μL of a 1% (w/v) casein solution allowing incubation for 30 min. All incubation steps were made at room temperature, in a humid environment and in the darkness. After washing with deionized water, 5 μL of a 10 $\mu\text{g mL}^{-1}$ cAb were added to the bioelectrode surface allowing incubation for 1 h. Washing steps with deionized water and drying were performed after each reagent addition. Thereafter, 5 μL of IL-1 β standard solution (or the sample) were added and incubated for 1 h, followed by the addition of 5 μL of 0.5 $\mu\text{g mL}^{-1}$ biotin-dAb and incubation for 1 h. The resulting biotin-dAb-IL-1 β -cAb-IgG-MWCNTs/SPCE was washed with deionized water and with 0.1 M Tris buffer of pH 7.2. Next, 5 μL of a 5 $\mu\text{g mL}^{-1}$ AP-strept were added and allowed incubation for 30 min. The determination of IL-1 β was accomplished by dropping 50 μL of 5 mM 1-NPP solution in 50 mM Trizma buffer of pH 9.6 onto the surface of the AP-strept-biotin-dAb-IL-1 β -cAb-IgG-MWCNTs/SPCE, and allowing the reaction to proceed for 5 min. The analytical readout was obtained by differential pulse voltammetry (DPV) using $\Delta E = 50$ mV and $v = 10$ mV s $^{-1}$ by measuring the peak current at +0.4 V vs Ag pseudo-reference electrode. Unless otherwise indicated, the presented data corresponded to the average of at least three replicates, and the confidence intervals were calculated for $\alpha = 0.05$.

2.3.5. Sample analysis

A Salivette[®] collection device (Sarstedt) was used to collect saliva samples from a volunteer after rinsing the mouth thoroughly with water and chewing a cotton swab for 5 min. After this, the swab saturated with saliva was inserted into a vial, sealed with the cap and centrifuged for 10 min at 3000 rpm. The determination of IL-1 β was performed immediately by applying the procedure described above using a 5- μL aliquot of the undiluted sample.

3. Results and discussion

As it is shown in **Schematic 1**, the preparation of the AP-strept-biotin-dAb-IL-1 β -cAb-IgG-MWCNTs/SPCE immunosensor implied the use of synthesized aMWCNTs and ethynylated IgG to prepare IgG-aMWCNTs conjugates by CuAAC reaction upon cuprous catalyst electrochemical generation at the electrode surface. Aliquots from aMWCNTs (step 1) and CuSO₄ containing also ethynylated IgG (step 2) were deposited on the surface of a SPCE, and a constant potential of -0.4 V vs Ag pseudo-reference electrode was applied during 300 s (step 3). This method proved to be a fast, simple and clean way to prepare a suitable platform for the subsequent immobilization of the immunoreagents needed for the detection of IL-1 β . Once the immunosensor configuration was completed, the determination of IL-1 β was accomplished by dropping 1-NPP solution onto the surface of the electrode and measuring the peak current from differential pulse voltammograms recorded between -0.2 and +0.7 V vs Ag pseudo-reference electrode using

$\Delta E = 50$ mV and $v = 10$ mV s⁻¹. Such response corresponded to the electrochemical oxidation of 1-naphthol (1-NP), the enzymatic product of the 1-NPP hydrolysis reaction catalyzed by AP.

3.1. Optimization of the experimental variables

The variables affecting the electro-click CuAAC reaction, i.e. the type and the concentration of Cu (II) salt, the pH value and the composition and concentration of the buffer solution, the applied reduction potential and the time of application, the aMWCNTs loading onto SPCE, and the ethynylated IgG concentration, were optimized. As stated in the Experimental section, the method described by Le et al. [30] involving peryodate, with slight modifications, was applied for IgG ethynylation. This method was selected because of the claimed properties of this reagent able to oxidize 1,2-diols of carbohydrates existing in the Fc region of antibodies thus leading to the oriented modification of IgG. Moreover, the variables affecting the immunosensor performance such as the casein loading used as blocking agent and the time for incubation, the cAb loading onto IgG-MWCNT/SPCE and the time for incubation, the biotin-dAb loading onto IL-1 β -cAb-IgG-MWCNT/SPCE and the incubation time, and the AP-strept loading onto biotin-dAb-IL-1 β -cAb-IgG-MWCNT/SPCE and the time for incubation, were optimized. The results of the optimization studies are summarized in Table 1. Details are given in the Supporting Information and Figs. S1–S6.

3.2. Electrochemical characterization

The different steps involved in the preparation of the immunosensor were monitored by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) using 5 mM Fe(CN)₆^{3-/4-} as the redox probe in 5 mM KCl solution. The recorded traces are displayed in Fig. 1. The Nyquist plots recorded upon the successive SPCE modification steps are shown in Fig. 1A accompanied by the Randles circuitry describing the processes at the electrode. As expected, the electron transfer resistance (R_{CT}) measured with the bare SPCE (curve 1, $R_{CT} = 1654 \Omega$) was larger than that measured at the electrode modified with aMWCNTs (curve 2, $R_{CT} = 460 \Omega$) due to the higher conductivity of the modified electrode. Furthermore, a small increase in R_{CT} was measured after the electro-click reaction at the resulting IgG-MWCNTs/SPCE (curve 3, $R_{CT} = 515 \Omega$), whereas further incubation of cAb produced a notable increase in the R_{CT} value to 985 Ω due to the partially insulating barrier built on the electrode surface. These spectra fitted well to the Randles circuitry, the fitting errors for the spectra 1–4, expressed as percentage, being respectively: 3.0; 7.3; 8.7 and 15.7. Such circuit contains three resistances: R1 is the solution resistance, R2 is the resistance between the solution and the elec-

trode modified with the biomolecules, and R3 is the resistance between the solution and the electrode itself. Furthermore, Q1 is the coating capacitance and Q2 is the double layer capacitance.

Cyclic voltammograms (Fig. 1B), recorded at a 100 mV s⁻¹ scan rate over the -0.6 to +1.5 V range, showed as the SPCE modification with aMWCNTs provoked a noticeable increase in the oxidation and reduction peak currents of the redox probe with values of $i_{pa} = 120$ nA and $i_{pc} = 135$ nA, with respect to the unmodified electrode ($i_{pa} = 70$ μ A and $i_{pc} = 80$ μ A) suggesting a quasi-reversible behavior of the electroactive system at the modified electrode. These results are probably due to the increase in the active surface area in the presence of carbon nanotubes. In addition, the peak potentials values were 250 mV (E_{pa}) and 50 mV (E_{pc}), with a ΔE of 200 mV remarkably smaller than that observed at the bare SPCE (325 mV). The cyclic voltammogram recorded at the IgG-MWCNTs/SPCE showed slightly lower peak currents, while the immobilization of the capture electrode produced a decrease in the anodic and cathodic peak currents to 70 and 100 nA, respectively. Therefore, the results obtained by CV were fully consistent with those found by EIS, demonstrating the successful incorporation of the immunoreagents on the electrode surface involving the use of click chemistry for the preparation of the electrochemical platforms.

3.3. Analytical characteristics of the immunosensor

Fig. 2 displays the calibration plot for IL-1 β standards constructed with the developed immunosensor under the optimized working conditions. Error bars were calculated from the DPV oxidation peak currents recorded with three different immunosensors. The current vs. IL-1 β concentration followed the adjusted equations: $i_p, \mu A = (4.1 \pm 0.1) [IL-1\beta], ng mL^{-1} + (0.62 \pm 0.01)$ ($r^2 = 0.998$) in the 10–200 pg mL⁻¹, and $i_p, \mu A = (2.3 \pm 0.1) [IL-1\beta], ng mL^{-1} + (0.90 \pm 0.03)$ ($r^2 = 0.998$) between 200 and 1200 pg mL⁻¹ which were adequate for the determination of IL-1 β in clinical samples considering the expected concentrations at the ng mL⁻¹ level. A possible explanation for linearity in two ranges is the restricted amount of the capture antibody. When increasing the antigen concentration, the active sites of antibody become relatively scarce and, as a result, the sensitivity of the immunosensor decreases accordingly [31]. The LOD value, 5.2 pg mL⁻¹, was calculated according to the $3s_b/m$ criterion, where s_b was estimated as the standard deviation calculated from ten DPV responses obtained with the immunosensor in the absence of IL-1 β , and m was the slope of the lower range of the calibration plot. It is worth to mention that these analytical characteristics improve those claimed for commercial ELISA kits. As indicated in the Introduction section, for instance, the colorimetric sandwich-type ab46052 IL-1 beta Human ELISA Kit from Abcam (<https://www.abcam.com/human->

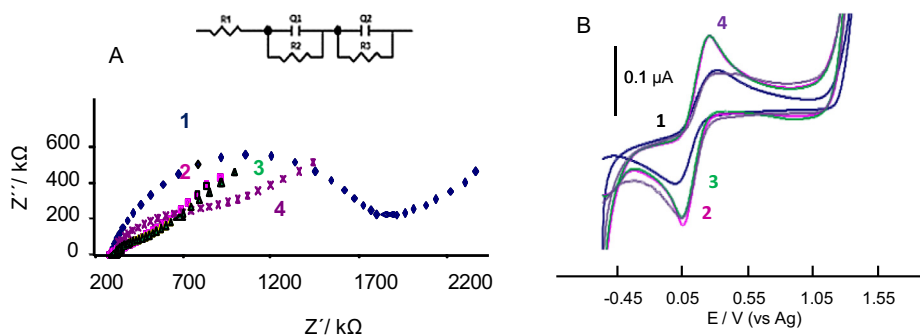


Fig. 1. Nyquist plots and the equivalent circuit (A) and cyclic voltammograms (B) recorded for 5 mM Fe(CN)₆^{3-/4-} 0.1 M KCl at bare SPCE (1), aMWCNTs/SPCE (2), IgG-MWCNTs/SPCE (3), and cAb-IgG-MWCNTs/SPCE (4).

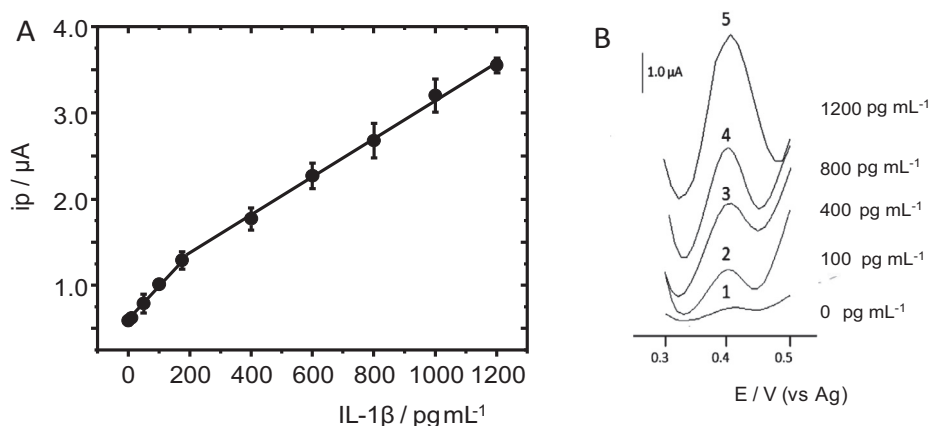


Fig. 2. Calibration plot for IL-1 β standards constructed with the AP-strept-biotin-dAb-IL-1 β -cAb-IgG-MWCNTs/SPCE immunosensor (A) and DP voltammograms for different IL-1 β concentrations (B). See the text for more information. Error bars estimated as triple of the standard deviation ($n = 3$).

[il-1-beta-elisa-kit-ab46052.html](https://www.abcam.com/products/assays/elisa-kits/IL-1-beta-elisa-kit-ab46052.html)), provides a logarithmic calibration graph with a linear range between 15.6 and 500 pg mL^{-1} , and a minimum detectable dose (determined by adding 2 s to the mean response ($n = 6$) from the zero standard) of 6.5 pg mL^{-1} IL-1 β . Moreover, the assay time extends up to 3 h 45 min while the immunosensor requires only 2 h 30 min.

The reproducibility of the voltammetric measurements was tested for IL-1 β concentrations of 0 and 1 ng mL^{-1} with 10 different immunosensors prepared on the same day. Relative standard deviation values, RSD, of 2.4% and 5.2%, respectively, were obtained. Furthermore, RSD values of 3.7% and 6.2% were obtained using 10 different immunosensors prepared in different days. Thus, these results demonstrated the good accuracy of the voltammetric measurements and indicated that the proposed method for the immunosensor preparation was reliable and reproducible.

The storage capability of the cAb-IgG-MWCNT/SPCE bioelectrodes was tested. Different immunosensors were prepared on the same day and stored at 4 $^{\circ}\text{C}$ in a humid environment. Then the immunosensors were used to measure 1 ng mL^{-1} IL-1 β on different days, according to the procedure described in Sections 2.3.4 and 2.3.5. A control chart was constructed (Fig. S7) by setting ± 3 s as control limits, where “s” is the standard deviation of the measurements ($n = 10$) carried out on the first day. The immunosensor responses remained within the control limits for at least 10 days (no longer storage times were tested).

The selectivity of the capture antibody was also evaluated by measuring the voltammetric responses in the absence and in the presence of other proteins such as bilirubin (BIL), transforming growth factor $\beta 1$ (TGF), leptin (LEP), interleukin-8 (IL-8), interleukin-6 (IL-6), hemoglobin (HB), cholesterol (CHL), bovine serum albumin (BSA), ghrelin (GHRL), and tumour necrosis factor α (TNF), all of them at the expected concentrations in the serum of healthy individuals. Fig. 3 shows that there were no significant differences between the currents measured in the absence or in the presence of these compounds, thus demonstrating the practical specificity of the immunosensor for the determination of IL-1 β .

3.4. Determination of IL-1 β in saliva

The analysis of saliva collected from a volunteer was performed by applying the procedure described in the Experimental section to 5- μL aliquots of undiluted sample. Considering the low concentration of IL-1 β that can be expected in saliva, a few tens pg mL^{-1} [32,33], the calibration in the lower range of 0–200 pg mL^{-1} was used. The possible existence of matrix effect in this biological fluid was evaluated by comparing the slope value of the calibration plot

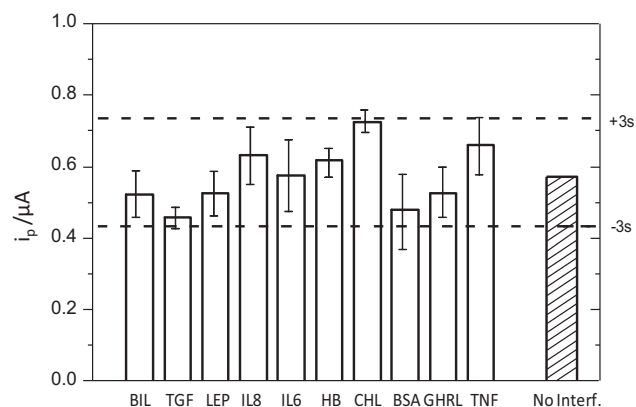


Fig. 3. DPV peak current responses measured with the immunosensor in the presence of 190 $\mu\text{g mL}^{-1}$ BIL; 500 pg mL^{-1} TGF; 30 pg mL^{-1} LEP; 30 pg mL^{-1} IL-8; 50 pg mL^{-1} IL-6; 50 $\mu\text{g mL}^{-1}$ HB; 50 $\mu\text{g mL}^{-1}$ CHL; 5 mg mL^{-1} BSA; 500 pg mL^{-1} GHRL; 100 pg mL^{-1} TNF. See the text for more information. Error bars estimated as triple of the standard deviation ($n = 3$).

constructed with IL-1 β standard solutions in 0.1 M PBS and in saliva spiked with the target protein within such interval. The obtained results revealed that the slope of the linear calibration graph constructed in spiked saliva was $(4.6 \pm 0.2) \times 10^{-3} \mu\text{A mL pg}^{-1}$. This value was statistically compared with that of the calibration graph constructed with IL-1 β standards, $(4.1 \pm 0.1) \times 10^{-3} \mu\text{A mL pg}^{-1}$ by applying the Student t -test. The t_{exp} value was 2.06, which is lower than the tabulated one, 2.306 ($n = 8$, $\alpha = 0.05$), indicating that no statistically significant differences existed between the slope values of both calibration plots. Therefore, the determination of IL-1 β could be carried out by interpolation of the current values measured in saliva samples into the external calibration plot. The mean IL-1 β concentration determined for six samples analyzed by triplicate was $54 \pm 2 \text{ pg mL}^{-1}$. This result is in good agreement with that obtained using ELISA, $51.8 \pm 0.5 \text{ pg mL}^{-1}$ [29], and with the reported data of the mean salivary IL-1 β concentration values found in healthy individuals tested as controls in monitoring of periodontal disease: 33.11 pg mL^{-1} [32] and 61 pg mL^{-1} [33].

4. Conclusions

The electro-click methodology was introduced in this paper for the preparation of an electrochemical immunosensor for IL-1 β cytokine. The methodology involves the binding of ethynylated

IgG to azide-MWCNTs electrode scaffolds by Cu(I) catalyzed-cycloaddition where the catalyst was electrochemically synthesized from CuSO₄ by application of −0.4 V vs Ag pseudo-reference electrode over just 300 s. This strategy resulted significantly faster and greener than the method usually employed to generate the catalyst via chemical reduction. In addition, it allowed oriented immobilization of the capture antibody and the further establishment of a sandwich-type immunoassay with biotinylated anti-IL-1 β and alkaline phosphatase-streptavidin conjugate. This approach coupled to differential pulse voltammetric transduction using the 1-NPP/1-NP system, provided a high sensitivity and precision. The achieved analytical characteristics improve those claimed for commercial ELISA kits with the important advantage that the assay time is, at least, more than one hour shorter. An excellent behaviour was observed in the determination of salivary IL-1 β with no sample treatment and by simple interpolation into a standard calibration plot.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioelechem.2020.107484>.

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SUPPLEMENTARY INFORMATION

Electro-click chemistry, a novel path for immunosensors design. Application to the detection of IL-1 β cytokine in saliva.

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1. Optimization of the experimental variables involved in the preparation of the AP-strept-biotin-dAb-IL-1 β -cAb-IgG-MWCNTs/SPCE immunosensor

The variables affecting the electro-click CuAAC reaction, i.e. a) the type and the concentration of Cu (II) salt; b) the pH value and the nature and concentration of the buffer solution; c) the reduction potential applied and the time of application, and d) the aMWCNTs loading onto SPCE and the ethynylated IgG concentration, as well as those affecting the immunosensor performance: e) the loading of casein used as blocking agent and the time for incubation; f) the loading of cAb onto IgG-MWCNT/SPCE and the time for incubation; g) the loading of biotin-dAb onto IL-1 β -cAb-IgG-MWCNT/SPCE and the incubation time, and h) the loading of AP-strept onto biotin-dAb- IL-1 β -cAb-IgG-MWCNT/SPCE and the time for incubation were optimized. The results obtained in the optimization studies are summarized in Table 1, and details are given in the following section and in Figures S1-S6. Error bars were estimated as triple of the standard deviation (n=3).

1.1 Optimization of the variables involved in the electro-click CuAAC reaction

The voltammetric responses of different immunosensors prepared according to the procedures described in the Experimental section with 10 $\mu\text{g mL}^{-1}$ cAb, 1 (specific, S) and 0 ng mL^{-1} (unspecific, N) IL-1 β , 1% (w/v) casein, 0.5 $\mu\text{g/mL}$ biotin-dAb, and 5 $\mu\text{g/mL}$ AP-strept were measured to evaluate the effect of the variables involved in the electro-click reaction.

1.1.1 Effect of the type and the concentration of Cu (II) salt

Figure S1 shows the results obtained in the measurement of the specific (S, black), unspecific (N, white) and the S/N ratio (red) to study the effect on the immunosensor response of using different inorganic cupric salts: sulfate, chloride, nitrate or acetate, as the substrate for further reduction of Cu (II) to cuprous ion for acting as the catalyst of the CuAAC reaction between alkynylated IgG and aMWCNTs on the electrode surface. As can be seen (Fig. S1a), both the highest specific and the lowest unspecific currents were obtained when cupric sulfate was used. Furthermore, in the presence of chloride, the immunosensor showed the poorest specific response, being these results in accordance with those reported by Hein et al. [1]. Indeed, sulfate and acetate salts are recommended for the reduction in situ of the Cu (II) ion, while chloride can be deleterious due to its inhibitory effect. Regarding the concentration of the selected salt, it can be observed in Fig. S1b that 5×10^{-3} mM CuSO_4 solution provided the best experimental conditions for further work.

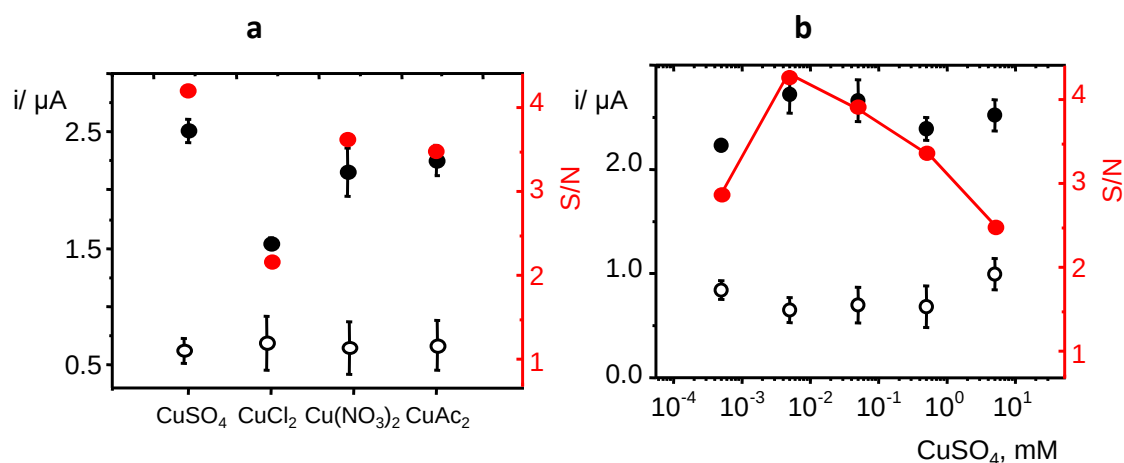


Figure S1. Effect of the type (a) and the concentration (b) of Cu(II) salt in the electro-click reaction. See the text and the main manuscript for more information.

1.1.2 Effect of the pH value, and the nature and concentration of the buffer solution

The experimental conditions of the solution dropped onto the aMWCNTs/SPCE, which contained ethynylated IgG and CuSO_4 were also optimized. Figure S2a shows the results obtained in the study of the pH effect in the 5 - 8 range using a 10 mM MES buffer solution that clearly demonstrated the optimal conditions at pH 7. This result agrees with that reported related to the recommended pH for bioconjugation by copper-catalyzed azide-alkyne click chemistry [2]. Once selected the pH value, the type and concentration of the buffer solution

were also optimized. As Figure S2b and S2c show, the best results were obtained using a 10 mM MES solution. It has been reported that buffer solutions containing species that may react with copper ions are not adequate. In our case, this could happen with citrate or phosphate, which form complexes often insoluble with Cu (II) and also with Tris buffer as it is prepared from the hydrochloride salt. Therefore, the results obtained agree with the published data [2] that recommend MES buffer (or MOPS, the equivalent propane sulfonic acid) as certainly suitable as they do not contain Cu-binding species [3].

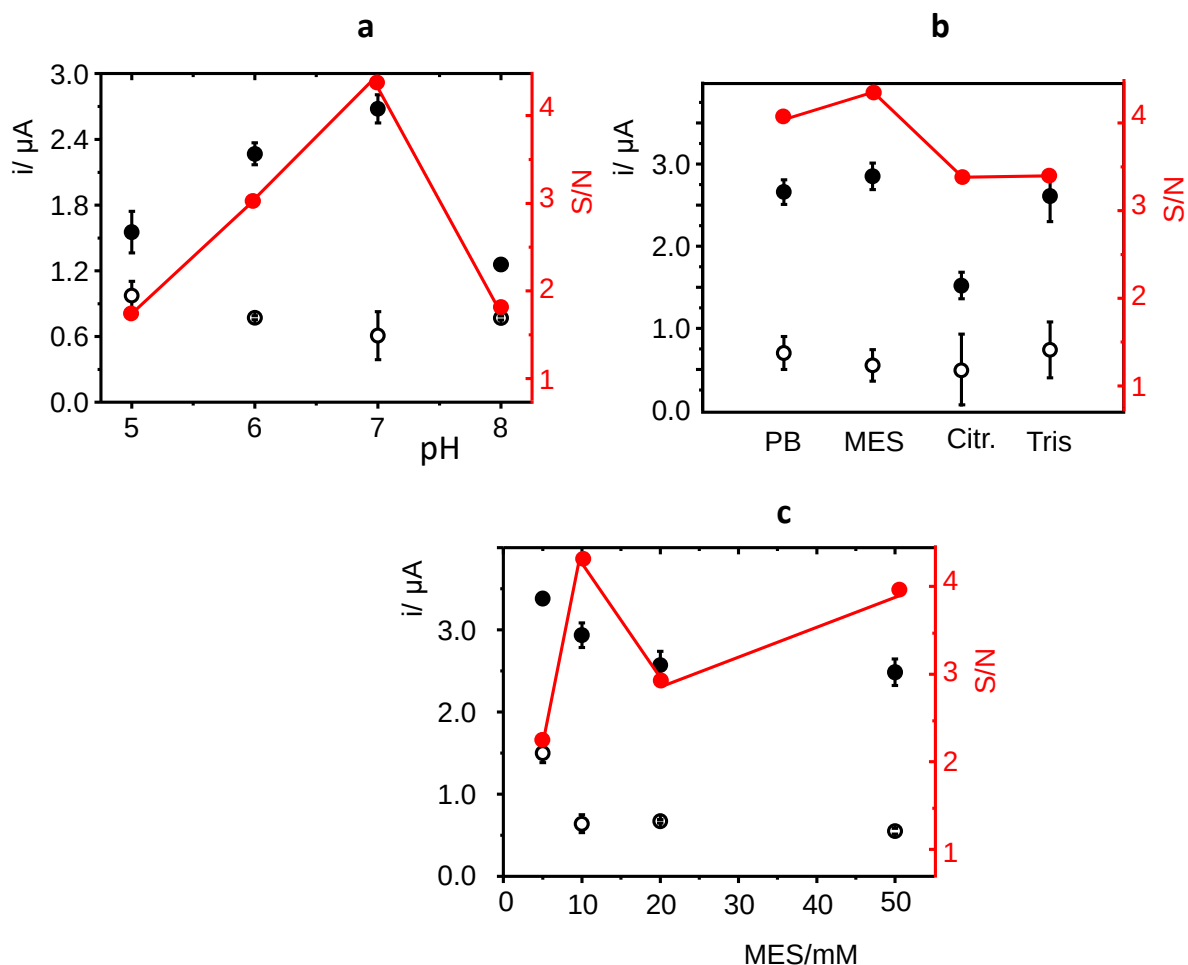


Figure S2. Effect of the pH value (a), and the nature (b) and concentration (c) of the buffer solution. See the text and the main manuscript for more information.

1.1.3 Effect of the reduction potential applied and the time of application

The reduction potential and the time of application onto the aMWCNTs/SPCE coated with the alkynylated IgG in the presence of CuSO_4 were optimized under the experimental conditions previously selected with the results shown in Figure S3. As can be seen (Fig. S3a), both

specific and unspecific responses of the immunosensor remain practically constant in the range of 0.0 to -0.4 V vs Ag pseudo-reference electrode and tend to decrease at more negative potential values. At the same time, S/N ratio reaches constant values up to -0.6 V. Taking this into account, a potential of -0.4 V was selected as the optimum to ensure that Cu (I) is formed at the electrode surface. This value is accords with the reported by Devaraj et al. [5] (-300 mV vs Ag/AgCl) for the electrochemical synthesis of Cu (I) catalyst used in electro-click reaction between azide-terminated self-assembled monolayers and ethynylferrocene onto a gold electrode. Furthermore, the time of application of the selected potential was also optimized (Fig. S3b). It can be seen that a time of 5 min is enough to ensure adequate response of the immunosensor.

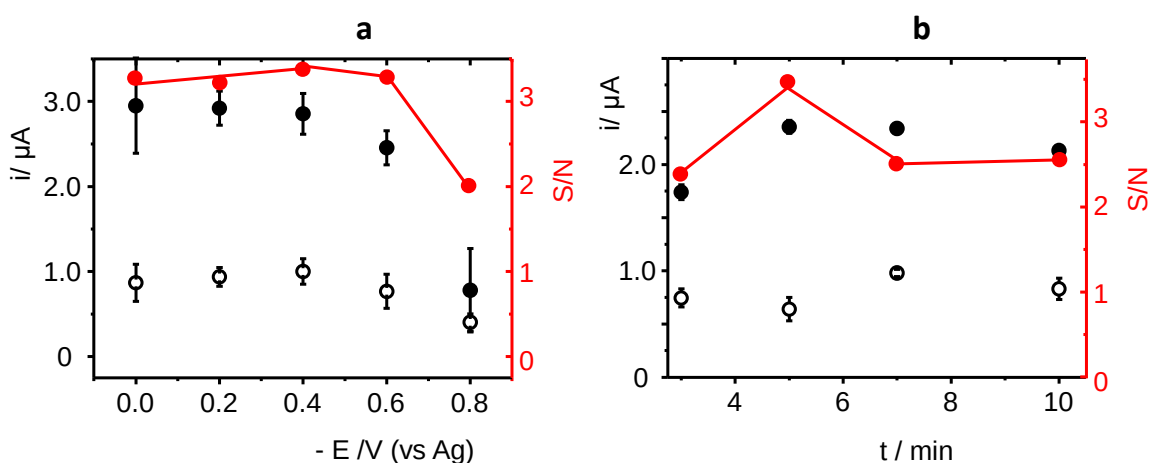


Figure S3. Effect of the reduction potential and the time of application. See the text and the main manuscript for more information.

1.1.4 Effect of the aMWCNTs loading onto SPCE and the ethynylated IgG concentration.

As described in the Experimental section, once modified the SPCE surface with aMWCNTs, a mixture solution of ethynylated IgG and CuSO_4 was dropped onto the resulting electrode. In this section, the loading of aMWCNTs and the concentration of the ethynylated IgG solution were optimized with the results shown in Figure S4. Regarding Fig. S4a, it is shown that 0.5 mg mL^{-1} resulted a suitable concentration of the aMWCNTs solution for the preparation of the aMWCNTs/SPCE. Furthermore, as Fig. S4b reveals, a 1/100 dilution of the ethynylated IgG solution provided the best S/N ratio and thus, it was also selected for the preparation of the immunosensor.

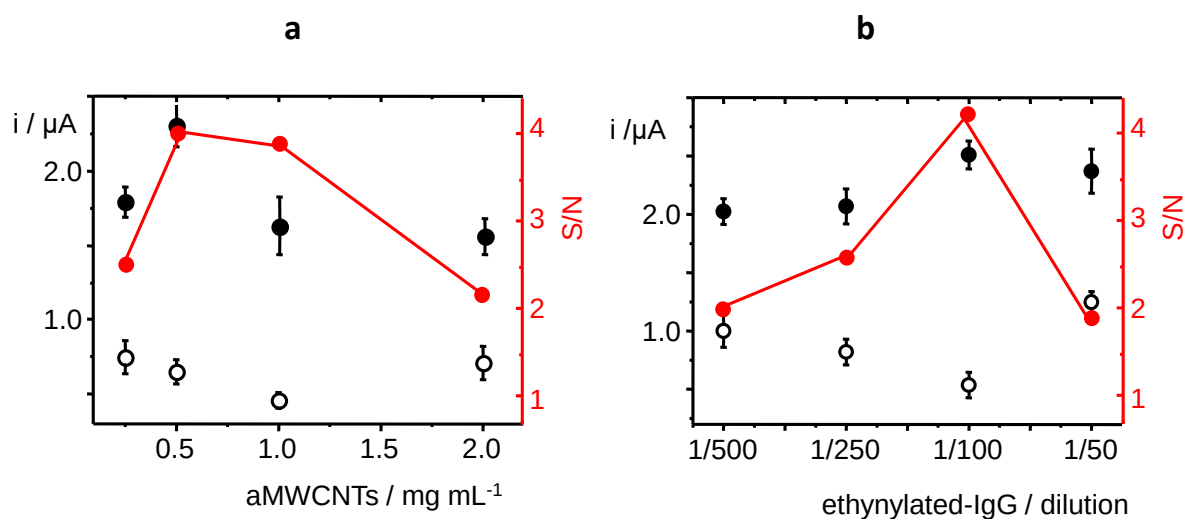


Figure S4. Effect of the dilution of the loading of aMWCNTs onto SPCE (a) and the ethynylated IgG concentration. See the text and the main manuscript for more information.

1.2. Optimization of the variables involved in the performance of the immunosensor

1.2.1. Effect of the loading of casein, the cAb concentration and the times for incubation

Figs. S5a and b show, respectively, the effect of the casein blocking agent concentration and the time for incubation onto the IgG-MWCNTs/SPCE on the immunosensor response. The largest S/N current ratio was found with 1% (w/v) casein solution and 30 min incubation. Regarding cAb concentration, results shown in Fig. S5c led us to choose a $10 \mu\text{g mL}^{-1}$ concentration for further work because the specific current decreased for larger loadings due to the steric hindrance of the antigen recognition that is produced when large amounts of capture antibodies are immobilized [22]. The corresponding incubation time (Fig. S5d) showed better S/N ratios for 60 min.

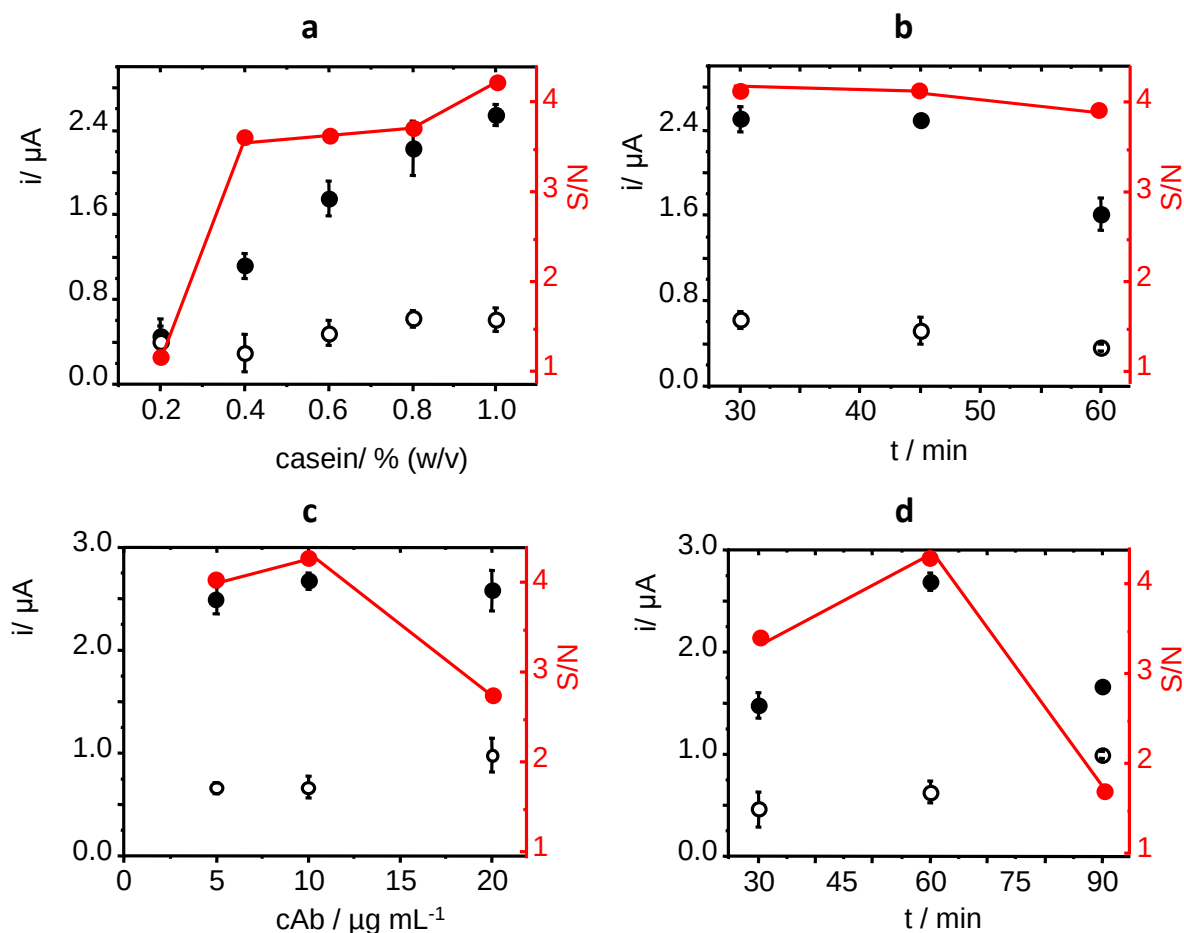


Figure S5. Effect of (a) the loading of casein used as blocking agent and (b) the time for incubation, (c) the loading of cAb onto IgG-MWCNT/SPCE and (d) the time for incubation. See the text and the main manuscript for more information.

2.2. Effect of the loadings of biotin-dAb and AP-strept, and the incubation times

Fig. S6a displays how the specific current provided by the immunosensor increased with the concentration of biotinylated antibody between 0.25 and 0.5 μ g mL⁻¹ and remains constant for a higher concentration. Thus, this loading was chosen for further studies. On the other hand, Fig. S6b shows that the best S/N ratio was attained for an incubation time of 30 min. Finally, the effect of the AP-Strept concentration and the time for incubation displayed in Fig. S6c and d led us to select 5 μ g mL⁻¹ and 30 min to get an effective binding with the biotinylated detector antibody.

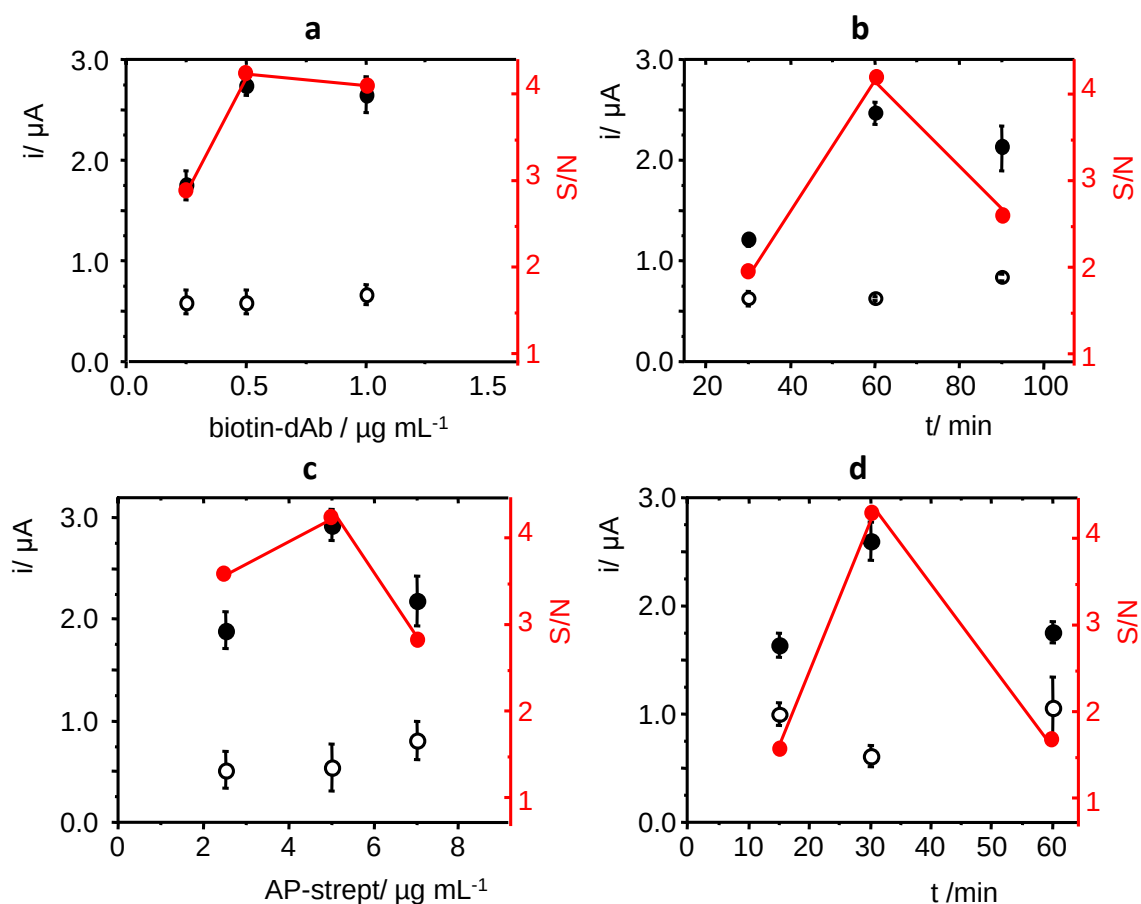


Figure S6. Effect of (a) the loading of biotin-dAb and (b) the incubation time, (c) the loading of AP-strept and (d) the incubation time. See the text and the main manuscript for more information.

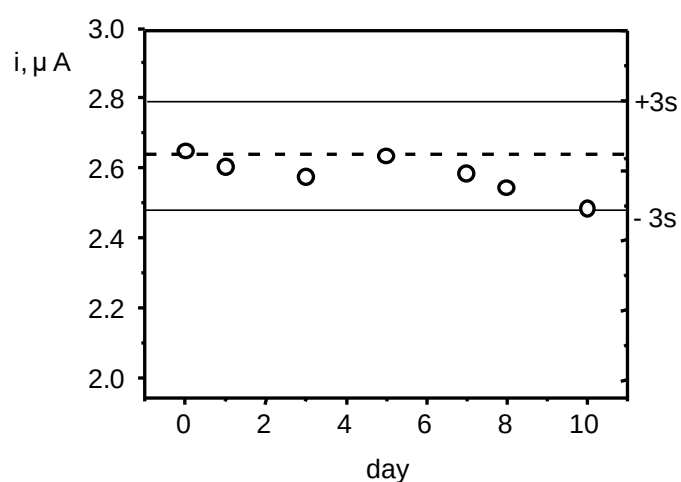


Figure S7. Control chart constructed to evaluate the storage stability of cAb-IgG-MWCNTs/SPCE. Each point corresponds to the mean value of three successive measurements for 1 ng mL^{-1} IL-1 β .

Table 1. Optimization of the variables involved in the preparation of the AP-strept-biotin-dAb-IL-1 β -cAb-IgG-MWCNTs/SPCE immunosensor.

Variable	Tested range or compound	Selected	Section*
Cu (II) salt	CuSO ₄ , CuCl ₂ , Cu(NO ₃) ₂ , CuAc ₂	CuSO ₄	1.1.1
CuSO ₄ concentration, mM	5 x 10 ⁻⁴ - 5	0.005	1.1.1
pH	5 - 8	7	1.2
Buffer solution	PB, MES, Cit, Tris	MES	1.2
Concentration of buffer solution, mM	5 - 50	10	1.2
Reduction potential E _{red} vs Ag, mV	- 800 - 0	- 400	1.3
Time of application of E _{red} , s	180 - 600	300	1.3
aMWCNTs loading, mg mL ⁻¹	0.25 - 2.0	0.5	1.4
Alkynylated IgG dilution	1/500 - 1/50	1/100	1.4
Casein concentration, % (w/v)	0.2 - 1.0	1.0	2.1
Incubation time of casein, min	30 - 60	30	2.1
cAb loading, μ g mL ⁻¹	5 - 20	10	2.1
Incubation time of cAb, min	30 - 90	60	2.1
Biotin-dAb loading, μ g mL ⁻¹	0.25 - 1.0	0.5	2.2
Incubation time of dAb, min	30 - 90	60	2.2
AP-strept loading, μ g mL ⁻¹	2.5 - 7.0	5.0	2.2
Incubation time of AP-strept, min	15 - 60	30	2.2

*The numbers indicate the section in the Supplementary Information where the corresponding variable was optimized.

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Electrochemical biosensor for the simultaneous determination of rheumatoid factor and anti-cyclic citrullinated peptide antibodies in human serum

PAPER

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Electrochemical biosensor for the simultaneous determination of rheumatoid factor and anti-cyclic citrullinated peptide antibodies in human serum

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This paper reports a dual electrochemical biosensor involving carboxylated- or neutravidin-functionalized magnetic microbeads and dual screen-printed carbon electrodes for the simultaneous determination of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCPA) autoantibodies used as biomarkers for the detection of rheumatoid arthritis autoimmune disease. Sandwich-type biosensors involving Fc fragments of IgG Fc(IgG) and biotinylated cyclic citrullinated peptide (CCP-biotin) to form CCP-biotin-Neutr-MBs for the specific immobilization of RF and CCPA, respectively, as well as conjugation with HRP-IgM and HRP-IgG for RF and CCPA, respectively, were prepared. Amperometric detection was performed at -0.20 V vs. Ag pseudo-reference electrode using the H_2O_2 /hydroquinone (HQ) system upon capturing the bioconjugates onto the corresponding working electrode (WE1 or WE2) of SPCdEs. The dual biosensor exhibits high sensitivity for RF and CCPA with LOD values of 0.8 and 2.5 IU mL^{-1} , respectively. The simultaneous determination can be completed in about two hours using a simple protocol and a sample volume (25 μL) four times smaller than that required by the ELISA method. The dual electrochemical biosensor was used for the determination of both target biomarkers in human serum.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease causing pain, swelling and stiffness, characterized by inflammation of the joints.^{1,2} Early diagnosis of RA is extremely important for applying suitable treatment to prevent irreversible joint damage. Serum of RA patients shows frequent presence of autoantibodies, with IgM-rheumatoid factor (RF) as the most prevalent, and anti-citrullinated protein antibodies (CPAs).³ RF is an antibody targeting the Fc fragment of the patient's own IgG molecules while CPAs comprise a group of highly specific RA antibodies, detected during the early stages of the disease, which recognize various peptides and proteins such as filaggrin or fibrinogen, whose arginyl residues are post-translationally transformed into citrullinyl residues. These autoantibodies play an important role in inducing inflammation and joint damage, releasing pro-inflammatory cytokines from monocytes/macrophages. It is reported that 70–80% of RA patients are CPA positive. Therefore, high levels of CPA are associated to bad prognosis of the disease.^{4,5} In

addition, most of patients with RA are also positive to RF, although this factor is not specific for RA as it is produced in a wide range of autoimmune rheumatic diseases and infections.^{6,7} Citrullinated fibrinogen, citrullinated α -enolase and mutated citrullinated vimentin can be used as the selective antigens for CPAs monitoring in RA diagnosis, but the most common antigen is cyclic citrullinated peptide (CCP), which is based on cyclic citrullinated sequences derived from filaggrin^{8,9} and is specific for anti-cyclic citrullinated peptide antibodies (CCPA).

The development of sensitive and accurate methods for the detection of disease biomarkers is an essential tool for achieving an effective early and reliable clinical diagnosis. In this context, multianalyte strategies provide more valuable information. Therefore, faster and cost effective multiplexed assays are of great significance. Regarding RA biomarkers, the development of reliable methodologies for the determination of RF and CPA autoantibodies is of great interest, as their monitoring is challenging due to the low concentration ranges found in biological samples. The established cut-off values are 14 IU mL^{-1} for RF and 20 IU mL^{-1} for CCPA.¹⁰ These biomolecules are usually determined individually by flocculation or agglutination of IgG-coated particles or cells (particularly in the case of RF), or by ELISA methods. Indeed, various colorimetric

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ELISA kits for the single determination of RF or CCPA are commercially available. As an example, the protocol involved in the RF IgM ELISA kit (RFM31-K01) from Eagle Biosciences Inc. for the determination of RF in serum [https://eaglebio.com/wp-content/uploads/data-pdf/rfm31-k01-rf-igm-elisa-assay-kit-insert.pdf] implies wells coated with IgG to bind with RF and detection with an HRP conjugated anti-human IgA. The assay time is around 1 h 45 min, and the logarithmic calibration plot (non-linear) ranges between 1 and 300 IU mL⁻¹. ELISA kits for the determination of CCPA use CCP-coated microtiter plates to bind the antibody followed by detection with HRP-labelled IgG. As an example, the protocol involved in the Medizym® anti-CCP 3860 CCP31-K01-A kit from Eagle Biosciences Inc. [https://eaglebio.com/wp-content/uploads/data-pdf/ccp31-k01-anti-ccp-elisa-assay-kit-package-insert.pdf], exhibits a logarithmic calibration plot with a non-linear dynamic range between 1 and 2000 IU mL⁻¹, and an assay time of about 2 h 15 min.

Regarding biosensors, a label free impedimetric immunosensor for the determination of RF was reported using the rare earths oxide Yb_xO_y sensing membranes and covalent immobilization of the autoantibody, providing a calibration range from 10⁻⁷ to 10⁻³ M (1.54–1.54 × 10⁴ IU mL⁻¹).¹¹ More recently, Chinnadayya *et al.*¹² proposed an impedimetric-interdigitated wave type microelectrode array (IDWμE) functionalized with a self-assembled monolayer (SAM) of thioctic acid for antigen immobilization and determination of RF in serum. The covalent immobilization of IgG Fc fragment onto the SAM modified electrode array provided a linear calibration plot between 1 and 200 IU mL⁻¹ and a LOD value of 0.6 IU mL⁻¹. Regarding the detection of CCPA, a multiwalled carbon nanotube-polystyrene based electrochemical transducer using as the recognition element a chimeric fibrin-filaggrin synthetic peptide covalently immobilized to the surface of the electrode was reported.¹³ Although these two latter biosensors were used for direct measurements in human serum, no data on the calibration plots and the analytical characteristics of the methods were reported.

In this paper, we report an electrochemical biosensor for the simple, fast and reliable simultaneous determination of RF and CCPA. Dual screen-printed carbon electrodes (SPCdEs) and carboxyl- or neutravidin-functionalized magnetic beads (cMBs or Neutr-MBs) were used as the substrates for the immobilization of the specific recognition elements for RF and CCPA, respectively. A Fc fragment of native human IgG protein (Fc(IgG)) was covalently immobilized onto cMBs and the target RF was sandwiched with a HRP-labelled detection antibody (HRP-IgM). In the case of CCPA, a biotinylated CCP peptide was immobilized onto Neutr-MBs conjugated to CCPA antibody and sandwiched with HRP-IgG. The MBs bearing the resulting bioconjugates (HRP-IgM-RF-Fc(IgG)-cMBs and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs) were magnetically captured on the SPCdE electrode surfaces (W1 and W2, respectively). Quantification of RF and CCPA was accomplished by amperometry at -0.2 V vs. Ag pseudo-reference electrode after addition of hydrogen peroxide in the presence of hydroquinone (HQ).

2. Experimental

2.1. Apparatus and electrodes

Amperometric measurements were made with a CH1 1030B potentiostat (Chemical Instruments) equipped with the CH1 1030B software. SPCEs (DRP-110) with a 4 mm-Ø carbon working electrode and dual SPCdEs (DRP-X1110) consisting of two elliptic carbon working electrodes with surface area of 5.6 mm² (Metrohm-DropSens), were used. These electrodes include a carbon counter electrode and a silver pseudo-reference electrode. The specific DRP-CAC and DRP-BICAC connection cables were also from Metrohm-Dropsens. Magnetic separation during the incubation/washing steps was performed using a DynaMag™-2 (DynaL Biotech ASA, Thermo Fisher) magnetic particle concentrator. A neodymium magnet (AIMAN GZ) was used to control the attraction of the modified MBs to the SPCE surface. A Velp Scientific Vortex was used for the homogenization of the solutions. An Optic Ivymen System (Comecta), a Crison model Basic 20+ pH-meter, an Elmasonic S-60 (Elma) ultrasonic bath, and an MPW-65R centrifuge from MPW (Med. Instruments) were also employed.

2.2. Reagents and solutions

Human IgG Fc fragment (Fc(IgG), ab90285), rheumatoid factor IgM (RF, ab178653), and peroxidase labelled anti-human IgM (HRP-IgM, ab178653) were from the anti-rheumatoid factor IgM ELISA kit (ab178653) from Abcam. Biotinylated cyclic cytrullinated peptide (CCP-biotin) was also used. Human cyclic cytrullinated peptide antibody (CCPA) and HRP labelled anti-human IgG antibody (HRP-IgG) prepared in buffered serum matrix were from the commercial ELISA kit DE7760 (Demeditec). Carboxyl-functionalized magnetic microbeads (cMBs, 2.8 μm, 10 mg mL⁻¹, Dynabeads® M-270 carboxylic acid) were purchased from Dynal Biotech ASA (Thermo Fisher Scientific). Neutravidin-modified-magnetic microparticles (Neutr-MBs, 1 μm-Ø, 10 mg mL⁻¹, SpeedBeads™) were from GE Healthcare. Deionized water was from a Millipore Milli-Q purification system (18.2 MΩ cm). Other reagents used were HQ and hydrogen peroxide (30%, w/v), both from Sigma.

Buffer solutions were: 25 mM MES of pH 5.0 prepared from 2-(*N*-morpholine) ethano-sulfonic acid (Gerbu); a 0.05 M potassium phosphate buffer of pH 6.0 (PB); PBS, consisting of a 0.05 M sodium phosphate buffer of pH 7.2 supplemented with 0.0920 g NaCl (Labkem) and 0.0020 g KCl (Scharlau) in 100 mL deionized water; PBST, prepared by addition of 0.1000 g Tween 20 (Aldrich) to 100 mL PBS.

2.3. Procedures

2.3.1. Preparation of the biosensor for the determination of RF. A 3 μL-aliquot of the cMBs suspension was transferred to a 1.5 mL eppendorf tube and two washings were made with 50 μL of 25 mM MES buffer solution of pH 5.0 by incubating for 10 minutes at 25 °C with constant stirring at 950 rpm. After each washing, the tube was placed in the magnetic concentrator for 2 min then removing carefully the supernatant. Thereafter, 25 μL of a solution containing 50 mg mL⁻¹ EDC

and NHSS were added to activate the carboxylic groups, allowing incubation for 30 minutes at 25 °C with constant stirring. Subsequently, two washings with 50 μL of 25 mM MES buffer solution of pH 5.0 were made, and 25 μL of a 25 $\mu\text{g mL}^{-1}$ Fc (IgG) solution in 25 mM MES buffer solution of pH 5.0 were added, allowing incubation for 30 min. Then, the bio-functionalized MBs were separated, the supernatant discarded, and two washings with 50 μL of 25 mM MES buffer solution of pH 5.0 were made. Next, 25 μL of the analyte (RF) solution (or the sample) were added and incubated for 60 minutes at 37 °C with constant stirring at 950 rpm. Then, the particles were separated using the magnetic separator, the supernatant discarded, and two further washings were performed with 50 μL of the MES buffer solution. 25 μL of the commercial HRP-IgM conjugate were added and incubated for 60 minutes at 25 °C with constant stirring at 950 rpm. Once the particles were magnetically separated, two washings were performed with 50 μL of the washing solution included in the anti-rheumatoid factor IgM ELISA kit. The HRP-IgM-RF-Fc(IgG)-cMBs conjugates were resuspended in 25 μL of a 0.05 M PB 6.0 buffer solution and kept in the eppendorf tube until the measurements were carried out.

2.3.2. Preparation of the biosensor for the determination of CCPA. A 3 μL aliquot of the Neutr-MBs suspension was deposited in a 1.5 mL microcentrifuge tube. The functionalized microparticles were washed twice with 50 μL PBST of pH 7.2. Each washing involved placing the tube in the magnetic concentrator for 2 min and further removing of the supernatant. Thereafter, the Neutr-MBs were incubated with 25 μL of CCP-biotin prepared in PBS of pH 7.2 for 30 min. After two washings with 50 μL of the same buffer, the supernatant was removed

and 25 μL of CCPA solution (or the sample) were incubated with the CCP-biotin-Neutr-MBs during 60 min. Thereafter, 25 μL of HRP-IgG solution were incubated for 60 min onto CCPA-CCP-biotin-Neutr-MBs. The resulting HRP-IgG-CCPA-CCP-biotin-Neutr-MBs bioconjugates were washed twice as indicated above and resuspended in 50 μL of 0.05 M PB (pH 6.0) just before the amperometric measurement.

2.3.3. Individual determination of RF or CCPA. The single amperometric determination of RF or CCPA was performed by depositing the HRP-IgM-RF-Fc(IgG)-cMBs or HRP-IgG-CCPA-CCP-biotin-Neutr-MBs bioconjugates on the SPCE working electrode surface previously placed on a housing containing an encapsulated neodymium magnet to ensure magnetic capture in a stable and reproducible manner. Then, the SPCE bearing the functionalized MBs was immersed in a measuring cell containing 10 mL of 0.05 M PB (pH 6.0) and 1 mM HQ solution prepared immediately before the electrochemical measurement. The current was measured in stirred solution after applying a constant potential of -0.2 V vs. Ag pseudo-reference electrode. Once the background current was stabilized (100 s approximately), 50 μL of the 0.1 M H_2O_2 solution were added to the electrochemical cell and the change in the recorded current as a consequence of the enzymatic reduction of H_2O_2 mediated by HQ was monitored once the steady state was reached (aprox. 200 s). The biosensor responses were measured as the difference between the steady state current and the background current.

2.3.4. Simultaneous determination of RF and CCPA. The procedures described above for the individual determination of RF and CCPA were also used for their simultaneous

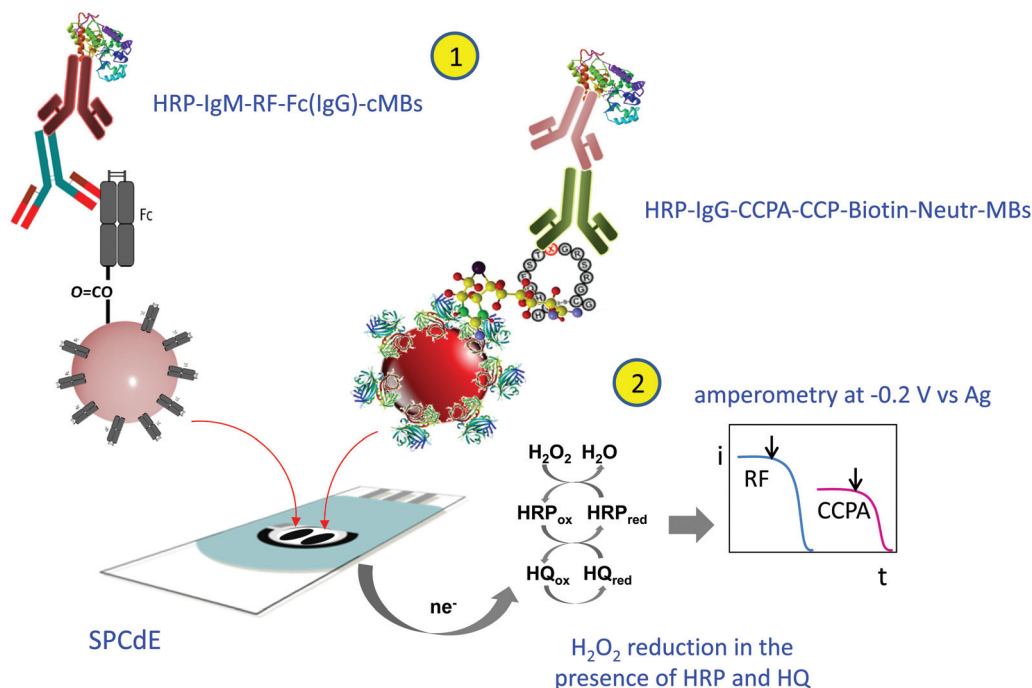


Fig. 1 Graphics showing (1) the addition of HRP-IgM-RF-Fc(IgG)-cMBs and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs conjugates to the SPCE and (2) the reactions involved in the amperometric detection.

amperometric determination through deposition of the HRP-IgM-RF-Fc(IgG)-cMBs or HRP-IgG-CCPA-CCP-biotin-Neutr-MBs on the W1 or W2 working electrode surface of the SPCdE, respectively (Fig. 1).

2.3.5. Determination of RF and CCPA in serum. The simultaneous determination of RF and CCPA in human serum was performed by applying the procedure described above to aliquots of undiluted serum samples and interpolation of the measured amperometric responses into the calibration plots of RF and CCPA standard solutions.

3. Results and discussion

As it is described in the Experimental section, hIgG Fc fragments (Fc(IgG)) immobilized onto carboxylated MBs were used for the specific binding of RF. Fc(IgG) possesses high affinity for RF and as demonstrated by Kanmert *et al.*,¹⁴ the use of Fc fragments implies that no other parts of IgG interfere with RF binding, thereby reducing the risk of false-positives, and also allowing for precise control of the Fc-antigens at the molecular level, which provides greater reproducibility. Regarding CCPA, Neutr-MBs were used as supports to immobilize biotinylated CCP for further binding with CCPA. The use of neutravidin-instead streptavidin- or avidin functionalized MBs has some advantages.¹⁵ Neutravidin possesses higher affinity toward

biotin, almost reaching the theoretical maximum activity ($14 \mu\text{g}$ biotin per mg protein).¹⁶ In addition, the nearly neutral charge of neutravidin minimizes non-specific interactions, thus providing calibration plots with smaller intercepts. Furthermore, since neutravidin is a deglycosylated form of avidin, the unions to lectins are practically undetectable.

3.1. Optimization of the experimental variables

The influence of the variables involved in the preparation of the RF and CCPA biosensors on the respective amperometric responses was evaluated. The working value of each variable was selected according to the criterium of a better specific-to-unspecific current (S/N) ratio. Regarding the biosensor for RF, the loading of Fc(IgG) and the time for incubation on cMBs were tested (Fig. 2). As it can be seen, a larger S/N ratio was found for $25 \mu\text{g mL}^{-1}$ Fc(IgG) (Fig. 2A), and for an incubation time of 30 min (Fig. 2B). Therefore, these values were selected for further work.

The effect of the HRP-IgM dilution on the amperometric responses of the biosensor is shown in Fig. 2C. The biosensor specific response decreases as the conjugate dilution factor increases while the nonspecific current remains almost constant. Thus, a larger S/N ratio was achieved using undiluted conjugate solution. Moreover, Fig. 2D shows that a larger S/N ratio was obtained for 60 min as the incubation time of HRP-IgM onto RF-Fc(IgG)-cMBs/SPCE.

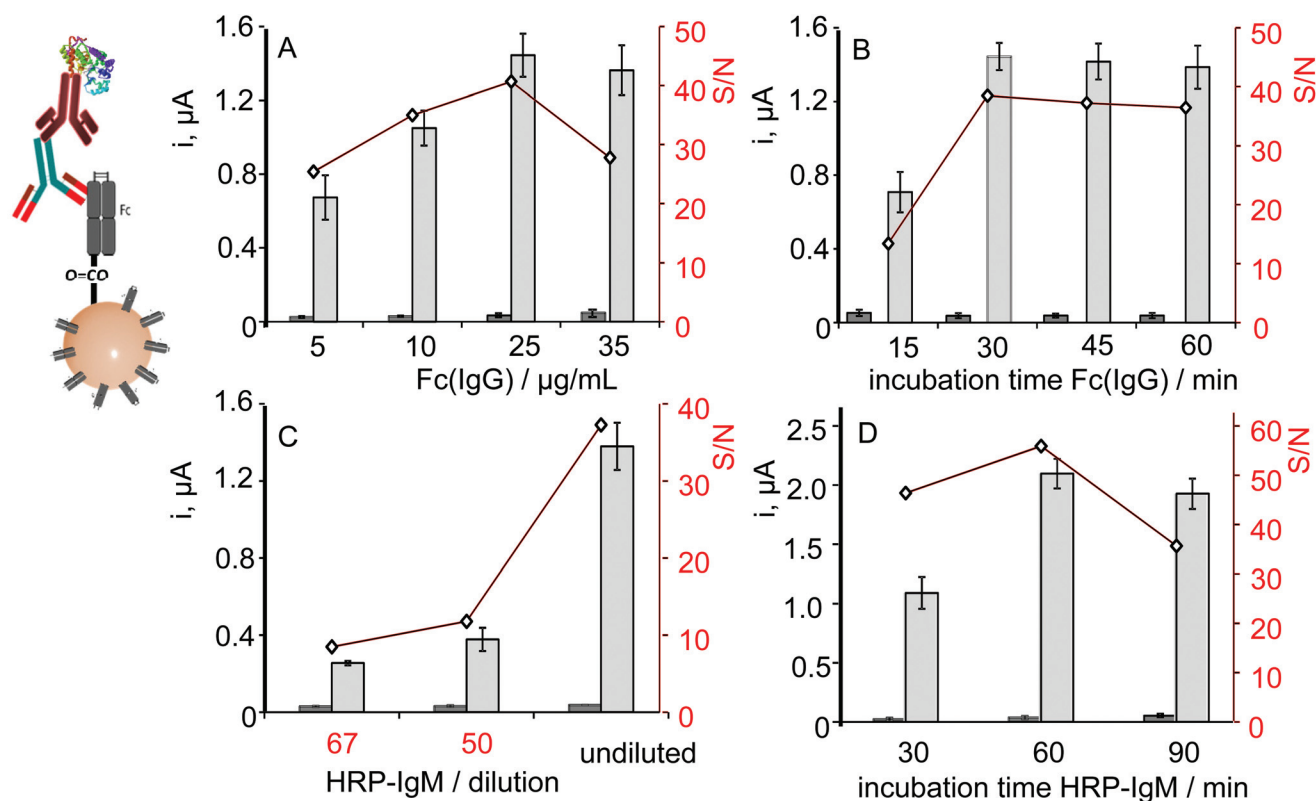


Fig. 2 Effect of loading (A) and incubation time (B) of Fc(IgG) onto cMBs /SPCE; dilution (C) and incubation time (D) of HRP-IgM immobilized onto RF-Fc(IgG)-cMBs/SPCE on the amperometric responses of the HRP-IgM-RF-Fc(IgG)-cMBs/SPCE biosensor. $3 \mu\text{L}$ cMBs; 0 U mL^{-1} (dark grey) or 300 U mL^{-1} (light grey) RF, 60 min.

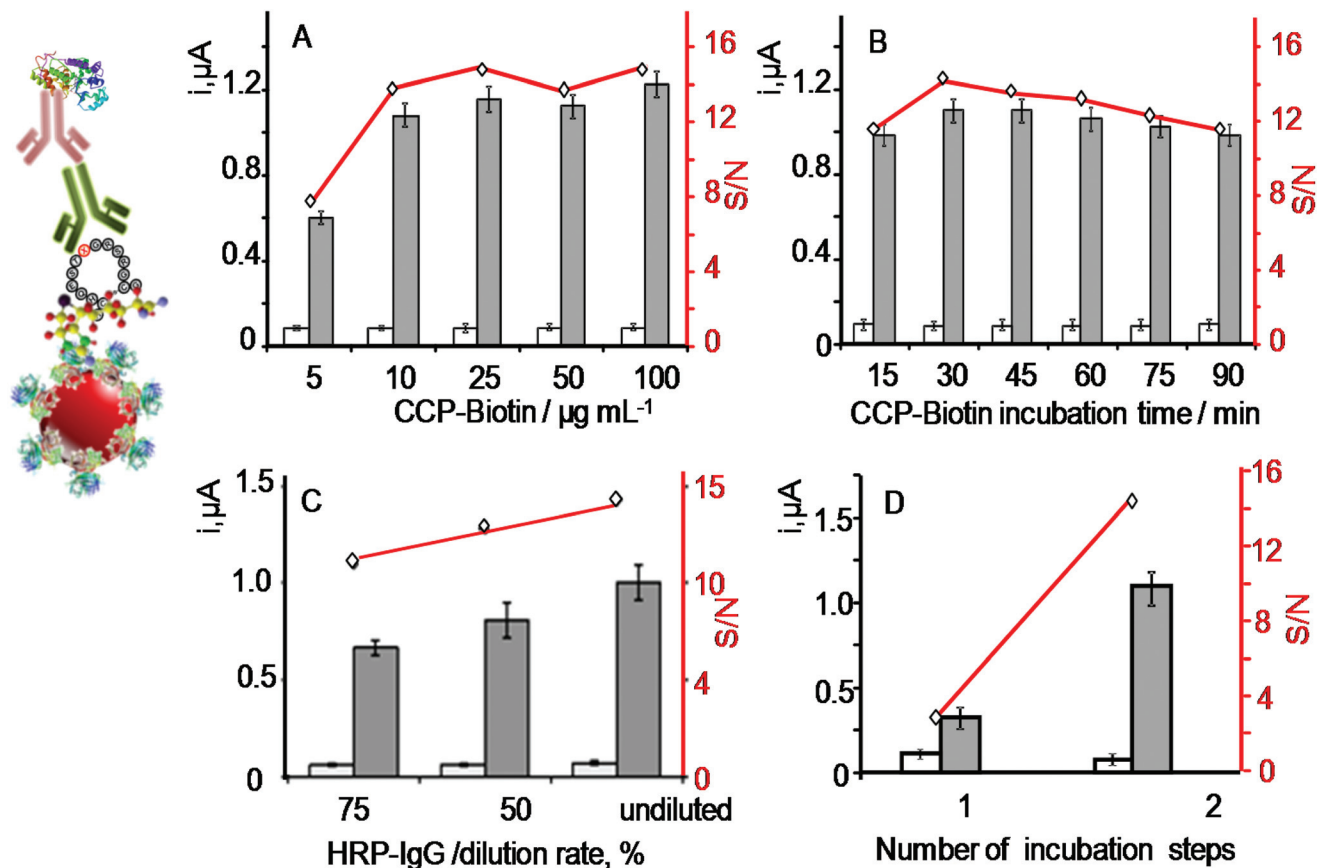


Fig. 3 Effect of: loading (A) and incubation time (B) of CCP-biotin onto Neutr-MBs/SPCE; dilution rate of HRP-IgG (C) immobilized onto CCPA-CCP-biotin-Neutr-MBs/SPCE; number of incubation steps involved in the biosensor preparation (D) on the amperometric responses of the HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCE biosensor. 3 μL Neutr-MBs; 0 U mL^{-1} (white) or 20 U mL^{-1} (grey) CCPA, 60 minutes.

The loading of CCP-biotin conjugate and the time for incubation on Neutr-MBs were optimized by measuring the amperometric responses of the HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCE biosensor. Fig. 3A shows as a concentration of 25 $\mu\text{g mL}^{-1}$ CCP-biotin provided a larger ratio between the current measured in the presence (S) of 20 U mL^{-1} CCPA and its absence (N). In addition, an incubation time of 30 min yielded a larger S/N ratio (Fig. 3B). The effect of the HRP-IgG dilution on the electrochemical response of the biosensor is depicted in Fig. 3C. The specific response decreased as the dilution factor increased while the nonspecific current kept practically constant. Therefore, a better S/N ratio was obtained when using undiluted conjugate solution. Moreover, 60 min showed to be an adequate incubation time of HRP-IgG onto CCPA-CCP-biotin-Neutr-MBs/SPC (results not shown).

In addition, the number of steps involved in the assay was checked in order to short the time needed for the preparation of the biosensor. Two different procedures were evaluated: (1) a single 60 min incubation step involving both the target CCPA and HRP-IgG solution on the CCP-biotin-Neutr-MBs, or (2) two steps involving 60 min incubation of CCP-biotin-Neutr-MBs in the CCPA solution and a further 60 min incubation of CCPA-CCP-biotin-Neutr-MBs in HRP-IgG solution. As it can be

seen in Fig. 3D, both a larger specific response and a better S/N ratio were obtained using two sequential incubations. This is more likely due to a smaller biorecognition efficiency of the target antibody and the peptide immobilized onto the Neutr-MBs due to the steric hindrance occurring when CCPA was mixed with HRP-IgG in homogeneous solution. A similar behavior was observed for sandwich-type immunosensors involving the use of biotinylated detection antibodies conjugated with strep-HRP.¹⁷

Table 1 summarizes the results obtained in the optimization studies. Other experimental conditions such as the composition of the $\text{H}_2\text{O}_2/\text{HQ}$ system or the detection potential were optimized previously.¹⁵ It is worth mentioning the high S/N values, near 50 and 15, achieved with the HRP-IgM-RF-Fc (IgG)-cMBs/SPCE and CCPA-CCP-biotin-Neutr-MBs/SPCE biosensors, respectively. This behavior is probably due to the use of Fc(IgG)-cMBs and Neutr-MBs conjugates as immobilizing substrates because of the special characteristics of Fc(IgG) and neutravidin commented above, as well as to the use of MBs implies that the reagents not specifically linked or the non-targeted components of the sample can be easily and efficiently removed by magnetically controlled washings thus leading to lower matrix effects.

Table 1 Experimental variables selected for the simultaneous determination of RF and CCPA with the dual biosensor

Target	Variable	Studied range	Selected value
RF	cMBs, μL	—	3
	Fc(IgG) loading, $\mu\text{g mL}^{-1}$	5–35	25
	Incub. time Fc(IgG), min	15–60	30
	HRP-IgM, dilution %	67, 50, undiluted	Undiluted
	Incub. time HRP-IgM, min	30–90	60
CCPA	Neutr-MBs, μL	—	3
	CCP-biotin loading, $\mu\text{g mL}^{-1}$	5–100	25
	Incub. time CCP-biotin, min	15–90	30
	HRP-IgG, dilution %	75, 50, undiluted	Undiluted
	Incub. time HRP-IgG, min	60; 1 or 2 steps	60; 2 steps

3.2. Analytical characteristics of the dual biosensor

Fig. 4 displays the calibration plots and some amperometric responses for RF and CCPA standards obtained with the SPCdE biosensor under the optimized working conditions summarized in Table 1. The current vs. RF concentration followed the adjusted equation: i , nA = $(848 \pm 36) \log([\text{RF}], \text{IU mL}^{-1}) - (315 \pm 59)$, with $R^2 = 0.995$, and a range of linearity extended between 3 and 300 IU mL^{-1} . The LOD value was calculated according to the $3S_b$ criterion, where S_b was estimated as the standard deviation expressed in concentration units from ten amperometric responses of the biosensor in the absence of antibody. The LOD value was 0.8 IU mL^{-1} . Regarding CCPA, the equation for the calibration plot is: i , nA = $(1409 \pm 54) \log[\text{CCPA}, \text{U mL}^{-1}] - (1179 \pm 113)$ ($R^2 = 0.996$). The range of linearity ranged between 10 and 1000 U mL^{-1} CCPA, with a LOD value of 2.5 U mL^{-1} .

The repeatability between the amperometric responses for both biomarkers was calculated using ten different dual immunosensors prepared at the same day. The RSD values for measurements without RF and CCPA were 6.1% and 5.4%, respectively, while RSD values calculated from solutions containing 300 IU mL^{-1} RF and 20 IU mL^{-1} CCPA were 6.8% and 5.7%. Similar RSD values were obtained from measurements made on different days in the absence or in the presence of both targets, which confirmed the reproducibility of the working procedure including the preparation of the sandwich immunocomplexes on the MBs, their magnetic capture on the SPCdEs and the dual amperometric detection.

The storage stability of Fc(IgG)-cMBs and CCP-biotin-Neutr-MBs was tested by preparing the same day various conjugates and storing them at 8 °C in 50 μL PBS. Different biosensors prepared using the stored conjugates were used for a daily control by measuring 300 IU mL^{-1} RF or 1000 IU mL^{-1} CCPA. The obtained results (not shown) revealed that the responses of the dual biosensor kept within the control limits set at ± 3 times the standard deviation of the measurements ($n = 3$) carried out the first day of the study, for the next 20 days.

Importantly, the obtained results allow the use of the dual biosensor for the determination of RF and CCPA at clinically relevant levels according to the cut-off values of 14 IU mL^{-1} for

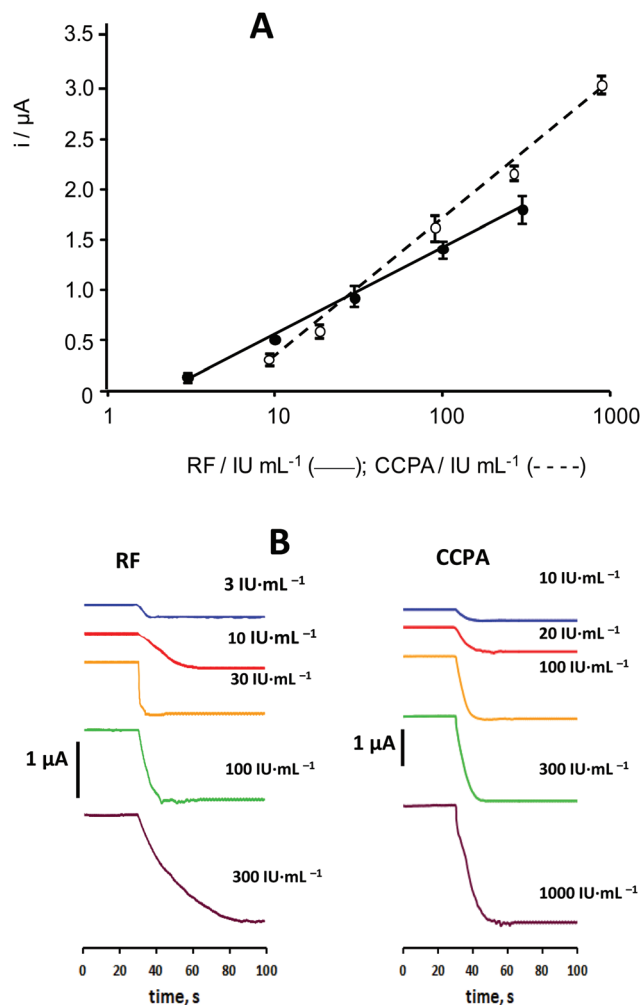


Fig. 4 (A) Calibration plots constructed with the dual biosensor for RF (black) and CCPA (white); (B) amperometric responses at the SPCdE biosensor. Working conditions are summarized in Table 1. Error bars estimated as triple of the standard deviation of three replicates.

RF and 20 IU mL^{-1} for CCPA.¹⁰ The comparison of the analytical characteristics achieved with the dual biosensor with those claimed for the individual assays using ELISA kits leads to the following discussion. The method using the RF IgM ELISA kit (RFM31-K01) from Eagle Biosciences [<https://eaglebio.com/wp-content/uploads/data-pdf/rfm31-k01-rf-igm-elisa-assay-kit-insert.pdf>] claims 1 IU mL^{-1} as the lowest RF concentration detected, while the anti-CCP ELISA assay kit 31-K01 [<https://eaglebio.com/wp-content/uploads/data-pdf/ccp31-k01a-anti-ccp-elisa-assay-kit-package-insert.pdf>] gives a CCPA concentration of 2 IU mL^{-1} as the functional sensitivity. These values are very similar to the LOD values achieved with the dual biosensor. Moreover, similar analytical characteristics were also found when the comparison is made with the few biosensors reported in the literature.^{11,12} Obviously, the main advantage of the biosensor developed in this work is the possibility of performing the simultaneous determination of two relevant rheumatoid arthritis biomarkers, which is not possible using the ELISA methodology and has not been described so far for

any other biosensor design for the target antibodies. In fact, the dual biosensor allows the determination of RF and CCPA in an assay time of two hours approximately, counting from the immobilization of the autoantibodies on the respective Fc (IgG)-cMBs and CCP-biotin-Neutr-MBs conjugates. An important issue is the simultaneous determination can be accomplished at similar sensitivity levels and LOD values than those claimed for the single determination of the target autoantibodies using other methodologies. In addition, only 25 μL of sample solutions are required in the dual assay in comparison with the 100 μL required by the ELISA method.

3.3. Selectivity

The selectivity of the dual biosensor was tested by comparing the amperometric responses measured for 0 and 30 IU mL^{-1} RF or 0 and 20 IU mL^{-1} CCPA standard solutions in the absence and in the presence of various proteins coexisting in serum samples. Fig. 5 shows that no significant interference was apparent in the determination of RF and CCPA in the presence of serum proteins at the expected concentration levels for healthy patients. These results evidenced the high specificity of the Fc(IgG) and CCP peptide employed for the recognition of the target compounds as well as the excellent selectivity of the amperometric transduction at the optimized working conditions.

A potential major risk in the preparation of multiplexed biosensors is the possible cross-talking between adjacent working electrodes. To check this, the amperometric responses

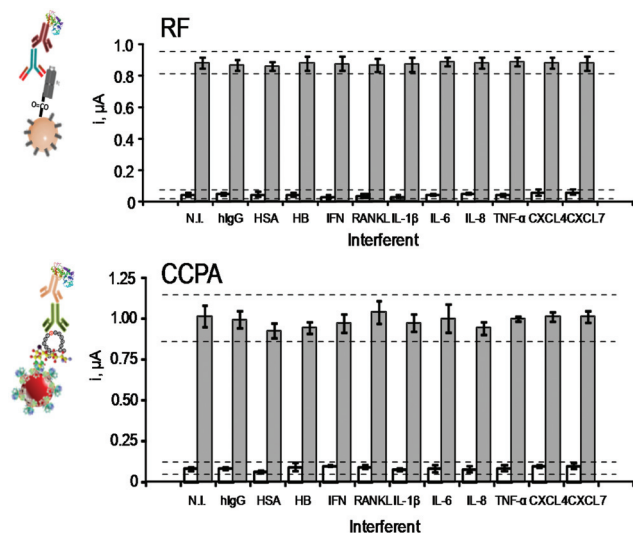


Fig. 5 Amperometric measurements obtained with the dual biosensor for 0 (white), and 30 IU mL^{-1} RF (up) or 20 IU mL^{-1} CCPA (down) (grey) prepared in the absence and in the presence of 1 mg mL^{-1} human IgG; 50 mg mL^{-1} human serum albumin (HSA); 5 mg mL^{-1} hemoglobin (HB); 100 pg mL^{-1} interferon gamma (IFN); 50 pg mL^{-1} receptor activator of nuclear factor- κB ligand (RANKL); 50 pg mL^{-1} interleukin 1 β (IL- β); 300 pg mL^{-1} interleukin 6 (IL-6); 300 pg mL^{-1} interleukin 8 (IL-8); 200 pg mL^{-1} tumor necrosis factor α (TNF- α); 200 pg mL^{-1} chemokine (C-X-C motif) ligand 4 (CXCL4) and 50 ng mL^{-1} chemokine (C-X-C motif) ligand 7 (CXCL7). Error bars are estimated as three times the standard deviation value of three replicates.

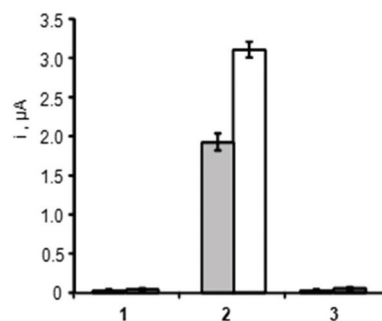


Fig. 6 Simultaneous amperometric responses measured with the HRP-IgM-RF-Fc(IgG)-cMBs/SPCdE (W1, grey) and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCdE (W2, white) biosensors for standard mixtures containing: (1) 0 IU mL^{-1} of both biomarkers; (2) 300 IU mL^{-1} RF + 1000 IU mL^{-1} CCPA; (3) 1000 IU mL^{-1} CCPA + 300 IU mL^{-1} RF. Error bars estimated as triple of the standard deviation of three replicates.

measured with different biosensors for different mixtures of RF and CCPA were compared (Fig. 6). As it is clearly shown, no significant cross-talking occurred between the two working electrode surfaces thus endorsing the viability of the dual bio-platform for the simultaneous determination of both biomarkers.

3.4. Simultaneous determination of RF and CCPA in human serum

The dual biosensor was applied to the quantification of RF and CCPA in human serum samples from Abcam (ab178653) and Demeditec (DE7760) provided by the respective ELISA kits as negative and positive controls for both targets. The possible existence of matrix effects was tested by comparing the slope values of the calibration plots constructed with RF or CCPA buffered standards solutions (Fig. 4) with those calculated from calibration plots prepared in serum by successive addition of standard aliquots to the samples. The application of the Student t test at a significance level of 0.05 and five degrees of freedom, provided $t_{\text{exp}} = 2.219 < t_{\text{tab}} = 2.571$ for RF, and $t_{\text{exp}} = 0.689 < t_{\text{tab}} = 2.571$ for CCPA, thus revealing the absence of matrix effect. Therefore, quantification was carried out by simple interpolation into the standards calibration plots.

Table 2 shows as the simultaneous determination of RF and CCPA with the dual biosensor provided concen-

Table 2 Determination of RF and CCPA in serum by means of the RF and CCPA dual biosensor and comparison with the results obtained with ELISA kits for the single determination of each autoantibody^a

	RF, IU mL^{-1}		CCPA, IU mL^{-1}	
	Biosensor ($n = 6$)	ELISA ($n = 3$)	Biosensor ($n = 6$)	ELISA ($n = 3$)
Control				
Negative	4.3 ± 0.5	4 ± 1	9 ± 1	9 ± 2
Positive	62 ± 6	64 ± 9	263 ± 5	265 ± 10

^a Mean value $\pm$ ts ($\alpha = 0.05$).

tration values in good agreement with those obtained by ELISA kits for the individual determination of each autoantibody involving colorimetric detection with the TMB/H₂O₂ system.

4. Conclusions

In this work, the first electrochemical dual biosensor for the simultaneous determination of RF and CCPA, two relevant biomarkers related with rheumatoid arthritis, is reported. The developed methodology relies on the preparation of sandwich type bioassays for the target compounds between Fe(IgG) or biotin-CCP captured onto carboxylated MBs (cMBs) or neutravidin-functionalized MBs (Neutr-MBs), respectively, and HRP-labeled detector antibodies. The dual biosensor implies the use of dual SPCEs as the electrochemical platform. High sensitivities were achieved with both IgG HRP-IgM-RF-Fc(IgG)-cMBs/SPCde and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCde biosensors with LOD values of 0.8 IU mL⁻¹ RFA and 2.5 IU mL⁻¹ CCPA, respectively. In addition, the biosensor exhibits a great selectivity. The quantification of both targets can be completed in about two hours (counting from the immobilization of the targets on the respective conjugates) involving a simple working protocol and a sample volume (25 µL for each biomarker) four times smaller than that required with ELISA kits. All these results clearly improve the characteristics of the ELISA methods, also taking into account that this technique only allows individual but not simultaneous determinations.

Conflicts of interest

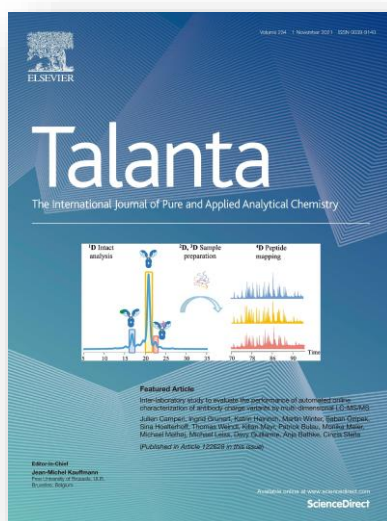
There are no conflicts to declare.

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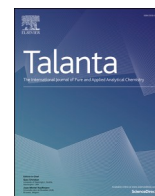
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Simultaneous determination of CXCL7 chemokine and MMP3 metalloproteinase as biomarkers for rheumatoid arthritis



Simultaneous determination of CXCL7 chemokine and MMP3 metalloproteinase as biomarkers for rheumatoid arthritis

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ABSTRACT

This paper reports the preparation of the first dual electrochemical immunosensor for the simultaneous determination of the CXCL7 chemokine and the MMP3 metalloproteinase as relevant biomarkers for the better diagnosis and monitoring of rheumatoid arthritis derived from the multiple biomarkers measurement. The developed immunosensor involves the use of carboxylated magnetic beads (MBs) and dual screen-printed carbon electrodes (SPdCEs). Sandwich-type configurations implied the covalent immobilization of specific anti-CXCL7 (cAb1) or anti-MMP3 (cAb2) capture antibodies onto MBs and the use of biotinylated detection antibodies with further labelling with HRP-Strept conjugates. The resulting MBS bioconjugates were magnetically captured on the respective working electrode of the SPdCE and the determination of the antigens was accomplished by measuring the amperometric responses of H₂O₂ mediated by hydroquinone (HQ) at a potential value of -0.20 V. The dual immunosensor provided calibration plots with linear ranges between 1 and 75 ng mL⁻¹ (CXCL7) ($R^2 = 0.997$) and from 2.0 to 2000 pg mL⁻¹ (MMP3) ($R^2 = 0.998$) with detection limits of 0.8 ng mL⁻¹ and 1.2 pg mL⁻¹, respectively. The assay took 2 h 20 min for the simultaneous determination of both biomarkers. The dual immunosensor was successfully applied to the analysis of human serum from positive and negative RA patients.

1. Introduction

Early detection and monitoring of diseases require sensitive, accurate and fast analytical methods and, ideally, easy to use even for patients themselves with no need for sample treatment. In this context, electrochemical immunosensors have demonstrated their usefulness and ability to detect and quantify a variety of biomolecules playing important roles as markers for diseases in biological samples such as serum, saliva, urine or tissues. A main advantage of these biosensors is the possibility of determining simultaneously different biomarkers in short time without impairing the essential features of sensitivity, selectivity and accuracy. This ability dramatically increases the knowledge of patients' state thus enhancing the opportunities for appropriate treatments to be applied at the right time [1].

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease affecting the articular joints and contributing to cartilage and bone damage [2]. While the etiology of RA is not completely understood, multiple studies show that autoantibodies and other biomarkers including chemokines and proteases, are elevated in patients even years

before RA development [3].

Chemokines are small chemoattractant cytokines that play a key role in the accumulation of inflammatory cells at the site of inflammation [4]. In particular, the 9.3 kDa peptide CXCL7, also known as NAP-2, is a platelet-derived growth factor belonging to the CXC chemokine subfamily which has been shown to be highly expressed in synovial fluid and serum of patients developing RA during the first 12 weeks. However, it is expressed at lower levels in RA with longer duration, this variation being useful to reflect local pathological changes [5].

Common methods for the determination of CXCL7 are enzyme-linked immunosorbent assays (ELISAs) usually involving sandwich formats using biotinylated antibodies conjugated to peroxidase-streptavidin (HRP-Strept). For instance, the Human CXCL7/NAP-2 DY393 kit from R&D Systems [6] is claimed to provide an absorbance vs. log [CXCL7] calibration plot with a logarithmic dynamic range between 15.6 and 1000 pg mL⁻¹. A 100 μ L sample volume is required and the assay time takes 4 h 40 min. Regarding immunosensors, to our knowledge, only one electrochemical immunosensor has been previously reported for the determination of this relevant chemokine [7]. It involved click

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chemistry-assisted immobilization of antibodies performed by reaction of azide functionalized multi-walled carbon nanotubes (MWCNTs) and ethynyl-IgG onto screen-printed carbon electrodes (SPCEs) followed by conjugation of capture antibody to IgG-MWCNTs. Differential pulse voltammetry transduction upon addition of 1-naphthyl-phosphate provided a linear calibration plot between 0.5 and 600 pg mL^{-1} CXCL7 and a LOD value of 0.1 pg mL^{-1} .

Matrix metalloproteinase-3 (MMP3), also known as stromelysin-1, is an enzyme involved in the degradation of various extracellular matrix components and in the destruction of cartilage and bone in RA [8]. The increased levels in serum of MMP3 in RA patients [9,10] is thought to originate from the synovium, where MMP3 concentration is around 250 times higher [11], and correlate with the number of joints affected [12]. Therefore, the MMP3 concentration has been proposed as a derived serum marker of inflammation in RA with reported cut-off values of 14 ng mL^{-1} in women and 34 ng mL^{-1} in men [8]. Although increased MMP3 levels in serum are not specific for RA [13–15], the measurement of the serum MMP3 content is considered as a useful scarcely invasive method to monitor synovial inflammation and pathologic processes underlying joint destruction in RA, where a few hundred ng mL^{-1} MMP3 in serum of patients may be found.

Various ELISA kits involving sandwich-type configurations with biotinylated detection antibodies and HRP-Strept conjugates are available for the determination of MMP3. The reported calibrations range from a few tens or hundreds to several thousand pg mL^{-1} and the assay times take between 1 h 30 min to 5 h. For instance, the R&D Systems Human Total MMP3 DuoSet ELISA Kit DY513 [16] provides a non-linear logarithmic plot with dynamic range from 31.3 to 2000 pg mL^{-1} . The assay time was 4 h 40 min. A similar method from Abcam (ab269371 Human MMP3 ELISA Kit) provides a non-linear logarithmic range between 25 and 1600 pg mL^{-1} , with a LOD value of 4.4 pg mL^{-1} and a much shorter assay time of 1 h 30 min [17]. Regarding immunosensors, very few reports are found in the literature. Among them, it is worth mentioning an electrochemical immunosensor involving vertically aligned single-walled carbon nanotubes (SWCNTs) and providing a logarithmic calibration plot over the 0.4–40 ng mL^{-1} MMP3 range with a LOD value of 400 pg mL^{-1} . The implementation of an amplification strategy using polymeric beads with multiple HRP labels resulted in a 0.4–40 pg mL^{-1} logarithmic calibration plot and a LOD of 4 pg mL^{-1} MMP3 [18].

As it is well known, electrochemical immunosensors involving functionalized magnetic beads (MBs) have demonstrated to be valuable bioanalytical tools because they combine the excellent capabilities of electrochemical transduction in terms of sensitivity, low cost and miniaturization ability with the special features of MBs as substrates for immobilization of bioreagents [19]. The large surface area and the number of functionalities onto MBs enable the immobilization of large antibody loadings which results in low detection limits. In addition, the magnetic properties of MBs greatly facilitate the preparation of electrochemical biosensors, especially the optimization of experimental conditions and detection steps, while minimize unspecific bindings on the electrode and decrease matrix effects, and the reagents that are not specifically linked or unwanted sample components can be easily and efficiently removed by magnetically controlled washing [20]. Furthermore, combination of MBs with the use of planar disposable electrodes such as SPCEs provides optimal conditions for electrochemical detection because it is possible to magnetically concentrate the bioconjugates on the working electrode surface allowing an efficient electron transfer which results in larger measured higher currents.

This paper reports the preparation of the first dual electrochemical immunosensor for the simultaneous determination of CXCL7 and MMP3 with the objective of providing more accurate data for diagnosis and monitoring of RA disease. The developed immunosensor involves the use of carboxylated MBs and dual screen-printed carbon electrodes (SPCEs). Sandwich-type configurations involved the covalent immobilization of specific anti-CXCL7 (cAb1) or anti-MMP3 (cAb2) capture

antibodies onto MBs. Once the immunoaffinity reactions occurred, the respective biotinylated detection antibodies, Biotin-anti-CXCL7 (Biotin-dAb1) or Biotin-anti-MMP3 (Biotin-dAb2), were used to sandwich the target analytes with further labelling with HRP-Strept conjugates. The resulting HRP-Strept-Biotin-dAb1-CXCL7-cAb1-MBs and HRP-Strept-Biotin-dAb2-MMP3-cAb2-MBs were magnetically captured on the corresponding working electrode (WE1 or WE2, respectively) of the SPdCE and the determination of the antigens was accomplished by measuring the amperometric responses of H_2O_2 mediated by hydroquinone (HQ) at a potential value of -0.20 V (vs Ag pseudoreference electrode). The dual immunosensor was successfully applied to the analysis of human serum from positive and negative RA patients.

2. Experimental

2.1. Apparatus and electrodes

Amperometric measurements were made with a CH1 1030B potentiostat (Chemical Instruments) equipped with the CH1 1030B software. SPCEs (DRP-110) with a 4 mm- $\varnothing$ carbon working electrode and dual SPCEs (DRP-X1110) consisting of two elliptic carbon working electrodes with surface area of 5.6 mm^2 (Metrohm-DropSens), were used. The SPCEs and SPCEs include a carbon counter electrode and a silver pseudo-reference electrode. The specific DRP-CAC and DRP-BICAC connection cables were also from Metrohm-Dropsens. Magnetic separation during the incubation/washing steps was performed using a DynaMag™-2 (Dyna Biotech ASA, Thermo Fisher) magnetic particle concentrator. A neodymium magnet (AIMAN GZ) was used to control the attraction of the modified MBs to the SPCE surface. A Velp Scientific Vortex was used for the homogenization of the solutions. An Optic Ivymen System (Comecta), a Crison model Basic 20+ pH-meter, and an Elmasonic S-60 (Elma) ultrasonic bath were also employed.

2.2. Reagents and solutions

The immunoreagents used for the preparation of CXCL7 and MMP3 immunosensors were all from R&D Systems. Mouse anti-CXCL7 [Ref. 840319] reconstituted in phosphate buffer saline solution of pH 7.4 (PBS), goat biotinylated antibody (Biotin-anti-CXCL7) [Ref. 840320] and human CXCL7 antigen [Ref. 840321] reconstituted in PBS of pH 7.4 supplemented with 1% bovine serum albumin (BSA) were used. Goat anti-human MMP3 capture antibody [Ref. 844920] reconstituted in PBS of pH 7.4, biotinylated goat anti-human MMP3 detection antibody [Ref. 844921] reconstituted in PBS of pH 7.4 supplemented with 1% BSA, and recombinant human MMP3 standard [Ref. 841045] were used. HRP-Strept conjugates were from Roche. 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and sodium N-hydroxysulfo-succinimide (Sulfo -NHS) from Acros Organics, hydroquinone (HQ), hydrogen peroxide (30%, w/v) and ethanolamine from Sigma-Aldrich, were also used.

Carboxyl-functionalized magnetic microbeads (cMBs, 2.8 μm , 10 mg mL^{-1} , Dynabeads® M – 270 carboxylic acid) were purchased from Dynal Biotech ASA (Thermo Fisher Scientific). Ascorbic acid (AA), human serum albumin (HSA), hemoglobin (HB) and human immunoglobulin G (hIgG), all from Sigma, citrullinated peptide antibody (CCPA) (Demeditec), rheumatoid factor (RF) (Abcam), ligand receptor activator nuclear factor κB (RANKL), human endoglin (CD105) and human PF4 (CXCL4), all from R&D Systems, were tested as potential interfering biomolecules. Deionized water was from a Millipore Milli-Q purification system (18.2 $\text{M}\Omega$ cm at 25 °C).

Buffer solutions used were: 25 mM MES of pH 5.0 prepared from 2-(N-morpholine) ethano-sulfonic acid (Gerbu); 0.05 M potassium phosphate buffer (PB) of pH 6.0 prepared from K_2HPO_4 (Merck) and KH_2PO_4 (Labkem), and 0.1 M sodium phosphate buffer (PB) of pH 8.0 prepared from NaH_2PO_4 and Na_2HPO_4 (Labkem). Phosphate buffer saline (PBS) of pH 7.4 containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , and

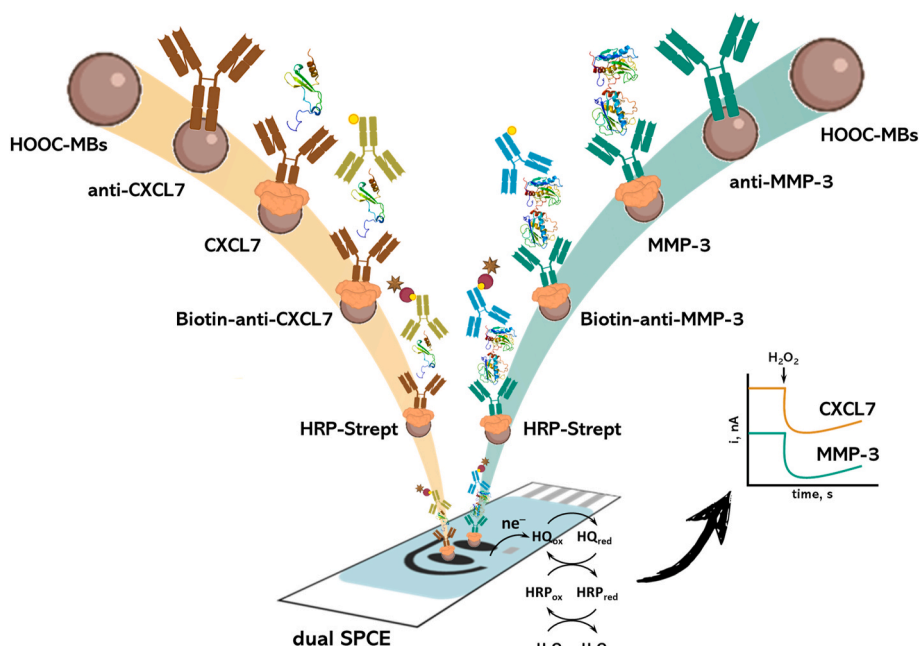


Fig. 1. Schematic display of the different steps involved in the preparation and functioning of the dual CXCL7 and MMP3 dual immunosensor.

1.5 mM KH_2PO_4 1.5 mM was from R&D Systems (Ref. 896036 DuoSet® Ancillary Reagent Kit 1).

The analysed samples were sera from healthy individuals and RA patients and were provided by Central BioHub® (Hennigsdorf, Germany).

2.3. Procedures

2.3.1. Preparation of the immunosensors for the determination of CXCL7 and MMP3

For each target protein, a 3 μL -aliquot of the carboxylated MBs suspension was transferred to a 1.5 mL eppendorf tube and two washings were made with 50 μL of 25 mM MES buffer solution of pH 5.0 by incubating for 10 min at 25 °C with constant stirring at 950 rpm. After each washing, the tube was placed in the magnetic concentrator for 2 min and the supernatant was removed carefully. All the incubations were performed at 25 °C under stirring at 950 min. Thereafter, 25 μL of a solution containing 50 mg mL^{-1} EDC and Sulfo-NHS were added to activate the carboxylic groups, allowing incubation for 30 min. Subsequently, two washings with 50 μL of 25 mM MES buffer solution of pH 5.0 were carried out and 25 μL of the respective capture antibody, anti-CXCL7 (Ab1, 10 $\mu\text{g mL}^{-1}$) or anti-MMP3 (Ab2, 15 $\mu\text{g mL}^{-1}$) were added and incubated for 45 min. Two further washings with 50 μL of 25 mM MES buffer solution of pH 5.0 were done, and the unreacted MBs activated groups were blocked by adding 25 μL of the 1 M ethanolamine solution in 0.1 M PB of pH 8.0. After an incubation of 30 min, the excess of ethanolamine was removed by washing twice with 50 μL 0.1 M PB of pH 8.0 and with 50 μL PBS of pH 7.4. Next, 25 μL of the corresponding CXCL7 or MMP3 solution or the sample were added and incubated for 60 min. Two additional washings with 50 μL of the PBS, pH 7.4, buffer solution were made and, thereafter, 25 μL of 0.25 $\mu\text{g mL}^{-1}$ Biotin-anti-CXCL7 (dAb1) or Biotin-anti-MMP3 (dAb2) were added and incubated for 60 min. Once the bioconjugates were magnetically separated, two washings steps were carried out with 50 μL of PBS pH 7.4 and, finally, 25 μL of 1/2000 diluted (CXCL7) or 1/500 diluted (MMP3) HRP-Strept were added. After incubation for 20 min, the HRP-Strept-Biotin-dAb1-CXCL7-cAb1-MBs or HRP-Strept-Biotin-dAb2-MMP3-cAb2-MBs conjugates were washed twice with 50 μL of the same buffer, re-suspended in 25 μL of a 0.05 M PB buffer solution of pH 6.0, and kept in the eppendorf

tube until the measurements were carried out.

2.3.2. Amperometric detection

Single amperometric measurements of CXCL7 or MMP3 were performed by depositing the HRP-Strept-Biotin-dAb1-CXCL7-cAb1-MBs or HRP-Strept-Biotin-dAb2-MMP3-cAb2-MBs bioconjugates on the SPCE working electrode surface previously placed on a housing containing an encapsulated neodymium magnet to ensure magnetic capture in a stable and reproducible manner. Then, the SPCE bearing the MBs-bioconjugates was immersed into a measuring cell containing 10 mL of 0.05 M PB of pH 6.0 and 1 mM HQ solution prepared in the same buffer immediately before the electrochemical measurement. The current was measured in stirred solution after applying a constant potential of -0.2 V vs. Ag pseudoreference electrode. Once the background current was stabilized (100 s approximately), 50 μL of 0.1 M H_2O_2 solution in 0.05 M PB of pH 6.0 were added to the electrochemical cell and the change in the recorded current due to the enzymatic reduction of H_2O_2 mediated by HQ was monitored once the steady state was reached (aprox. 200 s). The immunosensor responses were measured as the difference between the steady state current and the background current. The simultaneous amperometric determination of CXCL7 and MMP3 was performed by following the procedure described above through the deposition of the corresponding HRP-Strept-Biotin-dAb1-CXCL7-cAb1-MBs or HRP-Strept-Biotin-dAb2-MMP3-cAb2-MBs bioconjugates on the respective W1 or W2 working electrode surface of the SPCdE (Fig. 1).

2.3.3. Determination of CXCL7 and MMP3 in human serum

The simultaneous determination of CXCL7 and MMP3 in human serum was performed by applying the procedure described above to aliquots of 1/50 diluted in PBS of pH 7.4 serum samples. After amperometric measurements, current responses were interpolated into the calibration plots constructed with CXCL7 and MMP3 standard solutions.

3. Results and discussion

As described in the Experimental section, carboxylated MBs were used to immobilize the respective capture antibody for specific binding of CXCL7 and MMP3 biomarkers and to allow sandwich-type configurations (Fig. 1). Once the immunoaffinity reaction with the

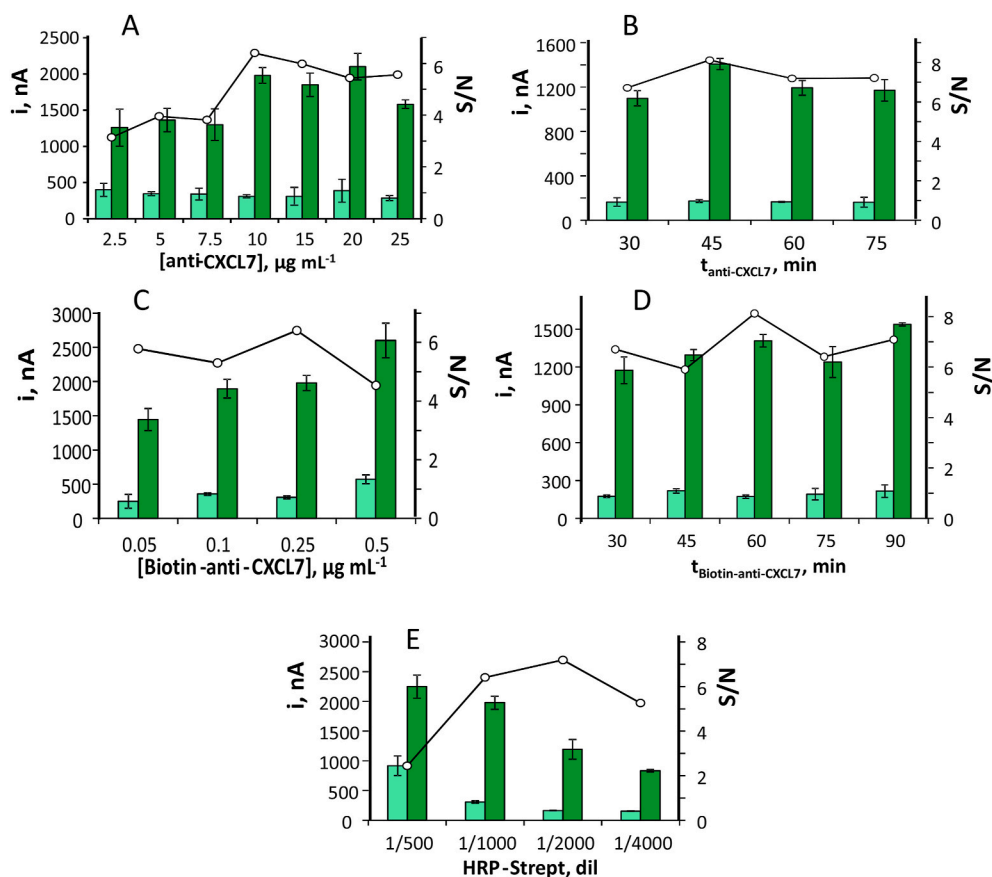


Fig. 2. Effect of the loading (A, C) and incubation time (B, D) of anti-CXCL7 (cAb1) (A, B) and Biotin-anti-CXCL7 (Biotin-dAb1) (C, D) onto cMBs, and effect of the HRP-Strept dilution (E) immobilized onto Biotin-dAb1-CXCL7-cAb1-MBs, on the amperometric responses measured with the developed immunosensor. 3 μL cMBs; 0 (light green) or 1 ng mL^{-1} (dark green) CXCL7, 60 min. Error bars were estimated as triple of the standard deviation of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

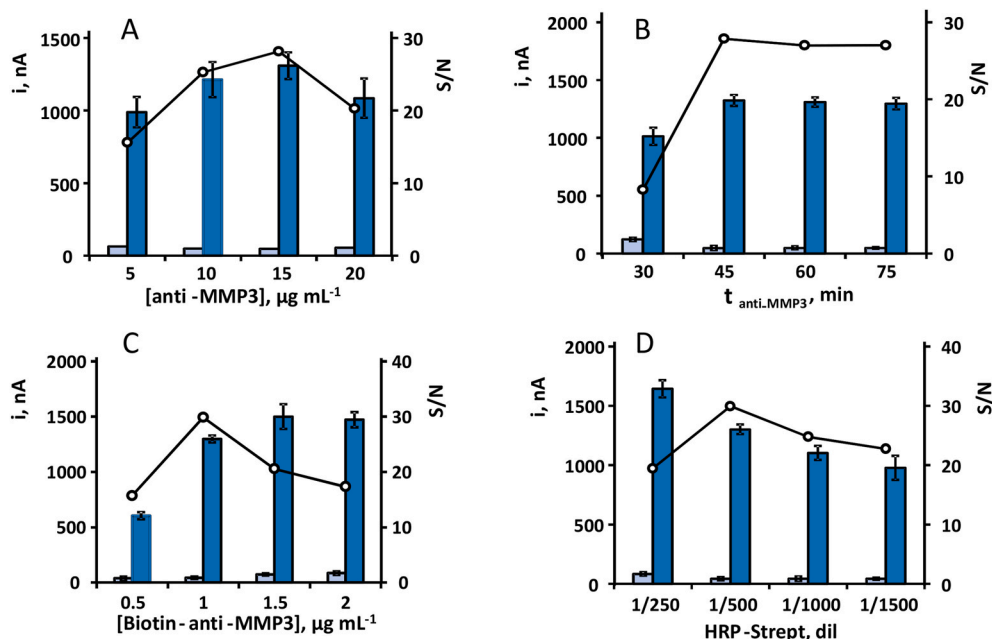


Fig. 3. Effect of the anti-MMP3 (cAb2) (A) and Biotin-anti-MMP3 (Biotin-dAb2) (C) loadings, the incubation time (B) of cAb2 on cMBs and the HRP-Strept dilution (D) on the amperometric responses of the HRP-Strept-Biotin-dAb2-MMP3-cAb2-MBs/SPCE immunosensor. 3 μL cMBs; 0 (light blue) or 2 ng mL^{-1} (dark blue) MMP3, 60 min. Error bars were estimated as triple of the standard deviation of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

corresponding target protein was accomplished, the respective biotinylated detection antibody was added to sandwich the analyte. After labelling with HRP-Strept conjugates, the resulting HRP-Strept-Biotin-dAb1-CXCL7-cAb1-MBs and HRP-Strept-Biotin-dAb2-MMP3-cAb2-MBs were captured on the corresponding working electrode of the SPdCE with a small magnet. The determination of the antigens was

accomplished by measuring the amperometric responses of H_2O_2 mediated by HQ at a potential value of -0.20 V (vs Ag pseudoreference electrode).

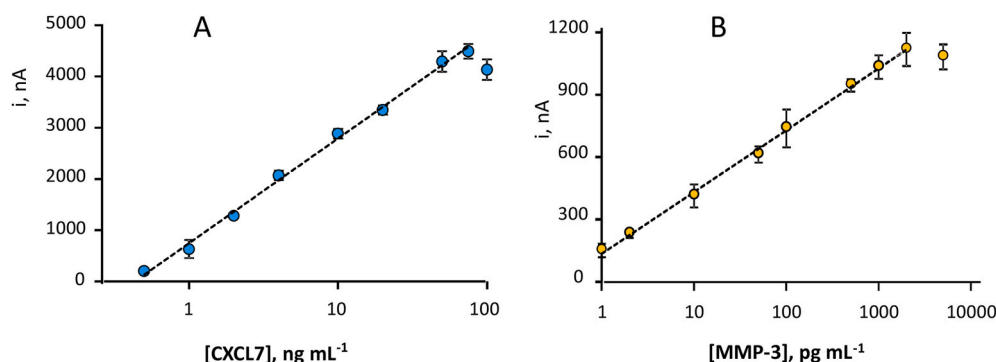


Fig. 4. Calibration plots for the simultaneous determination of CXCL7 (A) and MMP3 (B) biomarkers using the HRP-Strept-Biotin-dAb-CXCL7 (or MMP3)-cAb-MBs/SPCdE dual immunosensor. Error bars were estimated as triple of the standard deviation of three replicates.

3.1. Optimization of the experimental variables

The influence of the variables involved in the preparation of the CXCL7 and MMP3 immunosensors on the respective amperometric responses was evaluated for each target protein. Single SPCEs were employed for these studies. The chosen working value was selected according to the larger specific-to-unspecific current (S/N) ratio criterion. Results obtained are shown in Figs. 2 and 3, respectively. Regarding the effect of anti-CXCL7 (cAb1) loading and the time for incubation on MBs, Fig. 2A and B shows that larger S/N ratios occurred for 10 $\mu\text{g mL}^{-1}$ cAb1 and 45 min, respectively. Therefore, these values were selected for further work. The influence of the Biotin-anti-CXCL7 (Biotin-dAb1) conjugate loading and the time for incubation with CXCL7-anti-dAb1-MBs are displayed in Fig. 2C and D, respectively. A concentration of 0.25 $\mu\text{g mL}^{-1}$ Biotin-dAb1 provided a larger ratio between the currents measured in the presence of 1 ng mL^{-1} CXCL7 and its absence (Fig. 2C). In addition, an incubation time of 60 min yielded a larger S/N ratio (Fig. 2D). The effect of the HRP-Strept dilution on the electrochemical response of the immunosensor is shown in Fig. 2E. As expected, both the specific and nonspecific currents decreased as the dilution factor increased due to the lower amount of enzyme available. However, a better S/N ratio was found for 1/2000 diluted HRP-Strept. Moreover, 20 min showed to be an adequate incubation time of HRP-Strept conjugates onto Biotin-dAb1-CXCL7-cAb1-MBs (results not shown). It should be noted that under the selected conditions, the nonspecific response of the immunosensor is less than 14% of the specific response.

Regarding the optimization of the variables involved in the construction of the MMP3 immunosensor, the effect of the anti-MMP3 (cAb2) loading and the time for its incubation on MBs is shown in Fig. 3A and B, respectively. As it can be seen, larger S/N ratios were found for 15 $\mu\text{g mL}^{-1}$ cAb2 and 45 min, values selected for further work. The effect of Biotin-anti-MMP3 (Biotin-dAb2) loading (Fig. 3C) and the time for incubation with MMP3-anti-cAb2-MBs on the immunosensor response were also tested. A concentration of 0.25 $\mu\text{g mL}^{-1}$ Biotin-dAb2 provided larger ratio between the currents measured in the presence of 2 ng mL^{-1} MMP3 or in its absence. In addition, an incubation time of 60 min yielded larger S/N ratio (results not shown). The effect of the HRP-Strept dilution on the electrochemical response of the immunosensor is shown in Fig. 3D. Similarly to that occurred with CXCL7, both the specific and nonspecific currents decreased as the dilution factor increased, with a larger S/N ratio for 1/500 diluted HRP-Strept which was selected for the preparation of the MMP3 immunosensor. Moreover, 20 min showed to be an adequate incubation time of HRP-Strept conjugates onto Biotin-dAb2-MMP3-cAb2-MBs (results not shown). Importantly, the MMP3 immunosensor provided a nonspecific response less than 4% of the specific response at the working conditions selected for its the preparation.

The results obtained in the optimization of other variables involved in the preparation and functioning of the immunosensors are displayed

in the Supporting Information. As recommended by the application protocols of carboxylated MBs [21], 1 M ethanolamine solution prepared in 0.1 M PB of pH 8.0 was used to block the remaining active unreacted groups of MBs. Therefore, the effect of the incubation time of the ethanolamine solution with the cAb-MBs conjugates was checked between 0 (no blocking) and 45 min. Fig. S1 shows as similar results were obtained for both antigens. As expected, both specific and unspecific currents decreased with the incubation time due to the partial passivation of the electrode surface. A larger S/N ratio was found for 30 min which was selected for the preparation of the immunosensors. Furthermore, with the aim of simplifying the protocols involved in the preparation of immunosensors, the number of steps required to form the sandwich immunocomplexes onto the respective cAb-MBs was checked for each biomarker. Two different protocols were evaluated: (a) a single incubation step of the cAb-MBs in a mixture solution containing the target biomarker (CXCL7 or MMP3) and the respective Biotin-dAb detection antibody, and (b) an incubation step of the biomarker solution with the cAb-MBs followed by a subsequent incubation with the corresponding Biotin-dAb. The results obtained (not shown) were similar for both biomarkers and provided larger specific responses and S/N ratios when two sequential incubations were involved. This effect was attributed to a lower target recognition efficiency, due to a higher steric hindrance, when the biomarker was previously mixed with its corresponding Biotin-dAb [22].

All other experimental variables used in the preparation and functioning of the immunosensors such as the amount of cMBs, the applied potential (-0.20 V vs Ag pseudoreference electrode) and the concentration of H_2O_2 and HQ were the same than those optimized in previous works [23]. The tested working variables, including the checked ranges and the selected values, are summarized in Table S1 (Supporting information).

3.2. Simultaneous determination of CXCL7 and MMP3

The calibration plots for the simultaneous determination of CXCL7 and MMP3 are displayed in Fig. 4. The currents measured with the dual electrochemical platform involving magnetic capture of the immunoconjugates onto WE1 (CXCL7) and WE2 (MMP3) of the SPCdE for solutions containing both biomarkers showed a linear logarithmic dependence with the corresponding target protein concentration between 0.5 and 75 ng mL^{-1} for CXCL7, and from 1 to 2000 pg mL^{-1} for MMP3, according to the equations: $i, \text{nA} = (2041 \pm 47) \log ([\text{CXCL7}], \text{ng mL}^{-1}) + (746 \pm 52)$, $R^2 = 0.997$; $i, \text{nA} = (297 \pm 5) \log ([\text{MMP3}], \text{pg mL}^{-1}) + (135 \pm 10)$, $R^2 = 0.998$.

The obtained ranges cover more than two (CXCL7) or three (MMP3) orders of magnitude and are adequate to determine both biomarkers in clinical samples, considering their expected levels in serum of healthy individuals, close to 700 ng mL^{-1} CXCL7 [24] and of few tens of ng mL^{-1} MMP3 [8]. Furthermore, the limits of detection were calculated

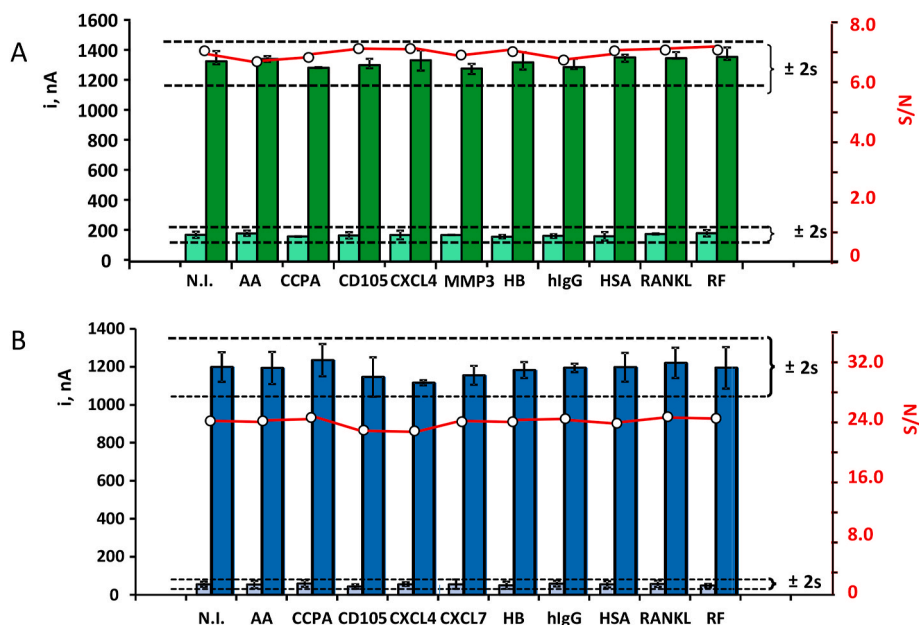


Fig. 5. Effect of the presence of $7 \mu\text{g mL}^{-1}$ AA, 100 IU mL^{-1} CCPA, 70 pg mL^{-1} CD105, 5 mg mL^{-1} HB, 1 mg mL^{-1} hlgG, 5 mg mL^{-1} HAS, 50 pg mL^{-1} RANKL, 100 IU mL^{-1} RF, and 2 ng mL^{-1} MMP3 (A) or 50 ng mL^{-1} CXCL4 (B), on the amperometric responses provided by the CXCL7 (A) and MMP3 (B) immunosensors for 0 (light bars) and 2 ng mL^{-1} CXCL7 (A) or MMP3 (B) standards (dark bars). Error bars were estimated as triple of the standard deviation of three replicates.

according to the $3 s_b/m$ criterion, where s_b was the standard deviation of the amperometric measurements ($n = 10$) carried out for the lowest concentration in the linear calibration plot, and m was the slope value of the corresponding calibration graph. The calculated values were 0.3 ng mL^{-1} CXCL7 and 0.8 pg mL^{-1} MMP3. Furthermore, the limits of quantification (LOQs) calculated as $10 s_b/m$ were 0.5 ng mL^{-1} CXCL7 and 1.1 pg mL^{-1} MMP3.

The analytical characteristics achieved with the dual immunosensor can be advantageously compared with other immunoassay techniques. In the particular case of MMP3, the linear calibration range is wider than those claimed for ELISA kits and the lowest measurable concentration is about thirty times smaller than that reported for R&D Systems [16] using the same bioreagents, and about twenty times smaller than that claimed with Abcam [17]. Moreover, the semi-logarithmic relationship observed between the measured current and the target analyte concentration is defined by a straight line instead of a curve. In addition, the calculated LOD value is about 3.5 times lower than that from Abcam's ELISA kit [17]. In respect to the comparison with the only immunosensor found in the literature for the determination of MMP3 [18], the analytical characteristics of the dual immunosensor are also better even when the immunosensor reported by Munge et al. used an amplification strategy. In fact, the LOD value reached with the dual immunosensor is more than three hundred times smaller, the calibration range extends through a wider concentration range and exhibits a slope value about 4 times larger.

Regarding CXCL7, the linear range achieved with the dual immunosensor extended at the ng mL^{-1} level, which is that required for the analysis of serum with no need for excessive dilutions. Moreover, a sample volume of $25 \mu\text{L}$, four times smaller than that required in the ELISA kits, and an assay time of $2 \text{ h } 50 \text{ min}$ versus almost 5 h counted in both cases after incubation of the capture antibody, are advantageous features of the dual immunosensor.

The intraday reproducibility of the measurements was evaluated by comparing the amperometric responses from different single immunosensors prepared on the same day in the absence and in the presence of 2 ng mL^{-1} of CXCL7 or MMP3. The obtained RSD values ($n = 5$) were 3.1 and 4.8% (CXCL7) or 4.7 and 4.9% (MMP3), respectively. In addition, the interday reproducibility was studied from the responses provided by different single immunosensors prepared on different days in the

absence and in the presence of 2 ng mL^{-1} of CXCL7 or MMP-3. In this case, RSD values ($n = 10$) of 3.7 and 4.0% (CXCL7) or 5.4 and 5.6% (MMP3), respectively, were obtained. These RSD values confirmed the reliability and the reproducibility in the preparation of the developed immunosensors.

The storage stability of cAb1-MBs and cAb2-MBs conjugates was also tested. Different cAb1-MBs/SPCEs and cAb2-MBs/SPCEs were prepared on the same day, stored under wet conditions at 4°C , and used each control day to construct the corresponding individual immunosensor and to measure 2 ng mL^{-1} CXCL7 or MMP3, respectively. The results obtained are displayed in Fig. S2 showing that the amperometric responses of the HRP-Strept-dAb1-CXCL7-cAb1-MBs/SPCE and HRP-Strept-dAb2-MMP3-cAb2-MBs/SPCE immunosensors remained within the control limits, set at ± 2 times the standard deviation of the respective mean current value provided by 10 different immunosensors for each target protein prepared the first day, for at least 25 days.

3.3. Selectivity

The effect of various non-target proteins that can be present in human serum on the amperometric responses of the immunosensors for the determination of CXCL7 and MMP3 was evaluated by comparing the currents measured for 0 and 2 ng mL^{-1} CXCL7, and 0 and 2 ng mL^{-1} MMP3 in the absence and in the presence of the tested compound at the concentration level expected in this type of clinical sample. Fig. 5 clearly shows that only very slight differences in the measured current values occurred in the presence of the potentially interfering biomolecule. In fact, in all cases, both specific and unspecific responses remained within the control limits set at ± 2 times the standard deviation of the corresponding mean current value ($n = 10$). Moreover, no significant differences in the calculated signal-to-noise (S/N) ratios were apparent in any case. These results evidenced that no significant interference occurred in the determination of CXCL7 and MMP3 in the presence of the tested proteins (see foot of Fig. 5) at the concentration levels expected for healthy individuals. The high selectivity observed suggested the high specificity of the antibodies employed for recognition of the target biomarkers. In addition, according to the specifications of the ELISA kits for CXCL7 and MMP3, the used immunoreagents do not exhibit cross-reactivity or interference in the presence of 50 ng mL^{-1} of

Table 1

Determination of CXCL7 and MMP3 in human serum of healthy individuals and patients diagnosed with RA using the developed dual immunosensor and single ELISA kits.

Group	Sample	CXCL7, ng mL ⁻¹		MMP3, ng mL ⁻¹	
		Immunosensor ^a	ELISA ^b	Immunosensor ^a	ELISA ^b
Healthy	8097	843 ± 70	832 ± 81	2.0 ± 0.5	2.0 ± 0.7
	8118	858 ± 32	860 ± 65	3.1 ± 0.7	3.1 ± 0.8
	8139	823 ± 48	821 ± 73	2.6 ± 0.6	3 ± 1
	8160	883 ± 57	891 ± 58	3.5 ± 0.5	3.4 ± 0.8
RA Patients	4285	1219 ± 47	1226 ± 51	38 ± 4	39 ± 5
	5650	1521 ± 100	1513 ± 72	40 ± 3	40 ± 6
	6499	1328 ± 60	1308 ± 65	15 ± 2	17 ± 4
	6501	1201 ± 69	1195 ± 57	18 ± 2	19 ± 3

Mean values ± ts/√n; α = 0.05

^a n = 4

^b n = 2

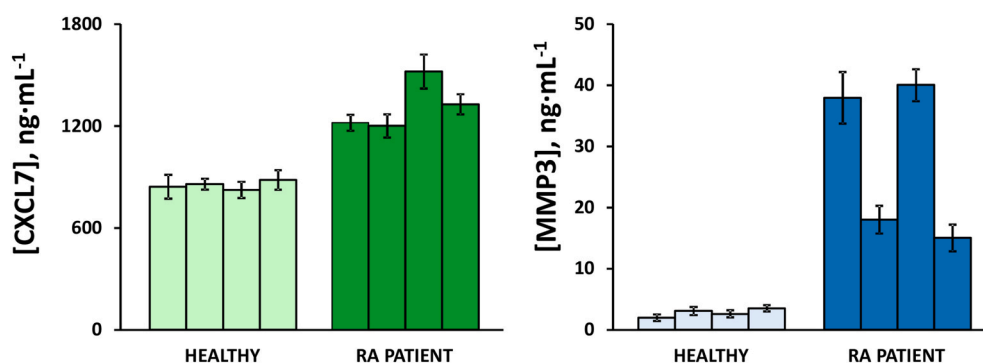


Fig. 6. CXCL7 (left) and MMP3 (right) concentrations in serum of healthy individuals (light coloured bars) and in patients diagnosed with RA (dark coloured bars). Error bars were estimated as triple of the standard deviation of three replicates.

each CXCL1, 2, 3, 5, 8, 9, 10 and 12 (CXCL7), or MMP-1, -2, -7, -8, -9, -12, and -13 (MMP3).

A potential major risk in the preparation of multiplexed biosensors is the possible cross-talking between adjacent working electrodes. To check this, the amperometric responses measured with dual immunosensors for different mixtures of CXCL7 and MMP3 were compared (Fig. S3). As it can be deduced, no significant cross-talking was apparent between the two working electrode surfaces thus endorsing the viability of the dual bioplatfrom for the simultaneous determination of both biomarkers.

3.4. Simultaneous determination of CXCL7 and MMP3 in human serum

The dual immunosensor was applied to the simultaneous determination of both biomarkers in human serum of healthy individuals and patients with rheumatoid arthritis. The possible existence of a matrix effect was checked by constructing calibration plots from aliquots of 1/50 diluted sera to which increasing concentrations of CXCL7 or MMP3 were added. The slope values: 2310 ± 120 nA per decade of concentration (CXCL7), and 296 ± 5 nA per decade of concentration (MMP3) were statistically compared with those of the calibration plots constructed with standard solutions of the biomarkers through the t-Student test. The experimental t values, $t_{\text{exp}} = 2.120$ and 0.657 for CXCL7 and MMP3, respectively, were lower than that tabulated, $t_{\text{tab}} = 2.228$, indicating that no significant matrix effect was apparent with the above-mentioned serum dilution factor. Therefore, the determination of the target biomarkers was carried out by interpolation of the amperometric responses in the diluted serum into the respective calibration plot prepared from CXCL7 and MMP3 standard solutions.

The results obtained with the dual immunosensor are summarized in Table 1 and displayed in Fig. 6. In addition, and for comparison, the results provided by ELISA method using the same immunoreagents are

included in Table 1.

As it can be seen, no significant differences were found between the concentrations obtained with the dual immunosensor and the ELISA method for the two biomarkers, being observed an excellent correlation between both methods as shown by Fig. S4. Moreover, these results were consistent with their expected levels according to the characteristics of the samples (indeed, CXCL7 concentrations in serum of healthy patients were recently reported as 850 ng mL⁻¹ [25]). Furthermore, it is clearly observed that CXCL7 was overexpressed in RA patients with levels approximately 1.5 times larger. Regarding MMP3, the results obtained in the analysis of serum from healthy individuals agreed also with literature data [8]. As expected, this biomarker was also overexpressed in RA patients with a MMP3 concentration between 6 and 14 times larger. A more detailed analysis revealed significant lower MMP3 concentrations for samples 6499 and 6501 in Table 1, which can be attributed to the fact that these samples come from treated patients for which remission of the disease was observed. Conversely, samples 4285 and 5650 were from patients showing swollen joints. Therefore, the determination of MMP3 allows monitoring synovial inflammation providing increased levels in serum, and pathologic processes underlying joint destruction in RA.

4. Conclusions

This paper describes the first electrochemical immunoplatfrom for the simultaneous determination of two relevant biomarkers in rheumatoid arthritis CXCL7 and MMP3. The dual immunosensor involves sandwich - type immunoassays using carboxylated MBs and specific antibodies to capture and detect the target biomarkers as well as amperometric transduction with the H₂O₂/HRP/HQ system. The electrochemical immunosensing platform is sensitive, with LODs of 0.3 ng mL⁻¹ and 0.8 pg mL⁻¹ for CXCL7 and MMP3, respectively, and highly

selective, this allowing the simultaneous determination of the target biomarkers in human serum using a simple protocol in shorter assay time (2 h 50 min) and requiring four times smaller volume of sample than ELISA methods. In this regard, it should be noted that this is the first time that an immunosensor has been used to determine CXCL7 and MMP3 in human serum from healthy individuals and RA patients, showing the results obtained the usefulness and efficiency of the dual developed electrochemical platform for clinical practice.

Credit author statement

Sara Guerrero: Conceptualization, Investigation, Writing, Review & Editing - original draft. **Lourdes Agüí:** Conceptualization, Investigation, Writing, Review & Editing - original draft. **Esther Sánchez-Tirado:** Conceptualization, Investigation, Supervision, Writing, Review & Editing - original draft. **Araceli González-Cortés:** Conceptualization, Investigation, Supervision, Writing, Review & Editing - original draft. And Funding acquisition. **Paloma Yáñez-Sedeño:** Conceptualization, Supervision, Writing, Review & Editing - original draft and Funding acquisition. **José M. Pingarrón:** Writing, Review & Editing - original draft and Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2021.122705>.

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SUPPORTING INFORMATION

Simultaneous determination of CXCL7 chemokine and MMP3 metalloproteinase as biomarkers for rheumatoid arthritis

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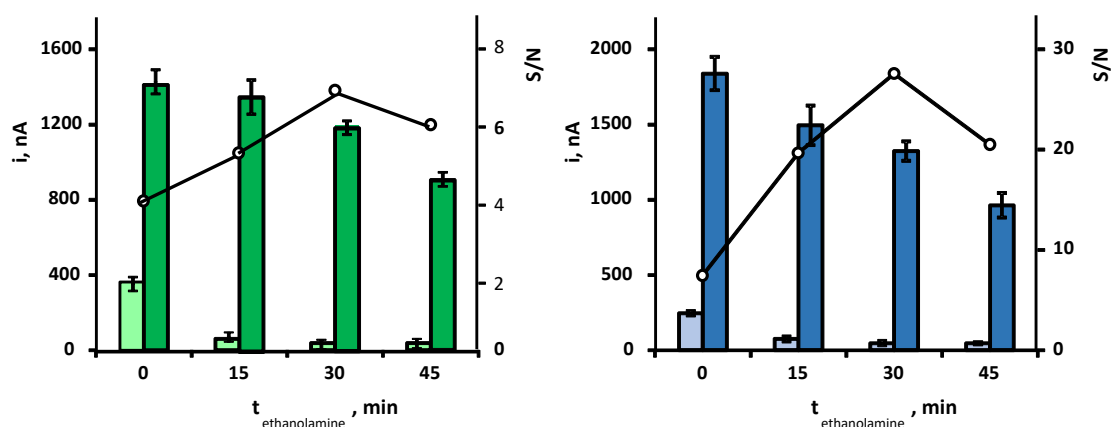


Figure S1.- Effect of the incubation time in the blocking step with 1 M ethanolamine in 0.1 M PB of pH 8.0 on the amperometric current of CXCL7 (green) or MMP3 (blue) immunosensors. Error bars were estimated as triple of the standard deviation of three replicates.

Table S1.- Optimization of the experimental variables affecting the amperometric response of the dual immunosensor for the determination of CXCL7 and MMP3

Variable	CXCL7		MMP3	
	Tested range	Selected value	Tested range	Selected value
cAb loading, $\mu\text{g mL}^{-1}$	2.5 - 25	10	5 - 20	15
Incubation time cAb, min	30 - 75	45	30 - 75	45
Biotin-dAb loading, $\mu\text{g mL}^{-1}$	0.05 - 0.5	0.25	0.5 - 2	1
Incubation time Biotin-Ab, min	30 - 90	60	30 - 90	60
HRP-Strept, dilution	1/500-1/4000	1/2000	1/250-1/1500	1/500
Incubation time, ethanolamine, min	0 - 45	30	0 - 45	30

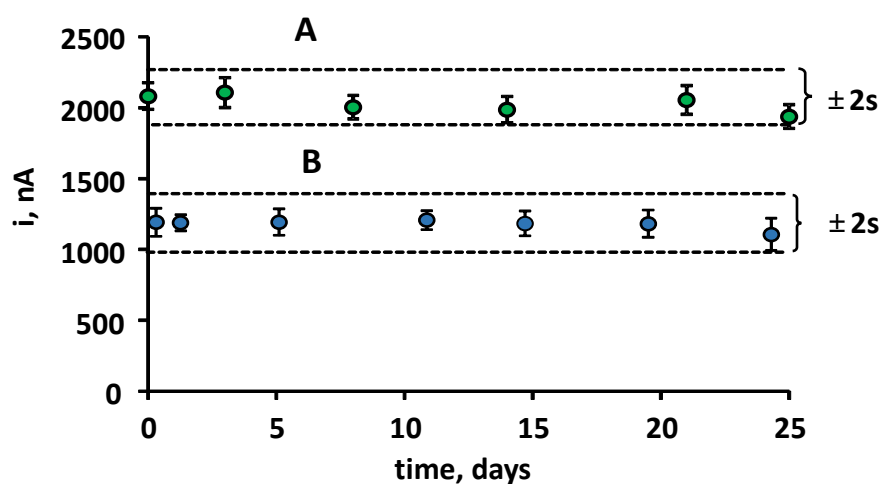


Figure S2.- Amperometric measurements obtained with individual immunosensors prepared with stored (under wet conditions at 4°C) cAb1-MBs (A) and cAb2-MBs (B), for 2 ng mL⁻¹ CXCL7 (A) and MMP3 (B) standards. Error bars estimated as triple of standard deviation of three replicates.

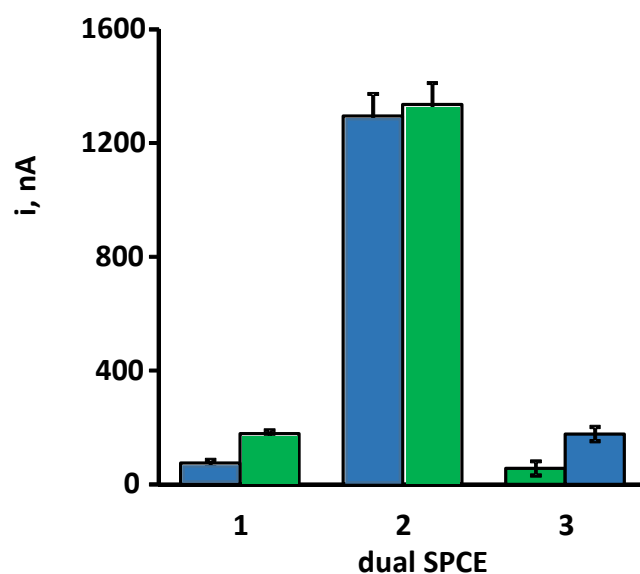


Figure S3.- Simultaneous amperometric responses measured with the HRP-Strept-Biotin-dAb1-CXCL7-cAb1-MBs/SPCdE (W1, CXCL7, green) and HRP-Strept-Biotin-dAb2-MMP3-cAb2-MBs/SPCdE (W2, MMP3, blue) for: (1) 0 ng mL⁻¹ CXCL7 (W1), 0 ng mL⁻¹ MMP3 (W2); (2) 2 ng mL⁻¹ CXCL7 (W1), 2 ng mL⁻¹ MMP3 (W2); (3) 2 ng mL⁻¹ MMP3 (W1), 2 ng mL⁻¹ CXCL7 (W2). Error bars were estimated as triple of the standard deviation of three replicates.

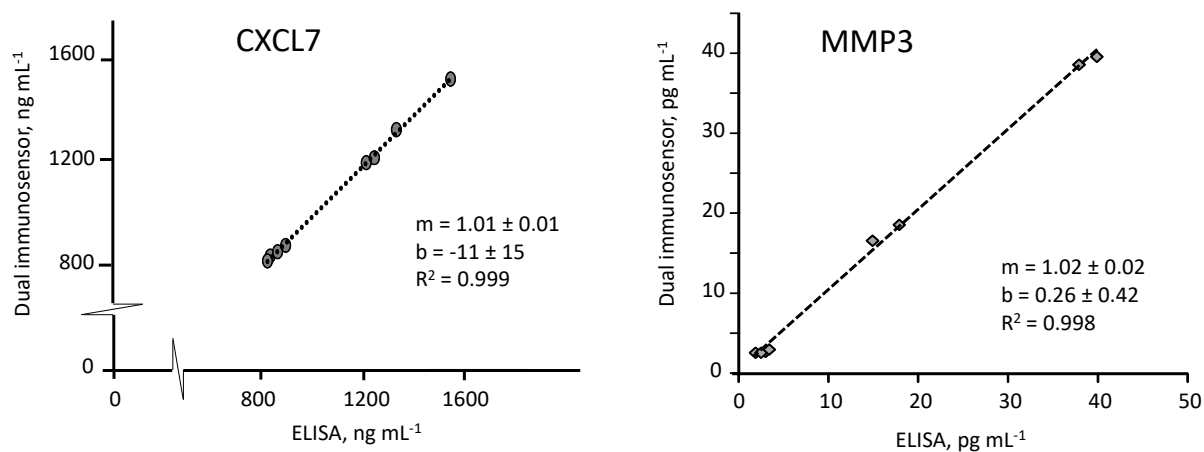
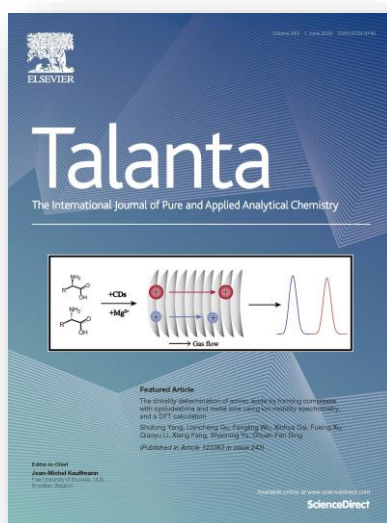
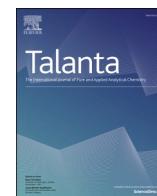


Figure S4. Correlation between the concentration of CXCL7 and MMP3 in human serum samples obtained with the developed dual immunoplatfrom and with individual ELISA kits involving the same immunoreagents (data summarized in Table 1).



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Monitoring autoimmune diseases by bioelectrochemical detection of autoantibodies.
Application to the determination of anti-myelin basic protein autoantibodies in serum of
multiple sclerosis patients



Monitoring autoimmune diseases by bioelectrochemical detection of autoantibodies. Application to the determination of anti-myelin basic protein autoantibodies in serum of multiple sclerosis patients

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ABSTRACT

This work reports an amperometric bioplatfrom for the determination of anti-myelin basic protein autoantibodies (anti-MBP), a relevant biomarker for multiple sclerosis (MS) autoimmune disease. The developed configuration involves the use of carboxylated magnetic microparticles (cMBs) where the protein for specific capture of the target autoantibodies was covalently attached. The immobilized anti-MBP were further conjugated with a secondary antibody labelled with horseradish peroxidase (HRP-anti-hIgG) and amperometric transduction was performed by adding hydrogen peroxide and using hydroquinone (HQ) as redox mediator. The cathodic current resulting from the reduction of the corresponding quinone was directly proportional to the logarithmic concentration of the target autoantibodies. The analytical performance of the developed method for the determination of anti-MBP is competitive in terms of sensitivity and range of linearity with that claimed for the only biosensor reported so far in the literature, as well as with commercially available ELISA kits showing a remarkably shorter assay time. The bioplatfrom was applied to the analysis of serum samples of healthy individuals and patients diagnosed with MS providing results in agreement with the ELISA methodology.

1. Introduction

Autoantibodies produced by the immune system act against our own cells or tissues as a first response to infections or as an indication of pathological processes. The presence of antibodies in serum can predict some illnesses well in advance of clinical symptoms or inform on the evolution of diseases. Especially important is the detection of autoantibodies in the field of autoimmune disorders where the evaluation of their type and concentration is currently used to assess the risk and progression of such disorders and to help in the finding of therapies to prevent or mitigate their impact on patients [1]. Some examples of autoantibodies associated to autoimmune diseases are anti-cyclic citrullinated peptide, rheumatoid factor, anti-aquaporin-4, or anti-nuclear antigens. Although the role of autoantibodies in the appearance and course of these diseases is not thoroughly known, however, their use as serum biomarkers constitutes a minimally invasive and relatively low-cost alternative compared to other methods of diagnosis requiring invasive protocols, highly qualified personnel and sophisticated instruments [2].

Systemic autoimmune diseases are a broad group of related illnesses where dysregulation of the immune system leads to the activation of immune cells to attack autoantigens resulting in inflammation and a variety of tissue damages [3]. Examples are rheumatoid arthritis (RA), systemic sclerosis (SLE), Sjögren's syndrome and multiple sclerosis (MS). Focusing on MS, this chronic inflammatory and neuro-degenerative autoimmune disease results in damage of myelin sheaths in the central nervous system (CNS). It is characterized by relapses, remissions and progression of disability over time [4] leading to inhibited muscle coordination and loss of cognitive functions among other neurological effects [5,6]. Therapeutic treatments can defer the course and symptoms of the disease if used at an early stage. Current diagnosis of MS relies on clinical examination supported by laboratory investigations including magnetic resonance imaging (MRI) to visualize lesions. Conversely with these time consuming and expensive methods, rapid monitoring of biomarkers is a useful tool in the diagnostic process. Particularly, autoantibodies play an important role in MS pathogenesis, and their detection can help to evaluate the disease status of patients and to predict the response to a specific therapy [7]. As demyelination is a

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key component of the MS disease progress, autoantibodies against myelin antigens have long been considered as the primary targets of the humoral autoimmune response in MS. In fact, anti-myelin basic protein (anti-MBP) emerging in MS patient's sera and cerebrospinal fluid (CSF) has been regarded as a biomarker of the disease progression [8]. However, despite the importance of this biomarker, the autoimmune response in MS is more complex and not only restricted to myelin but much more widespread thorough the brain [9].

Various ELISA kits for the determination of anti-MBP in serum are commercially available where the immunoassays are based on sandwich-type configurations. Most of them provide calibration plots with concentrations ranging between 3.12 and 200 ng mL⁻¹ anti-MBP, with minimum detectable dose (MDD), defined as the lowest concentration that differs from zero, of around 1 ng mL⁻¹ anti-MBP, and take assay times between 120 and 150 min (see as example [<http://www.cloud-clone.com/products/AEA539Hu.html>]). These immunoassays claim intra- and inter-assay precision levels estimated as variation coefficients (CVs) between 10 and 12%. Only the ELISA kit by MyBioSource [www.mybiosource.com/human-elisa-kits/myelin-basic-protein-autoantibody-anti-mbp/9398507] achieves ten-fold lower detection limit (0.1 ng mL⁻¹) and a calibration plot ranging between 0.25 and 8 ng mL⁻¹ anti-MBP. In this case, the assay time is shorter (80 min), but the CV, around 15%, is slightly worse.

Regarding immunosensors, only a label-free impedimetric biosensor for anti-MBP has been reported [10]. It consisted of a platinum electrode with MBP antigen immobilized on either gelatin or gelatin-TiO₂ nanoparticles. The impedimetric method involved measurements of relative impedance changes, provided calibration plots with two linear ranges extending up to 2500 ng mL⁻¹ and a detection limit of 0.15 ng mL⁻¹. In this context, electrochemical biosensors are a good alternative for the determination of anti-MBP due to their high sensitivity, simplicity of use, low cost, and miniaturization capacity which makes them ideal tools for point of care detection. Recently, our group has reported biosensors for the determination of anti-dsDNA autoantibodies [11], antibodies against aquaporin-4 [12], and for the simultaneous determination of rheumatoid factor and anti-cyclic citrullinated peptide antibodies [13].

In this paper, we report the first magnetic microbeads (MBs)-based bioplatfrom for the determination of anti-MBP autoantibodies. The developed strategy involves the use of carboxylated MBs (cMBs) as solid substrate to carry out the covalent immobilization of MBP followed by the specific capture of target antibodies against this protein. In this design, the properties of MBs, improving the biomarker isolation and preconcentration from the clinical samples thus minimizing unspecific adsorptions, are exploited. The anti-MBP-MBP-MBs immunocomplexes were prepared in homogeneous solution through a single incubation step and then labelled with a secondary antibody conjugated to the horseradish peroxidase enzyme (HRP-anti-hIgG). The resulting HRP-anti-hIgG-anti-MBP-MBP-MBs magnetic bioconjugates were magnetically captured on the working surface of a screen-printed carbon electrode (SPCE). This protocol avoids the need for electrode surface modification by applying several steps to achieve an efficient and stable incorporation of the recognition element and preparation of the immunoconjugate. The electrochemical detection was performed amperometrically at a potential of -0.2 V vs Ag pseudo-reference electrode by addition of H₂O₂ as the enzyme substrate using hydroquinone (HQ) as the redox mediator. Thus, the electrochemical reduction of the quinone generated in the enzyme reaction was measured to monitor the immunorecognition reaction, the cathodic current variation resulting directly proportional to the logarithm of anti-MBP concentration in a range between 0.05 and 50 ng mL⁻¹. The achieved LOD value was 0.016 ng mL⁻¹ which is ten times lower than that claimed for the impedimetric immunosensor [10]. The developed bioplatfrom was successfully applied to the determination of anti-MBP in serum of healthy individuals and patients diagnosed with MS autoimmune disease.

2. Experimental

2.1. Apparatus and electrodes

A magnetic concentrator DynaMag-2 (Cat. No. 12321D, Dynabeads®, Invitrogen-ThermoFisher Scientific), a Bunsen AGT-9 Vortex, a Thermo-Shaker MT100 (Universal Labortechnik), a P-Selecta (Cencom) centrifuge, a Crison model Basic 20+ pH-meter, an Elmasonic S-60 (Elma) ultrasonic bath and a magnetic stirrer MS01 (ELMI, Ltd.) were employed. All the amperometric measurements were made at room temperature using a model CHI812B (CH Instruments, Inc.) potentiostat controlled by the CHI812B software. Screen-printed carbon electrodes (SPCEs, DRP110, 4-mm Ø) and a specific cable connector (DRP-CAC) from Metrohm-DropSens were used. The measurements were performed in stirring solutions using 10 mL glass electrochemical cells from Pobel, where the SPCEs placed on a homemade polymethylmethacrylate (PMMA) casing with a neodymium magnet (AIMAN GZ) embedded, were immersed. ELISA absorbance readings were made in a Sunrise™ Tecan microplate reader with the Magellan V 7.1 software.

2.2. Reagents and solutions

Human eukaryotic myelin basic protein (MBP) from Cloud-Clone Corp. (Ref. EPA539Hu61) reconstituted in 20 mM Tris +150 mM NaCl buffer of pH 8.0 was used. Human monoclonal antibody to MBP (anti-MBP) from Cloud-Clone Corp (Ref. MAA539Hu28) and HRP-labelled goat anti-human IgG (HRP-anti-hIgG) from Abcam (Ref. ab97225) were also used. Carboxyl-functionalized magnetic microbeads (cMBs, 2.8 µm, 10 mg mL⁻¹, Dynabeads® M - 270 carboxylic acid) were purchased from Dynal Biotech ASA (Thermo Fisher Scientific). N-(3-dimethyl-aminopropyl)-N'-ethyl-carbo-diimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), ethanolamine, hydroquinone (HQ) and hydrogen peroxide (H₂O₂) (30% v/v) were provided from Sigma-Aldrich.

Ascorbic acid (AA, Sigma-Aldrich), uric acid (UA, Sigma-Aldrich), anti-cyclic citrullinated peptide antibody (CCPA, Demeditec DE7760 ELISA kit), endoglin (CD105, R&D Systems Inc. DY1097 ELISA kit), chemokine (C-X-C motif) ligand 7 (CXCL7, R&D Systems Inc. DY393 ELISA kit), platelet-factor 4 chemokine (CXCL4, R&D Systems Inc. DY795 ELISA kit), hemoglobin (Hb, Sigma-Aldrich, Cat. No. H7379), human immunoglobulin (hIgG, Sigma-Aldrich, Ref. I2511), human serum albumin (HSA, Sigma-Aldrich, Cat. No. A1653), matrix metalloproteinase 3 (MMP-3), rheumatoid factor (RF, Abcam 178,653 ELISA kit), and ligand receptor activator nuclear factor-κB (RANKL, R&D Systems Inc. DY626 ELISA kit) were tested as potential interfering compounds.

Buffer solutions were: a 25 mM MES of pH 5.0 prepared from 2-(N-morpholine) ethane-sulfonic acid (Scharlab), a 50 mM potassium phosphate buffer of pH 6.0 (PB), and PBS, consisting of a 100 mM sodium phosphate buffer of pH 7.4 supplemented with 0.0920 g NaCl (Labkem) and 0.0020 g KCl (Scharlab) in 100 mL deionized water. Deionized water was from a Millipore Milli-Q purification system (18.2 MΩ cm).

Sera from healthy and MS patients were purchased from Central BioHub® Specimens (Central BioHub GmbH, Hennigsdorf, Germany). All samples were stored at -80 °C until use.

2.3. Preparation of the magnetic bioconjugates

The steps involved in the preparation of bioconjugates on the MBs (Fig. 1) were performed in 1.5 mL microcentrifuge tubes using the magnetic concentrator and by placing the tubes during 2 min for removing the supernatants. 3-µL aliquots of the carboxylated magnetic particles suspension were washed twice with 50 µL of 25 mM MES buffer solution of pH 5.0 and incubated for 10 min at 25 °C with constant shaking at 600 rpm. Then, the particles were separated, the supernatant

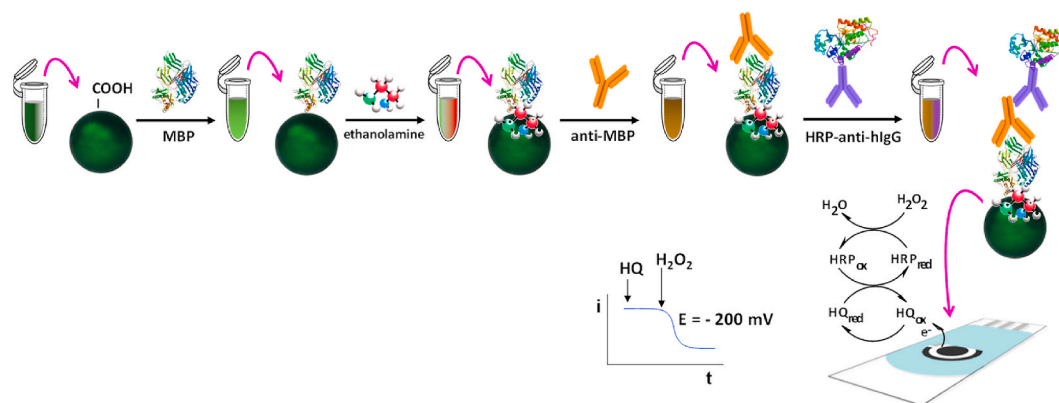


Fig. 1. Schematic display of the different steps involved in the construction of the magnetic MBs based immunosensor for anti-MBP involving MBP immobilization onto cMBs and specific conjugation with the target antibody followed by HRP-anti-hIgG conjugation and amperometric detection in the presence of H_2O_2 /HQ system.

was discarded, and 25 μL of a 50 mg mL^{-1} solution of EDC/NHSS in 25 mM MES buffer solution of pH 5.0 were added for activation of the carboxylic groups, allowing incubation for 30 min. Upon washing twice with 50 μL of 25 mM MES buffer solution of pH 5.0, 25 μL of a 20 $\mu\text{g mL}^{-1}$ MBP solution prepared in the 25 mM MES buffer of pH 5.0, were added and incubating for 45 min at 25 $^\circ\text{C}$ by shaking at 600 rpm. The resulting magnetic bioconjugates were washed twice with 50 μL of 25 mM MES buffer solution of pH 5.0. Thereafter, blocking of the possible free remaining reactive groups was made by incubation of the MBP-MBs conjugates with 50 μL of a 1 M ethanolamine solution for 60 min at 25 $^\circ\text{C}$. The excess of blocking solution was eliminated, a washing step with 50 μL of 100 mM PBS pH 7.4 was performed, and 25 μL of the anti-MBP standard solutions or the sample were added and incubated for 60 min at 25 $^\circ\text{C}$. After washing with the same buffer, 25 μL of 0.5 $\mu\text{g mL}^{-1}$ HRP-anti-hIgG in 100 mM PBS of pH 7.4 were added and incubated for 15 min at 25 $^\circ\text{C}$, shaking at 600 rpm and washed again as in the previous step.

2.4. Amperometric detection

Once the SPCE was placed on the homemade PMMA casing provided with the embedded neodymium magnet, the HRP-anti-hIgG-anti-MBP-MBP-MBs suspension (50 μL) were cast onto the working electrode surface. Then, the potentiostat was connected, the casing-electrode assembly immersed in the electrochemical cell containing 10 mL of 50 mmol L^{-1} PB (pH 6.0) and 100 μL of freshly prepared 0.1 mol L^{-1} HQ solution, and a constant potential of -0.20 V vs. Ag pseudo-reference electrode was applied. The current was recorded under stirring conditions. Upon stabilization of the background current (approximately 100 s), 50 μL of a 0.1 mol L^{-1} H_2O_2 solution freshly prepared in PB, pH 6.0 were added to the cell, and the cathodic current due to the electrochemical reduction of the quinone formed in the enzymatic reduction of H_2O_2 mediated by HQ was recorded until the steady state was reached. Amperometric signals were calculated as the difference between such steady state currents and the background currents. The given values are the mean of three replicates and the error bars were estimated as three times the standard deviation of each set of replicates ($\alpha = 0.05$).

3. Results and discussion

The fundamentals of the biosensor and the electrochemical reactions involved in the determination of anti-MBP are depicted in Fig. 1. As described in the Experimental section, the covalent immobilization of MBP onto activated cMBs was followed by blocking with ethanolamine and the selective capture of the target antibody further conjugated with a HRP-labelled secondary antibody (HRP-anti-hIgG). Subsequently, the MBs bearing the bioconjugates were magnetically captured on the SPCE

previously placed on the magnet-holding block. The ensemble was transferred to the electrochemical cell where the biorecognition events were amperometrically monitored. As it is schematically illustrated, the detection mechanism involves the enzymatic reduction of H_2O_2 by HRP and the regeneration of the reduced HRP by HQ. The oxidized form of the mediator, benzoquinone (BQ), is reduced at the applied constant potential (-0.20 V vs. Ag pseudo-reference electrode) on the electrode.

3.1. Optimization of the experimental variables

The different variables affecting the analytical performance of the HRP-anti-hIgG-anti-MBP-MBP-MBs/SPCE immunosensor were optimized. Larger S/N ratios between the currents measured in the presence (S) of 1 ng mL^{-1} anti-MBP and in the absence (N) of the target antibody were taken as the selection criterion for each tested variable, which were loading and time for incubation of MBP protein onto activated HOOC-MBs, and loading and time for incubation of HRP-anti-hIgG onto anti-MBP-MBP-MBs. As will be commented on later, other experimental variables such as the amount of MBs, the type of blocking agent, working pH, detection potential and concentration of H_2O_2 and HQ for the amperometric measurements were the same that those recommended in protocols for application of the materials used and/or optimized in previous works [14,15]. The results of these studies are shown in Fig. 2 (a–d) and summarized in Table 1.

Fig. 2a shows the results obtained in the optimization of the MBP loading on the previously activated cMBs. The specific amperometric current increased with the MBP loading over the 10–20 $\mu\text{g mL}^{-1}$ range and decreased for larger loading probably as consequence of the sterically hindered binding of the immobilized MBP with a large amount of anti-MBP. Furthermore, the unspecific responses (white bars), obtained in absence of MBP, remained practically constant over the whole tested range. Accordingly, a value of 20 $\mu\text{g mL}^{-1}$ MBP was selected for further work. Fig. 2b shows as both the specific and unspecific responses did not vary significantly over the 30–75 min range of incubation times tested. Nevertheless, a slightly larger S/N ratio was found for 45 min which was then selected. The effect of HRP-anti-hIgG loading conjugated onto anti-MBP-MBP-MBs on the biosensor response was checked over the 0.25–1.0 $\mu\text{g mL}^{-1}$ range (Fig. 2c). As expected, both the specific and unspecific responses increased with the amount of enzyme-labelled secondary antibody. As the increase of the response in the absence of anti-MBP was much smaller, a larger higher S/N ratio was observed for 0.50 $\mu\text{g mL}^{-1}$, which was chosen for further studies. Regarding the incubation time for HRP-anti-hIgG (Figs. 2d), 15 min were sufficient to achieve a practically constant specific signal. Due to the slight increase in the unspecific signal, a larger S/N ratio was reached for 15 min.

In addition, the need for a blocking step to quench the non-reacted activated carboxylic acid groups was evaluated. Ethanolamine, as the

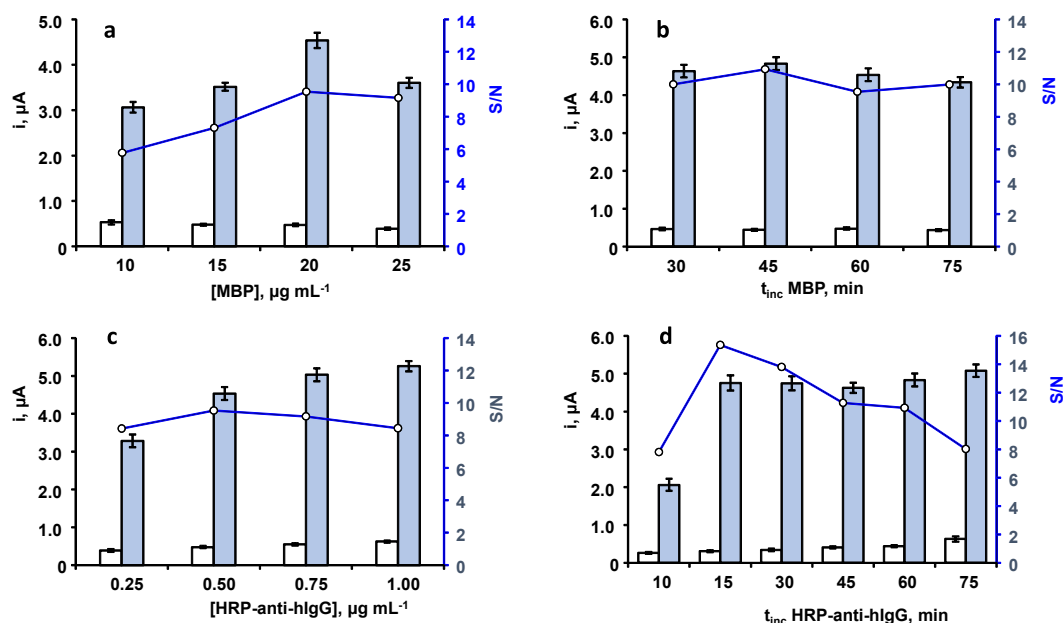


Fig. 2. Effect of the MBP loading (a) and incubation time (b) onto MBs, and of the amperometric responses obtained with the developed bioelectrochemical platform. 3 μL cMBs; 25 μL 50 $\mu\text{g mL}^{-1}$ EDC/NHSS, 30 min; 25 μL 1 M ethanolamine, 60 min; 25 μL 0 (white) or 1 (blue) ng mL^{-1} anti-MBP, 60 min; 25 μL MBP, 10–25 $\mu\text{g mL}^{-1}$ (a); 20 $\mu\text{g mL}^{-1}$ (b–d), 60 min (a,c), 30–75 min (b), 45 min (d); 25 μL 0.5 $\mu\text{g mL}^{-1}$ HRP-anti-hIgG (a,b) 60 min; 0.25–1.0 $\mu\text{g mL}^{-1}$ (c), 10–75 min (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Optimization of the experimental variables involved in the preparation of the HRP-anti-hIgG-anti-MBP-MBP-MBs bioconjugates used for the amperometric determination of anti-MBP.

Variable	Tested range	Selected value
MBP, $\mu\text{g mL}^{-1}$	10–25	20
MBP incubation time, min	30–75	45
HRP-anti-hIgG, $\mu\text{g mL}^{-1}$	0.25–1.0	0.5
HRP-anti-hIgG incubation time, min	10–75	15
Number of incubation steps	1–2	2

blocker agent recommended in the protocol for Dynabeads® M – 270 carboxylic acid, was utilized for such purpose by incubating the MBP-MBs in 1 M ethanolamine for 60 min. The obtained results (not shown) revealed that the S/N ratio was practically twice larger when the remaining active sites of the MBP-MBs were blocked, due to the clear decrease in the nonspecific response using blocked MBs as well as to the slight increase in the specific signal in the presence of anti-MBP. Regarding the reagents and pH used for the electrochemical detection, these variables were optimized previously [14,15]. Additions of H_2O_2 used as the enzyme substrate at pH 6.0 provided larger amperometric responses at a detection potential of -0.2 V (vs Ag pseudo-reference electrode) in the presence of HQ as the redox mediator. This common electroactive co-substrate was selected because of its fast enzymatic oxidation and the facile electrochemical reduction of the corresponding quinone, which is the oxidized form produced in the enzyme reaction. Furthermore, with the aim of simplifying the protocol involved in the MBs modification and thus reducing the assay time, the number of incubation steps for the preparation of the MBs conjugates was also checked. The different tested procedures implied independent incubations in 25 μL of 1 ng mL^{-1} anti-MBP followed by incubation in 25 μL of 0.5 $\mu\text{g mL}^{-1}$ HRP-anti-hIgG solution, or a single incubation step in a mixture solution containing 25 μL of 2 ng mL^{-1} anti-MBP and 25 μL of 1.0 $\mu\text{g mL}^{-1}$ HRP-anti-hIgG. In this latter protocol, the mixture solution was previously pre-incubated in the Eppendorf microtube, and then it was incubated with the MBs for 60 min. The obtained results (not shown) revealed that the S/N ratio was more than twice larger when

anti-MBP and HRP-anti-hIgG were immobilized in different steps probably due to the less efficient capture of the target anti-MBPs when they are previously conjugated with the labelled secondary antibody.

3.2. Analytical characteristics for the determination of anti-MBP

Fig. 3 shows as the calibration plot for anti-MBP standards exhibits a linear dependence between the measured cathodic current and the logarithm of antibody concentration over the 0.05–50 ng mL^{-1} ($R^2 = 0.997$) range, following the equation: i , $\mu\text{A} = (3.19 \pm 0.07) \log ([\text{anti-MBP}], \text{ng mL}^{-1}) + (4.54 \pm 0.06)$. The limits of detection and determination were calculated according to the $3\text{sb}/m$ and $10\text{sb}/m$ criteria, respectively, where sb is the standard deviation of 10 amperometric measurements obtained for 0.0 ng mL^{-1} anti-MBP and m the slope of the calibration plot. The calculated values were 0.016 and 0.054 ng mL^{-1} , respectively. When compared with the analytical characteristics claimed

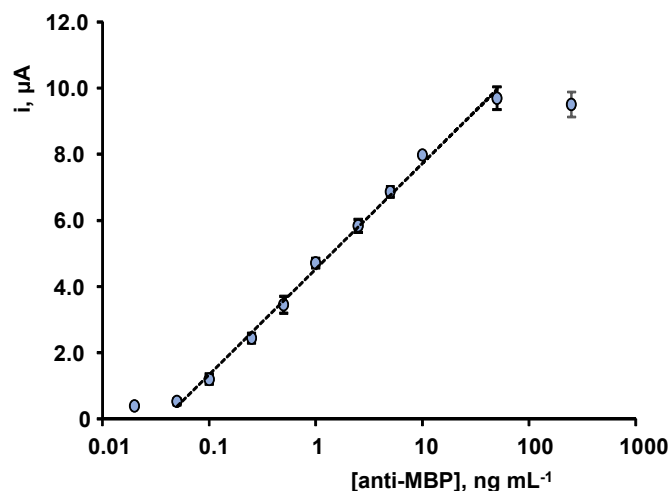


Fig. 3. Calibration plot for anti-MBP constructed with the HRP-anti-hIgG-anti-MBP-MBP-MBs/SPCE biosensor.

for commercial ELISA kits for the determination of anti-MBP, it can be deduced that the bioplatfrom is competitive in terms of sensitivity and dynamic range of calibration. In fact, the LOD value is 62.5 times smaller than the LLD (lower limit of detection) of the kit that uses the same reagents [http://www.cloud-clone.com/manual/ELISA-Kit-for-Anti-Myelin-Basic-Protein-Anti-body-Anti-MBP-E99509Hu.pdf], 1 ng mL^{-1} . Moreover, the LOD value is one order of magnitude lower than that reported for the only biosensor for anti-MBP reported in the literature (0.15 ng mL^{-1}) [10]. In addition, the developed biosensor exhibits a wider dynamic range for the calibration plot than that obtained with the ELISA kit, and starts at a concentration level more than sixty times lower ($0.05\text{--}50 \text{ ng mL}^{-1}$ vs. $3.12\text{--}200 \text{ ng mL}^{-1}$). Regarding the assay time, the developed bioplatfrom requires 1 h 15 min from the prepared MBP-MBs vs. 150 min needed for the ELISA kit. This analytical performance demonstrates the suitability of the biosensor for the rapid determination of anti-MBP in clinical samples containing small concentrations of the autoantibody.

The reproducibility of the amperometric responses obtained in the absence and in the presence of 1 ng mL^{-1} anti-MBP was tested using five different biosensors prepared in the same day. The relative standard deviation (RSD) values calculated were 4.5% and 4.8%, respectively. Moreover, the RSD values calculated using biosensors prepared in different days were 4.6% and 5.3%, respectively. These results indicated a good reproducibility of the whole experimental procedure involving the preparation of the bioconjugates on the MBs, their magnetic capture onto SPCE, and the amperometric transduction. These RSD values improve those claimed for the ELISA kit, for which precision values for intra-assay and inter-assay of CV < 10% or 12%, respectively, are reported.

The storage stability of the MBP-MBs conjugates (stored at 8°C in Eppendorf tubes containing $25 \mu\text{L}$ of 100 mM PBS of pH 7.4) was tested by preparing various immunosensors which were used for measuring the amperometric responses for 1 ng mL^{-1} anti-MBP in different days once the blocking step and the incubation with HRP-anti-hIgG conjugate were carried out. The results obtained (not shown) revealed that the initial amperometric response maintained within the control limits set at ± 3 times the standard deviation of three measurements carried out on the first day, for at least fifteen days (no longer times were tested) after the MBP-MBs storage. This good storage stability improves the results reported for the impedimetric biosensor for anti-MBP where the response decayed to around 75% of the initial response after 10 days due to the deformation of the modifier-film structure [10].

3.3. Selectivity

Various proteins, endoglin (CD105), chemokine (C-X-C motif) ligand 7 (CXCL7), platelet-factor 4 chemokine (CXCL4), hemoglobin (HB), human immunoglobulin (hIgG), human serum albumin (HSA), matrix

metalloproteinase 3 (MMP-3), ligand receptor activator nuclear factor- κB (RANKL), as well as other autoantibodies such as anti-cyclic citrullinated peptide antibody (CCPA), and rheumatoid factor (RF), and other compounds such as ascorbic acid (AA) and uric acid (UA), which can be present in samples containing anti-MBP, were tested as potential interfering substances for the determination of the target autoantibody with the developed immunosensor. To evaluate the effect of these substances on the anti-MBP determination, the amperometric responses for 0 and 1 ng mL^{-1} anti-MBP in the absence and in the presence of each tested compound at a concentration corresponding approximately to the normal level in serum of healthy individuals. Fig. 4 shows that no significantly different currents were measured in all cases with the mean values within the $\pm 3 \times$ standard deviation range of the current measured in the absence of interferent.

3.4. Determination of anti-MBP in serum samples

The developed bioplatfroms were applied to the analysis of serum samples from healthy individuals and from patients diagnosed with MS obtained from Central Biohub® Biospecimens. To test the potential existence of matrix effect, the slope value corresponding to the calibration plots constructed with standards, $3.19 \pm 0.07 \mu\text{A}$ per decade of autoantibody concentration, was compared with the slope value of the calibration graph constructed in 10-fold diluted sera over the $0.5\text{--}3.0 \text{ ng mL}^{-1}$ anti-MBP concentration range, $3.32 \pm 0.02 \mu\text{A}$ per decade of concentration. The statistical comparison of both slope values according to the t -test, $t_{\text{exp}} = 1.808 < t_{\text{tab}} = 2.262$ ($\alpha = 0.05$), indicated the absence of matrix effect under the mentioned conditions. Therefore, the concentration of anti-MB was quantified in the diluted serum by means of a simple interpolation of the responses obtained for the sera into the calibration graph prepared with standards. Importantly, the serum samples did not require any other treatment except the ten-fold dilution

Table 2
Determination of anti-MBP in serum of healthy individuals and MS patients using the HRP-anti-hIgG-anti-MBP-MBP-MBs/SPCE immunosensor.

Sample	Reference	Immunosensor, ng mL^{-1} ($n = 4$) ^a	ELISA kit, ng mL^{-1} ($n = 4$) ^a
Healthy individual	S468087	0.50 ± 0.07	0.48 ± 0.05
Healthy individual	S468160	0.54 ± 0.04	0.51 ± 0.03
MS patient	EM338745	2.71 ± 0.02	2.7 ± 0.1
MS patient	EM337913	2.05 ± 0.06	2.0 ± 0.1
MS patient	EM395952	2.44 ± 0.04	2.42 ± 0.06
MS patient	EM354878	2.43 ± 0.05	2.38 ± 0.05
MS patient	EM398619	1.67 ± 0.04	1.7 ± 0.1

^a Mean value $\pm ts/\sqrt{n}$, $\alpha = 0.05$.

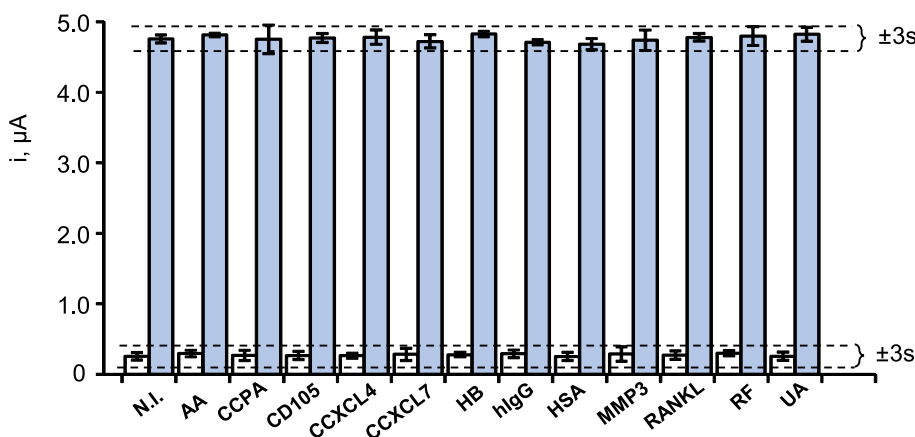


Fig. 4. Effect of the presence of $7 \mu\text{g mL}^{-1}$ AA, 100 IU mL^{-1} CCPA, 70 pg mL^{-1} CD105, 50 ng mL^{-1} CXCL4, 50 ng mL^{-1} CXCL7, 5 mg mL^{-1} HB, 1 mg mL^{-1} hIgG, 5 mg mL^{-1} HSA, 2 ng mL^{-1} MMP-3, 50 pg mL^{-1} RANKL, 100 IU mL^{-1} RF, $100 \mu\text{g mL}^{-1}$ UA on the amperometric responses obtained for 0 (white) or 1 ng mL^{-1} anti-MBP with the HRP-anti-hIgG-anti-MBP-MBP-MBs/SPCE immunosensor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in 100 mM PBS solution of pH 7.4.

Table 2 compares the results obtained with the developed immunosensor with those provided by an ELISA kit. An excellent agreement was found with a great correlation between both sets of data, with the slope and intercept values of the corresponding correlation plot of 0.997 ± 0.008 and -0.02 ± 0.03 respectively ($R^2 = 0.999$). As expected, significantly larger concentrations of anti-MBP were found in serum of patients with MS compared to those of healthy individuals. Moreover, these results agree with those reported in the literature on the hyperexpression of the autoantibody in these samples [10].

4. Conclusions

In this work, we report the first amperometric bioplatfrom developed to date for the determination of anti-MBP, an autoantibody biomarker of multiple sclerosis autoimmune disease. The proposed design involves the immobilization of MBP protein onto carboxylated MBs for the selective capture of the target autoantibody followed by enzymatic labelling with HRP-anti-hIgG. The amperometric responses were measured after magnetic capture of HRP-anti-hIgG-anti-MBP-MBs conjugates onto SPCEs and using the H_2O_2 /HQ system. The analytical performance of the bioplatfrom, with high reproducibility and selectivity, and achieving a low detection limit of 0.016 ng mL^{-1} , improves notably the performance claimed for the only immunosensor for anti-MBP reported so far, as well as that claimed using the ELISA methodology. The suitability and applicability of the developed bioplatfrom has been demonstrated by analyzing the anti-MBP endogenous content in serum from patients diagnosed with MS, with no sample treatment except a 10-fold dilution, providing results in agreement with those obtained by the ELISA method.

Credit author statement

S. Guerrero: Methodology, Investigation. E. Sánchez-Tirado: Methodology, Investigation, Writing – original draft, review. L. Agüí: Methodology, Investigation. A. González-Cortés: Conceptualization, Supervision, Writing – review & editing. Paloma Yáñez-Sedeño: Conceptualization, Supervision, Resources, Writing – review & editing, Funding acquisition. José M. Pingarrón: Supervision, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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