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# Electrochemical immunosensor for the determination of the cytokine interferon gamma (IFN-γ) in saliva

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ABSTRACT

A simple, fast and sensitive amperometric immunosensing method for the determination of the clinically relevant cytokine interferon gamma (IFN-γ) in saliva complying the requirements demanded for this kind of sample is reported. The target analyte was sandwiched between a specific capture antibody covalently immobilized on a screen-printed electrode functionalized by the diazonium salt grafting of *p*-aminobenzoic acid, and a biotinylated detector antibody labeled with a streptavidin-horseradish peroxidase conjugate. The amperometric responses measured at - 0.20 V vs Ag pseudo-reference electrode upon addition of hydrogen peroxide in the presence of hydroquinone as the redox mediator allowed a calibration plot with a linear range between 2.5 and 2000 pg $mL^{-1}$ and a low limit of detection (1.6 pg $mL^{-1}$) to be obtained. In addition, a good selectivity against other non-target proteins was achieved. The developed method was validated by analyzing a WHO 1st International Standard for IFN-γ. In addition, the immunosensor was used for the determination of the endogenous IFN-γ in saliva with results in excellent agreement with those obtained by a commercial ELISA kit.

## 1. Introduction

Human IFN-γ is a potent multifunctional cytokine primarily secreted by natural killer T cells as part of the innate immune response to intracellular pathogens. Originally characterized by its antiviral activities, IFN-γ also exerts anti-proliferative, immunoregulatory, and proinflammatory activities, and plays a central role in the progression of a variety of inflammatory and autoimmune diseases. For instance, increased IFN-γ production is associated with rheumatoid arthritis [1] and multiple sclerosis [2]. Therefore, because of this relevance, the development of sensitive, rapid and accurate methods for the determination of IFN-γ is crucial for monitoring the progress of these diseases. Among the available methods, ELISA kits for IFN-γ are usually employed in clinical practice providing suitable sensitivity for application to serum samples where concentrations of IFN-γ at the level of ng $mL^{-1}$ [3] are expected. Typically, such immunoassays involve sandwich-type configurations with horseradish peroxidase (HRP)-labeled secondary antibodies and colorimetric detection. Logarithmic calibration plots with linear ranges between tens and thousands pg $mL^{-1}$ IFN-γ and minimum detectable dose (MDD), calculated as the concentration corresponding to the mean response of the zero standard +2s, of various units of pg $mL^{-1}$, are claimed. An important drawback of ELISA methods is the long time required for the immunoassay, extending from 3 h [https://www.thermofisher.com/elisa/product/IFN-gamma-Human-ELISA-Kit/EHIFNG]to4.5h[https://www.rndsystems.com/products/human-ifn-gamma-quantikine-elisa-kit_dif50].

Electrochemical affinity biosensors for IFN-γ have also been reported. Aptasensors with different detection strategies [4–11], which were applied to serum [7,8] and cell culture [6,9], exhibit linear ranges for the calibration plots in the ng $mL^{-1}$ range [7,10], with limits of detection ranging between few pg $mL^{-1}$ [8] and ng $mL^{-1}$ [4,5]. Regarding electrochemical immunosensors, few papers have been found in the literature. However, these immunosensors exhibit, in general, a higher sensitivity than aptasensors, with linear calibration plots in the pg $mL^{-1}$ range and LOD values of few pg $mL^{-1}$ [12] or even lower [3,13,14], and have been applied to the analysis of serum samples. Label-free approaches [12] using electrochemical impedance spectroscopy (EIS), or sandwich-type configurations using CdS NPs as label tags and stripping voltammetry of dissolved nanoparticles [13,14] have been reported.

As it can be realized, there is a relatively high number of methods involving affinity interactions for the determination of IFN-γ in serum. However, there are no methods for the determination of this analyte in saliva, despite the variation of cytokine levels in this fluid is considered a helpful tool in the diagnosis and treatment of inflammatory or immune diseases and particularly in the case of oral diseases [15]. The analysis of saliva has already demonstrated a high potential diagnostic

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value with the distinctive advantage over other clinical samples that it can be repeatedly collected noninvasively [16] thus providing a cost-effective approach for disease monitoring and screening of large populations [17].

The concentration of IFN-γ in saliva of healthy individuals is around various units of pg $mL^{-1}$, which makes it necessary to develop sensitive methods for this type of sample. With this objective, we report in this work a simple and reliable electrochemical immunosensor for the determination of IFN-γ, which comply with the clinical requirements of sensitivity and accuracy demanded for application to saliva. The rational design of the immunosensor involves the immobilization of capture antibodies onto a disposable screen-printed carbon electrode (SPCE) functionalized by grafting of the diazonium salt of *p*-aminobenzoic (*p*-ABA). A sandwich-type immunoassay was implemented using biotin-anti-IFN and conjugation with peroxidase-labeled streptavidin (HRP-Strept). Amperometric measurements were carried out upon adding hydrogen peroxide solution to the electrode surface in the presence of hydroquinone (HQ) as the redox mediator. The developed method was validated by application to a WHO 1st International Standard for IFN-γ (National Institute for Biological Standards and Control, NIBSC), and the analytical usefulness of the immunosensor was demonstrated by analyzing real saliva samples from volunteers with results in excellent agreement with those obtained by using a commercial ELISA kit.

## 2. Experimental

### 2.1. Apparatus and electrodes

Screen-printed carbon electrodes (SPCEs, 110 DRP, DropSens-Metrohm) consisting of a 4-mm ∅ working electrode, a silver pseudo-reference electrode and a carbon counter electrode, were used as platforms for the preparation of the immunosensor. A PGSTAT 101 potentiostat from Autolab controlled by Nova 1.6 electrochemical software (EcoChemie B·V.) was employed for grafting of *p*-ABA onto SPCEs. EIS measurements were carried out using an Autolab type III potentiostat controlled by the FRA2 software (EcoChemie). Amperometric measurements were made with a CHI 1030B potentiostat (CH Instruments) controlled by CHI 1030B software. An Elmasonic Se60 (Elma) ultrasonic bath, a Vortex homogenizer (Velp Scientifica) and a Crison Basic 20+ pHmeter were also used. All electrochemical experiments were performed at room temperature.

### 2.2. Reagents and buffer solutions

Human anti–IFN–γ capture antibody (anti-IFN), human biotinylated anti–IFN–γ antibody (biotin-anti-IFN), and human IFN-γ were from R&D Systems. All these reagents were included in the DuoSet ELISA (Cat. No. DY285B, from R&D Systems, https://www.rndsystems.com). Human IFN-γ capture antibody was reconstituted with 250 μL of 0.01 M phosphate buffer saline solution (PBS) of pH 7.4 containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$ and 1.5 mM $KH_2PO_4$. Anti-IFN solutions were prepared by appropriate dilution with 25 mM 2-(N-morpholino) ethanesulfonic acid (MES, from GERBU Biotechnik GmbH, https://www.gerbu.de) buffer solution of pH 5.0. Biotinylated anti-human IFN-γ was reconstituted with 500 μL of 0.01 M PBS solution of pH 7.4 supplemented with 1% (w/v) bovine serum albumin (BSA, GERBU Biotechnik GmbH, https://www.gerbu.de). IFN-γ standard solutions were prepared in the same medium.

Sodium nitrite (Panreac) and *p*-aminobenzoic acid (*p*-ABA, Acros) were used to modify SPCEs by electrochemical grafting of diazonium salt. Moreover, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC, Acros Organics) and N–hydroxy-sulfosuccinimide (sulfo-NHS, Sigma-Aldrich) were also used as activation agents for carboxyl groups confined at the electrode surface. For this purpose, 100 mM EDC and sulfo-NHS solutions, each in 25 mM MES buffer solution of pH 5, were used. HRP-Strept (Roche) solutions were prepared in PBS of pH 7.4 supplemented with 1% bovine serum albumin (BSA). The blocking agent was a commercial 2% (w/v) BSA solution prepared in 0.01 M PBS. 0.05 M $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (Sigma) solutions were prepared in 0.01 M PB of pH 7.4. Hydrogen peroxide (Aldrich, 30% (w/w)) and hydroquinone (HQ, Sigma) were also employed. Transforming growth factor-β (TGF-β1), tumor necrosis factor alfa (TNF-α), interleukin 1 beta (IL-1β) and receptor activator for nuclear factor κ B (RANKL) from R&D Systems, BSA from Gerbu human immunoglobulin G (IgG), hemoglobin, glucose, ascorbic acid and uric acid, all from Sigma, interleukin 6 (IL-6) and interleukin 8 (IL-8) from Abcam, https://www.abcam.com. were tested as potential interfering compounds. All other chemicals used were of analytical reagent grade. All solutions were prepared with deionized water obtained from a Milli-pore Milli-Q purification system (18.2 MΩ cm at 25 °C).

A WHO 1st International Standard for IFN-γ (National Institute for Biological Standards and Control, NIBSC, code: 87/586), containing 12.5 ng $mL^{-1}$ IFN-γ was used as a reference material for validation of the developed method. Moreover, saliva samples from volunteers obtained and pre-treated as described in section 2.3.3 were analyzed.

### 2.3. Procedures

#### 2.3.1. Preparation of HRP-strept-biotin-anti–IFN–IFN-γ-anti-IFN/SPCE immunosensor

In a first step, SPCEs were modified by electrochemical grafting of *p*-ABA diazonium salt. To do that, 20 mg of *p*-ABA were dissolved in 2 mL 1 M HCl and cooled with ice. The diazonium salt was synthesized by adding 2 mM $NaNO_2$ aqueous solution dropwise to this solution (38 μL for each 200 μL) upon constant stirring. Next, 40 μL of the resulting solution were casted onto the SPCE and ten successive cyclic voltammetric scans were carried out between 0.0 and −1.0 V ($\upsilon$ = 200 mV $s^{-1}$). The as prepared modified electrodes were washed thoroughly with water and allowed drying in air (this process takes less than 1 min). Thereafter, the electrode surface confined carboxyl groups were activated by adding 10 μL of EDC/sulfo-NHS (0.1 M each) prepared in MES buffer solution of pH 5.0 and allowing incubation for 30 min at 25 °C in a humidified chamber. After rinsing with water, 5 μL of a 15 μg $mL^{-1}$ anti-IFN solution prepared in MES buffer of pH 5.0 were added and left to react for 1 h. Next, the electrodes were rinsed with water and blocking of the remaining active sites was made by adding 10 μL of a 1% BSA solution in PBS, allowing incubation for 30 min. Thereafter, 5 μL of standard IFN-γ solutions or the samples were placed onto the electrode surface and incubated for 60 min. Then, 5 μL of biotin-anti–IFN–γ solution (1.25 μg $mL^{-1}$) were dropped onto the resulting electrode and incubated for 45 min followed by the addition of 5 μL of HRP-Strept solution (1/500 diluted in PBS pH 7.4) and incubating for 20 min. Washings with PBS were included between these steps and the immunosensor was kept with a drop of a 0.05 M PB solution of pH 6.0 until the measurements were made.

#### 2.3.2. Amperometric measurements

Amperometric measurements were performed by adding 45 μL of a 1 mM HQ solution in 0.05 M PBS of pH 6.0 onto the surface of the immunosensor horizontally positioned. A detection potential of −200 mV vs Ag pseudo-reference electrode was applied and, once the background current was stabilized, (around 50 s), 5 μL of 50 mM $H_2O_2$ solution were added and allowed standing for 200 s to complete the enzyme reaction. The steady state current corresponding to the electrochemical reduction of benzoquinone was used as the analytical readout.

#### 2.3.3. Samples analysis

Glass ampoules containing lyophilized samples of a biological reference standard for IFN-γ (NIBSC, code: 87/586) were stored at −20 °C. According to the instructions provided by the supplier, the

solid from each ampoule was reconstituted in 0.5 mL of sterile distilled water. Then, the ampoule was rinsed with 0.4 mL of the same water and diluted up to a volume of 1.0 mL to obtain a final aqueous solution containing the cytokine at a 250 IU/mL (12.5 ng $mL^{-1}$) concentration. The determination of IFN-γ was performed after a 1000-fold dilution with PBS by applying the procedure described in Section 2.3.2. The measured currents were interpolated into the linear portion of the calibration plot constructed with IFN-γ standard solutions.

Saliva from volunteers was collected in the morning. Volunteers were asked not to eat, brush their teeth, or use mouth rinse at least 2 h before sample collection. A Salivette® collection device (Sarstedt) was used. Briefly, once rinsed the mouth thoroughly with water, a cotton swab was inserted into the mouth and chewed for 1 min. Then, the swap saturated with saliva was inserted into the vial, sealed with the cap and centrifuged for 10 min at 3000 rpm. The determination was carried out immediately by applying the procedure described in Section 2.3.2 to 5 μL of undiluted saliva. Similarly to that stated above, the amperometric responses were interpolated into the calibration plot constructed with standard solutions.

## 3. Results and discussion

Fig. 1 shows a scheme of the steps involved in the preparation of the HRP-Strept-biotin-anti–IFN–IFN-γ-anti–IFN–Phe/SPCE immunosensor as well as the reactions involved in the amperometric detection. The proposed strategy takes advantage of the good performance of grafted electrochemical scaffolds for covalent immobilization of biomolecules. As it is known [18], modification of carbon electrodes through free radical grafting allows the formation of compact and stable organic monolayers with specific functionalities. In this case, the electrochemical reduction of *p*-ABA diazonium salt onto SPCEs provided a dense layer of surface confined carboxylic moieties on which the capture antibody was immobilized by covalent binding once they were activated with the EDC/sulfo-NHS system. As it can be seen in step 1, the diazonium salt was synthesized by diazotization reaction followed by cyclic voltammetry. The experimental conditions used in this step were those previously optimized and reported [19]. Upon covalent immobilization of the capture antibody (step 2), the remaining free active sites were blocked with BSA (step 3). After addition of the target IFN-γ, a sandwich type immunoassay was implemented involving biotin-anti-IFN and HRP-Strept (step 4). The affinity reaction was amperometrically monitored by adding $H_2O_2$ in the presence of HQ as the redox mediator (step 5).

### 3.1. Optimization of the experimental variables involved in the preparation of the immunosensor

The effect of the different variables involved in the preparation of the immunosensor for IFN-γ on the amperometric responses was tested. The checked variables included: a) the anti-IFN antibody loading onto Phe-SPCE and the corresponding incubation time; b) the type and concentration of blocking agent; c) the biotin-anti-IFN loading onto IFN-γ-anti–IFN–Phe-SPCE and the incubation time; and d) the concentration of HRP-Strept. A larger ratio between the currents measured in the presence or in absence of the target cytokine (specific-to-unspecific current ratio) was taken as the selection criterion for each tested variable. The results of the optimization studies are shown in Fig. 2 and summarized in Table 1.

Fig. 2a shows the effect of the capture antibody loading on the immunosensor response. In the experiments, 5 μL of 5–20 μg $mL^{-1}$ anti-IFN solutions were incubated onto the grafted SPCE once the carboxylic groups were activated with the EDC/sulfo-NHSS solution and allowing 60 min for incubation. A larger specific-to-unspecific current ratio (S/N) was obtained for 15 μg $mL^{-1}$ antibody, this value being selected for the preparation of the immunosensor. The lower specific current observed for larger antibody concentrations can be attributed to the steric hindrance for the antigen recognition when large amounts of the capture antibody are immobilized [20]. Using the selected loading,

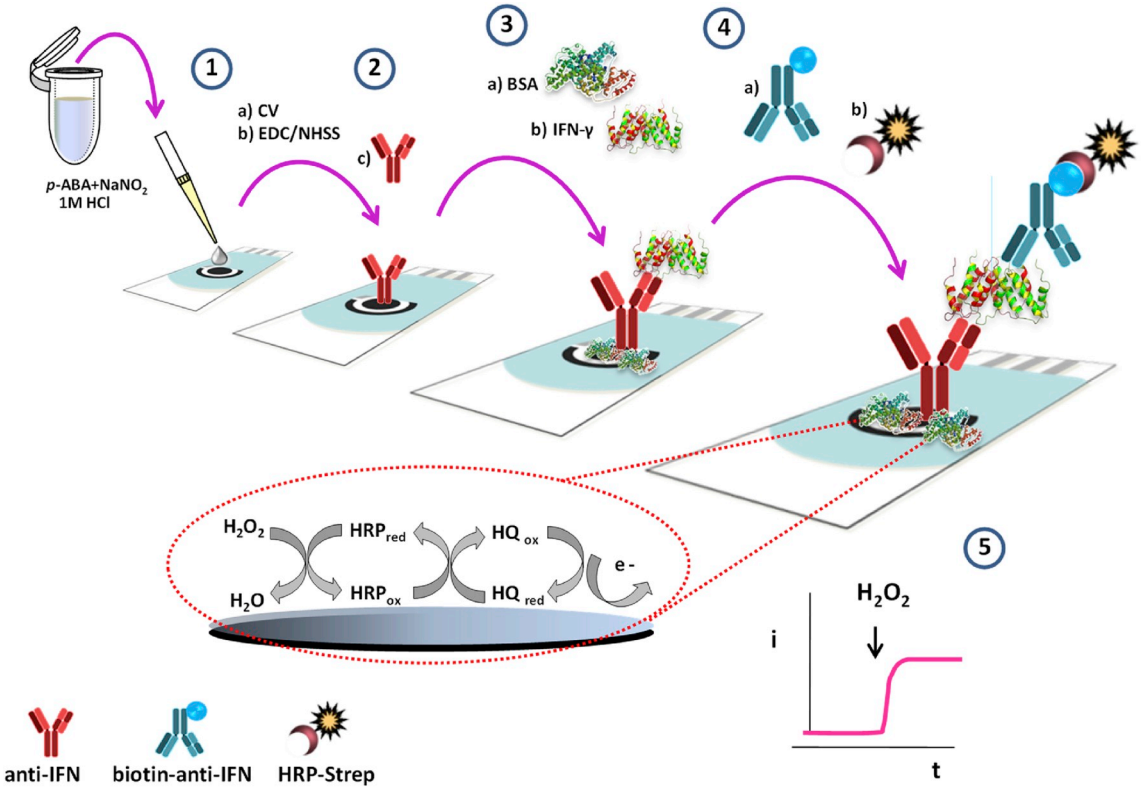



**Fig. 1.** Schematic picture of the steps involved in the preparation of the electrochemical immunosensor for the determination of IFN-γ. See the text for more information.

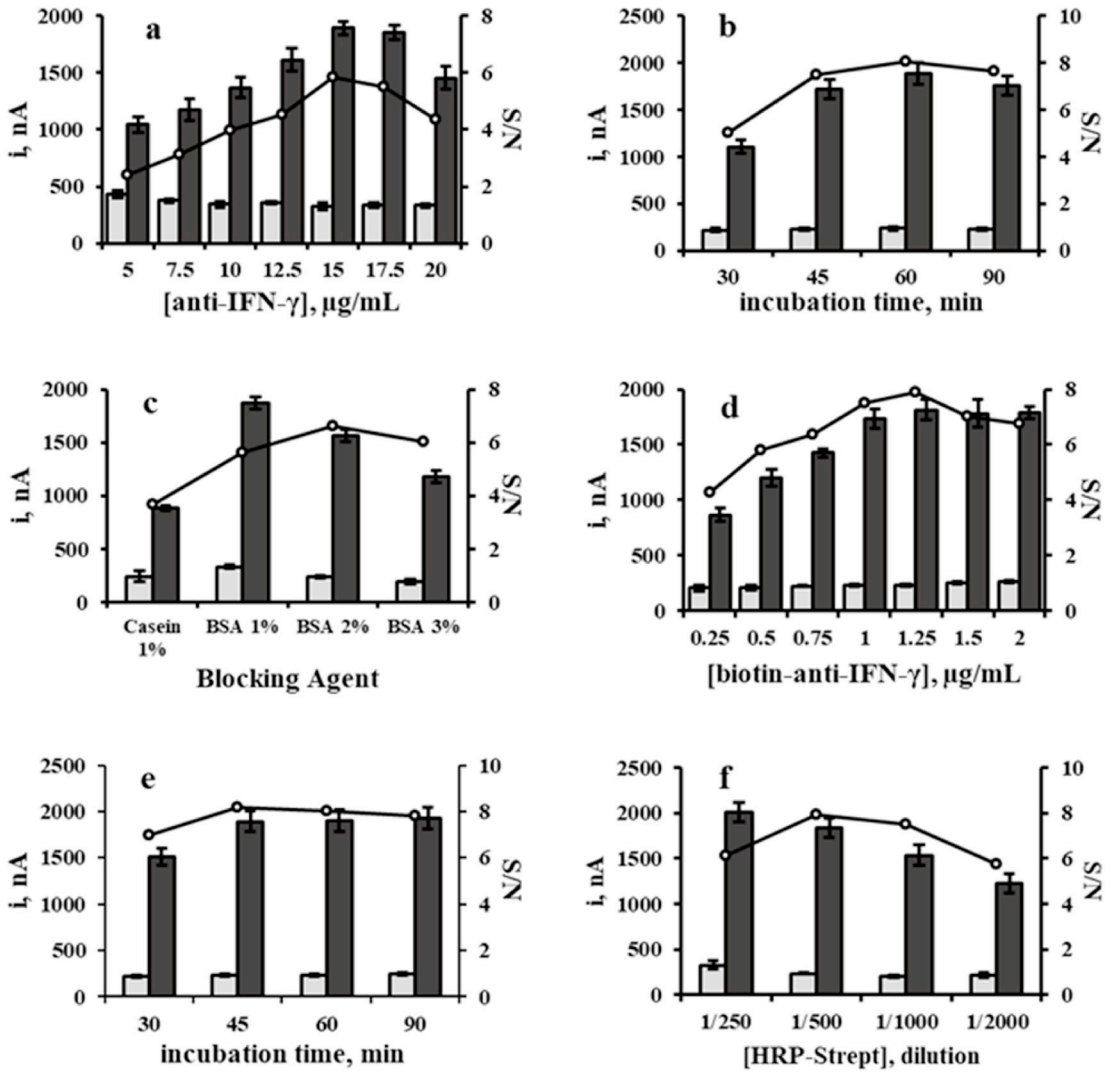



**Fig. 2.** Amperometric responses measured with the HRP-Strept-biotin-anti–IFN–IFN-γ-anti–IFN–Phe-SPCE immunosensors in the absence (light grey) and in the presence of 1.0 ng $mL^{-1}$ IFN-γ (dark grey) as a function of: the anti-IFN loading (5–20 µg $mL^{-1}$) (a); the anti-IFN incubation time (30–90 min) (b); the type and concentration of blocking agent (1–3% (w/v)) (c); the biotin-anti-IFN loading (0.25–2 µg $mL^{-1}$) (d); the biotin-anti-IFN incubation time (e); and the HRP-Strept conjugate dilution factor (1/2000–1/250) (f). Error bars estimated as triple of the standard deviation (n = 3).

**Table 1**
Optimization of the experimental variables involved in the preparation of an amperometric immunosensor for IFN-γ.

| Variable | Tested range (or compound) | Selected value |
|---|---|---|
| [anti-IFN], µg/mL | 5–20 | 15 |
| $t_{inc}$ anti-IFN, min | 30–90 | 60 |
| blocking agent | casein, BSA | BSA |
| [BSA], % (w/v) | 1–3 | 2 |
| [biotin-anti-IFN], µg/mL | 0.25–2 | 1.25 |
| $t_{inc}$ biotin-anti-IFN, min | 30–90 | 45 |
| HRP-Strept, dilution | 1/250–1/2000 | 1/500 |

optimization of the anti-IFN incubation time in the 30–90 min range led us to choose 60 min for further work (Fig. 2b).

Blocking of the free active sites on the electrode surface was carried out by testing casein and BSA solutions as blocking agents due to their well-recognized ability to minimize. Fig. 2c shows that larger S/N ratios were achieved using 2% (w/v) BSA. 30 min incubation was shown to be sufficient for an effective blocking.

The effect of the biotin-anti-IFN concentration was evaluated over the 0.25–2.0 µg $mL^{-1}$ range. Fig. 2d shows as the specific current increased with the biotinylated detector antibody concentration up to 1.25 µg $mL^{-1}$ and levelled off for larger concentrations. Since the S/N current ratio exhibited also a larger value for this concentration, it this selected for further work. Regarding the effect of the incubation time with the biotin-anti-IFN solution, Fig. 2e shows that a larger S/N ratio was achieved for 45 min which was selected for the immunosensor preparation.

The effect of HRP-Strept dilution on the response of the immunosensor was checked by preparing different immunosensors using the previously optimized experimental conditions and ranging the dilution factor between January 2000 and 1/250. The results displayed in Fig. 2f led us to select a 1/500 dilution for the construction of the immunosensor.

### 3.2. Characterization studies by electrochemical impedance spectroscopy

Once the experimental variables were optimized, the different steps involved in the immunosensor preparation were characterized by EIS using 2 mM $Fe(CN)_6^{4-/3-}$ prepared in 0.05 M PB of pH 7.4 as the redox probe. The corresponding Nyquist plots and the equivalent circuits are displayed in Fig. 3a and b. As expected, modification of the bare SPCE with *p*-ABA diazonium salt provoked a large increase in the electron

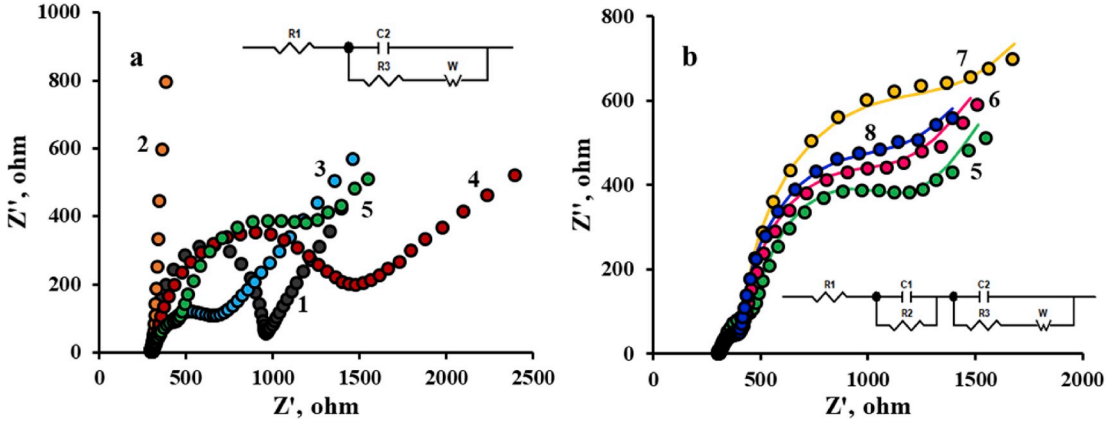



**Fig. 3.** Nyquist plots recorded in a 2 mM $Fe(CN)_6^{3-/4-}$ 0.1 M PB solution of pH 7.4 and equivalent circuits for: bare SPCE (1); *p*-ABA-SPCE (2); *p*-ABA-SPCE activated with EDC/sulfo-NHS (3); anti–IFN–Phe-SPCE (4); anti–IFN–Phe-SPCE blocked with BSA (5); IFN-γ-anti–IFN–Phe-SPCE (6); biotin-anti–IFN–γ–IFN–anti–IFN–Phe-SPCE (7); HRP-Strept-biotin-anti–IFN–IFN-γ-anti–IFN–Phe-SPCE (8). 1 ng $mL^{-1}$ IFN-γ; range of frequencies, $10^5$–0.040 Hz; open circuit. Lines in Fig. 2B were obtained by fitting the experimental data to the equivalent circuit displayed using Zview impedance software.

transfer resistance, Rct, from 646 to 4187 Ω, due to the electrostatic repulsion between the anionic redox probe and the negatively charged carboxylate groups present on the electrode surface at the working pH. Activation with EDC/sulfo-NHS gave rise to a dramatic decrease in the Rct value (497 Ω) because of the neutralization of the surface confined -$COO^-$ groups upon reaction with the carbodiimide/succinimide system. As expected, a further significant increase in Rct occurred upon anti-IFN immobilization (1143 Ω), which is attributed to the partially insulating barrier on the electrode surface produced by the presence of the antibody. It is worth mentioning that subsequent incubation of the anti-–IFN–Phe-SPCE bioelectrode in the BSA blocking solution provided a Nyquist diagram showing two different semicircles (Fig. 3b), which is due to the presence of two differentiated layers on the electrode surface. Consequently, this status should be defined by an equivalent circuit at least constituted by two RC semi-circuits. The resistance, $R_{ct2}$, corresponding to the second semicircle of the Nyquist plot, described the electron transfer through the immobilized substances including BSA, the biotinylated detector antibody, the antigen and HRP-Strept. As it can be seen, the represented R1 (R2C1) (C2 [R3W1]) circuit fitted well with the experimental results as demonstrated by the overlapping of the experimental points in the Nyquist plots in Fig. 2B with the respective lines obtained using the Zview impedance software [21]. As expected, the resistance of the second semicircle increased after incubation with the antigen solution. Further increase occurred after incubation with the biotinylated detector antibody solution due to the blocking effect of both proteins toward the interfacial electron transfer and also to the repulsion between biotin (dissociated at the working pH) and the negatively charged redox probe in solution. However, a remarkable decrease was observed upon incubation with the HRP-Strept solution, probably because of the partial neutralization of the biotin negative charges as a consequence of the interaction with streptavidin [22].

### 3.3. Analytical figures of merit of the immunosensor

Fig. 4a displays the calibration plot for IFN-γ standard solutions constructed with the developed immunosensor under the optimized working conditions. Error bars were calculated from measurements carried out with three different immunosensors prepared in the same manner. Some typical recorded amperograms are also displayed in Fig. 4b. The steady state current vs. logarithm of IFN-γ concentration plot followed the adjusted equation i, nA = (591 ± 12) log [IFN-γ], pg/mL + (121 ± 25) ($r^2$ = 0.997) with a range of linearity extending between 2.5 and 2000 pg $mL^{-1}$ IFN-γ. This range covers almost three orders of magnitude and is adequate for the determination of IFN-γ according to the expected concentrations in serum [3] and saliva [16]. Importantly, the cut-off concentration for LTBI (latent tuberculosis infection) in serum has been reported to be 15 pg $mL^{-1}$ [23]. The obtained LOD value, 1.6 pg $mL^{-1}$, was calculated according to the 3 × $s_b$ criterion, where $s_b$ was estimated as the standard deviation, expressed in concentration units, calculated from ten amperometric responses of the immunosensors prepared in the absence of IFN-γ. The limit of quantification, calculated as 10 × $s_b$ was LOQ = 5.3 pg $mL^{-1}$.

When these analytical figures of merit are compared with those claimed for commercial ELISA kits using similar immunoreagents, some noticeable differences are apparent. The dynamic range (non-linear) of the calibration plot reported for the Duo Set ELISA kit from R&D Systems (Cat. No. DY285B) is narrower, encompassing from 15.6 to 1000 pg $mL^{-1}$ IFN-γ, and no LOD or MDD value is provided (https://resourcesrndsystems.com/pdfs/datasheets/dy285.pdf. ). In addition, the required sample volume is 100 μL, twenty times larger than that needed with the immunosensor, and the assay time takes over 4 h 40 min, which is remarkably longer than the time taken with the immunosensor, 2 h 30 min (counting in both cases since the immobilization of the capture antibody). However, it must be said that the ELISA method can be performed in a 96 well plate at the same time. On the other hand, when the comparison is made with respect to other reported electrochemical immunosensors, the conclusions are not so clear since they use different strategies (label-free or sandwich-type with signal amplification) and transduction techniques (SWASV, DPV, or EIS). However, we can say that, in general, the range of linearity exhibited by the developed immunosensor is among the widest ones, and the achieved LOD value among the lowest ones. For example, the sandwich-type immunosensor reported by Wang et al. [13] involving the immobilization of the capture antibody onto magnetic particles, the use of CdS NPs/AuNPs as carrier tags for detector antibodies, and stripping voltammetry for the dissolved cadmium, provides a calibration plot with a non-linear dynamic range extending only over 0.4 and 40 pg $mL^{-1}$. In addition, it requires a much larger sample volume (100 μL). Regarding the LOD, the value achieved with our design is slightly lower than that obtained by Ruecha et al. (3.4 pg $mL^{-1}$) using a label-free impedimetric immunosensor where the capture antibody was immobilized onto a PANI/graphene screen-printed paper electrode. However, our LOD is notably larger than that reported by Zhang et al. (0.048 pg $mL^{-1}$) [3] using HRP-dAb-AuNPs tags for signal amplification. Nevertheless, the assay time is much longer, 4 h, and the required sample volume is four times larger.

The reproducibility of the amperometric measurements was tested by recording the responses in the absence and in the presence of 1 ng $mL^{-1}$ IFN-γ with five different immunosensors prepared in the

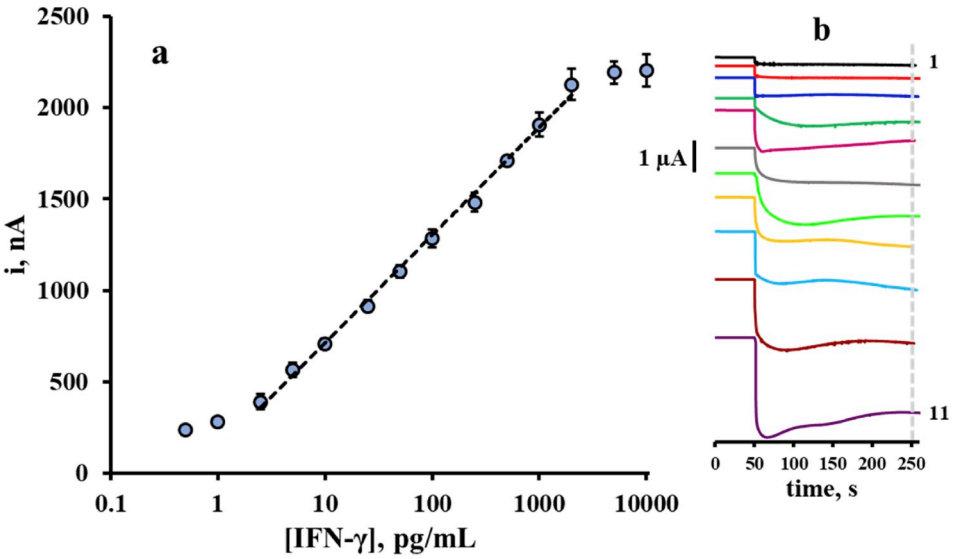



**Fig. 4.** A) Calibration plot constructed for IFN-γ with the HRP-Strept-biotin-anti–IFN–IFN-γ-anti–IFN–Phe-SPCE amperometric immunosensor. Error bars were estimated as triple of the standard deviation of measurements (n = 3). B) - Amperograms recorded with the immunosensor for different IFN-γ concentrations in pg $mL^{-1}$: (1) 0; (2) 2.5; (3) 5; (4) 10; (5) 25; (6) 50; (7) 100; (8) 250; (9) 500; (10) 1000; (11) 2000 pg $mL^{-1}$.

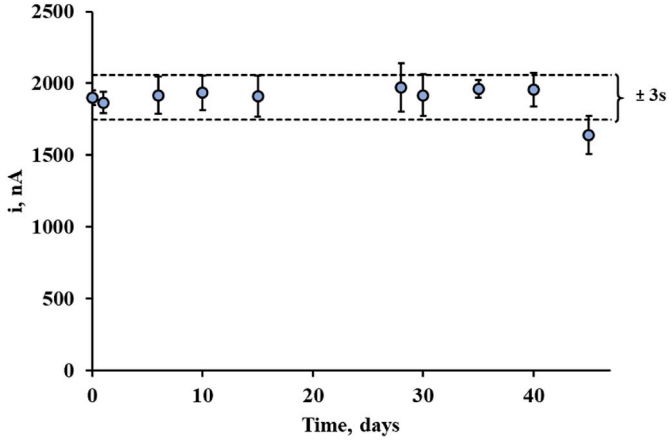



**Fig. 5.** Control chart constructed for checking the storage stability of the anti–IFN–Phe/SPCEs. Amperometric measurements corresponded to those recorded for 1 ng $mL^{-1}$ IFN-γ. Error bars estimated as triple of the standard deviation (n = 3).

same manner and on the same day. The relative standard deviation (RSD) values were 1.4 and 1.8%, respectively. Furthermore, RSD values of 2.1 and 2.6% were found for measurements made with five different immunosensors prepared in different days in the absence and in the presence of the same concentration of IFN-γ, respectively. These RSD values show well the suitability and reproducibility of the protocols involved in the immunosensor preparation and in the amperometric transduction. The storage stability of the prepared anti–IFN–Phe-SPCEs conjugates was tested by preparing different immunosensing platforms on the same day and storing them dry at −20 °C. The stored biosensors were used to measure 1 ng $mL^{-1}$ IFN-γ on different days. Fig. 5 shows the control chart constructed by setting the average current measured with 10 different immunosensors prepared the first working day as the central value, and the upper and lower limits of control at ± 3 × SD of this central value. As it can be observed, the amperometric responses remained inside the control limits for 40 days, thus showing the excellent storage stability of the prepared anti–IFN–Phe-SPCE immunosensing platforms.

### 3.4. Selectivity

The potential interference caused by the presence of other proteins on the amperometric quantification of IFN-γ with the developed immunosensor was checked by recording the responses for 0 and 1 ng $mL^{-1}$ IFN-γ in the absence and in the presence of the following non-target compounds: 7 µg $mL^{-1}$ ascorbic acid (AA), 5 mg $mL^{-1}$ bovine serum albumin (BSA), 1 mg $mL^{-1}$ glucose (Glu), 5 mg $mL^{-1}$ hemoglobin (HB), 5 mg $mL^{-1}$ human immunoglobulin G (IgG), 50 pg $mL^{-1}$ interleukin 1 beta (IL–1β), 300 pg $mL^{-1}$ interleukin 6 (IL–6), 300 pg $mL^{-1}$ interleukin 8 (IL–8), 100 pg $mL^{-1}$ receptor activator of nuclear factor kappa-B ligand (RANKL), 50 pg $mL^{-1}$ transforming growth factor β1 (TGF-β1), 200 pg $mL^{-1}$ tumoral necrosis factor alpha (TNF-α) and 100 µg $mL^{-1}$ uric acid (UA). These

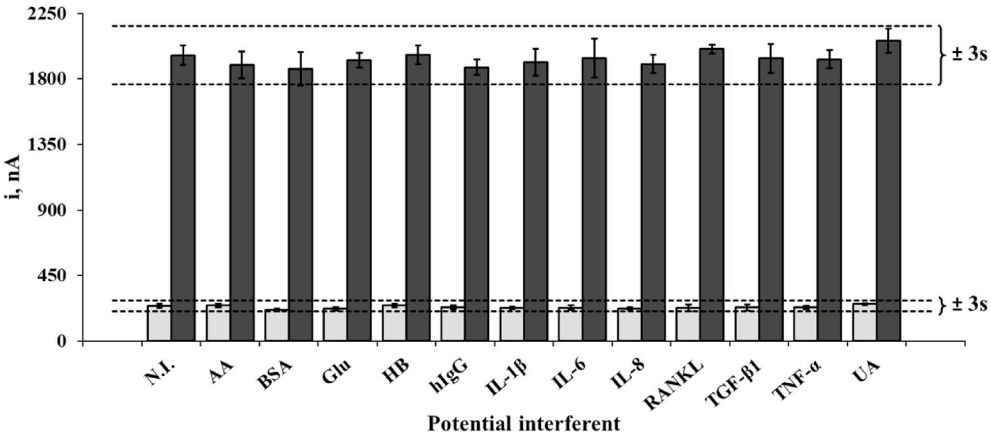



**Fig. 6.** Amperometric responses measured with HRP-Strept-biotin-anti–IFN–IFN-γ-anti–IFN–Phe-SPCE immunosensors for 0 (light grey) and 1 (dark grey) ng $mL^{-1}$ IFN-γ standards in the absence and in the presence of 7 µg $mL^{-1}$ AA, 5 mg $mL^{-1}$ BSA, 1 mg $mL^{-1}$ Glu, 5 mg $mL^{-1}$ HB, 5 mg $mL^{-1}$ human IgG, 50 pg $mL^{-1}$ IL–1β, 300 pg $mL^{-1}$ IL–6, 300 pg $mL^{-1}$ IL–8, 100 pg $mL^{-1}$ RANKL, 50 pg $mL^{-1}$ TGF-β1, 200 pg $mL^{-1}$ TNF-α, and 100 µg $mL^{-1}$ UA. See the text for more information.

**Table 2**
Determination of IFN-g in a reference material and in saliva samples using the HRP-Strept-biotin-anti–IFN–IFN-γ-anti–IFN–Phe/SPCE immunosensor.

| Biological International Standard | | | Student *t*-test |
|---|---|---|---|
| **Sample** | **Immunosensor (n = 9), pg/mL** | **ELISA (n = 6), pg/mL** | |
| NIBSC 87/586 | 12.5 ± 0.2 | 12.3 ± 0.5 | $t_{exp}$ = *1.732*; $t_{tab}$ = *2.160* |
| ***Saliva*** | | | *Student t-test* |
| **Sample** | **Immunosensor (n = 6), pg/mL** | **ELISA (n = 3), pg/mL** | |
| 1 (non-smoker) | 8.4 ± 0.3 | 8.2 ± 0.4 | $t_{exp}$ = *1.822*; $t_{tab}$ = *2.365* |
| 2 (non-smoker) | 7.3 ± 0.2 | 7.1 ± 0.3 | $t_{exp}$ = *1.774*; $t_{tab}$ = *2.365* |
| 3 (smoker) | 7.6 ± 0.2 | 7.5 ± 0.3 | $t_{exp}$ = *1.477*; $t_{tab}$ = *2.365* |
| 4 (non-smoker) | 7.2 ± 0.3 | 7.2 ± 0.5 | $t_{exp}$ = *1.443*; $t_{tab}$ = *2.365* |

concentrations are those expected according to the literature in healthy individuals. The results summarized in Fig. 6 allowed us to conclude that no significant interference was produced by any of the potential interfering biomolecule tested.

### *3.5. Determination of IFN-γ in a biological international standard and in saliva*

Validation of the HRP-Strept-biotin-anti–IFN–IFN-γ-anti–IFN–Phe/SPCE immunosensor was performed by analyzing a Biological International Standard (BIS) for IFN-γ containing a 12.5 ng $mL^{-1}$ IFN-γ certified concentration. In addition, the endogenous concentration of IFN-γ was quantified in saliva samples collected from four volunteers following the procedure described in Section 2.3.3. The potential occurrence of matrix effects was evaluated in both types of sample by statistically comparing the slope values of the calibration plots constructed by successive additions of IFN-γ standard aliquots to the samples (BIS, and Saliva 1,2,3 and 4) with the slope value of the calibration graph obtained using IFN-γ standard solutions in PBS. The corresponding regression equations were:

BIS: i, nA = (590 ± 12) log [IFN-γ], pg $mL^{-1}$ + (121 ± 25)
Saliva 1: i, nA = (571 ± 16) log [IFN-γ], pg $mL^{-1}$ + (125 ± 20)
Saliva 2: i, nA = (628 ± 24) log [IFN-γ], pg $mL^{-1}$ + (56 ± 29)
Saliva 3: i, nA = (630 ± 29) log [IFN-γ], pg $mL^{-1}$ + (56 ± 35)
Saliva 4: i, nA = (656 ± 47) log [IFN-γ], pg $mL^{-1}$ + (17 ± 58)

The application of the Student t-test by comparing with the equation calculated with standard solutions, i, nA = (591 ± 12) log [IFN-γ], pg/mL + (121 ± 25), provided $t_{exp}$ values 0.186 (BIS) and 0.993, 1.398, 1.277 and 1.352 for saliva samples 1 to 4, respectively. These $t_{exp}$ values were, in all cases, lower than the tabulated one, $t_{tab}$ = 2.228 for α = 0.05 and n = 10. Therefore, the determination of IFN-γ in BIS and saliva can be performed directly by interpolation of the amperometric current measured with the immunosensor for an aliquot of the undiluted sample, into the calibration plot constructed with standard solutions. The concentrations found in the analysis of BIS and saliva from two smoker and two non-smoker individuals are shown in Table 2. In addition, they are compared with the results provided by the commercial ELISA kit DuoSet ELISA, R&D Systems, Cat. No. DY285B. As it can be seen, an excellent agreement was found with the certified material as well as with the results obtained by the ELISA method. It is worth mentioning that the IFN-γ concentrations found in saliva also agree with the mean concentration value of 6.41 ± 2.53 pg $mL^{-1}$ reported in the literature for saliva of healthy individuals [16]. It is important to remark that the immunosensor can be applied to the clinical determination of this cytokine without sample treatment and in a total assay time of approximately half the time required using the ELISA kit involving the same immunoreagents.

## 4. Conclusions

A disposable amperometric immunosensor using SPCEs as electrochemical platform and diazonium salt grafting of *p*-aminobenzoic acid for the covalent immobilization of the capture antibody was prepared for the determination of interferon gamma (IFN-γ). The implemented sandwich-type immunoassay, involving a biotin-labeled detector antibody and HRP-streptavidin conjugate, allows obtaining a calibration plot for IFN-γ with a wider linear range and a much shorter assay time and a remarkably smaller sample volume than the corresponding analytical characteristics claimed for ELISA methodology. A LOD value of 1.6 pg $mL^{-1}$ was achieved which is adequate for the determination of IFN-γ in clinical samples. The immunosensor was employed for the determination of the biomarker in saliva with no sample treatment and results in good agreement with those obtained by using a commercial ELISA kit. The analytical performance exhibited by this new amperometric immunosensor of simple operation including disposability, as well as the possibility of using pocket-size electrochemical instrumentation makes it an attractive alternative for the development of automated POC systems for on-site determination of salivary IFN-γ.

## 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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