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Special Collection

Paving the Way for Reliable Alzheimer's Disease Blood Diagnosis by Quadruple Electrochemical Immunosensing

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Alzheimer's disease (AD), the most common neurodegenerative disorder, demands new cost-effective and easy-to-use strategies for its reliable detection, mainly in the preclinical stages. Here, we report the first immunoplatform for the electrochemical multidetermination of four candidate protein biomarkers in blood, neurofilament light chain (NfL), Tau, phosphorylated Tau (p-Tau) and TAR DNA-Binding Protein 43 (TDP-43). It involves implementation of sandwich-type immunoassays and enzymatic labelling with horseradish peroxidase (HRP) on the surface of magnetic microbeads (MBs). Amperometric detection is performed after depositing the magnetic immunoconjugates on disposable quadruple transduction platforms by monitoring the enzymatic reduction of H_2O_2 mediated by hydroquinone (HQ). The immunoplatform achieved LOD values smaller than the content of target biomarkers in plasma of healthy subjects, with RSD values < 5 %, and lower cost and shorter assay time (60–90 min) than other available methodologies and was applied to the analysis of plasma from healthy controls and AD patients.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide and the leading cause of dementia in the elderly.[1] AD has an estimated prevalence of 10–30 %, affects 45 million individuals worldwide and is expected to increase more than triple by 2050 due to population aging.[2,3] The burden of disease, increased mortality and high societal costs associated with dementia make the development of new prevention and treatment modalities a main public health priority.[4] Despite immense research efforts and significant current advances in therapeutic pathways to improve the diagnosis and management of AD,[5–7] the precise molecular events and biological pathways underlying the disease are still not fully understood.[8] This fact, unfortunately, leads to an unreliable diagnosis of AD in the pre-dementia stages, resulting in the late application of therapeutic actions to delay cognitive impairment and neuronal injury.[9,10] Therefore, strategies for identification and quantification of clinical biomarkers at the pre-clinical stage of AD is a challenging area in modern neuromedicine to apply effective treatment therapies to slow the disease progression and prevent its development before the brain deteriorates.[6,7,11]

The hallmarks pathologies of AD are the accumulation of β-amyloid (Aβ) protein in extracellular plaques and abnormal forms of Tau protein in neurofibrillary tangles (NFTs) within neurons, promoting oxidative stress, chronic neuroinflammation, synapse dysfunction and ultimately neuronal death.[1,12,13] Current clinical trials for differential AD diagnosis use measurements of Aβ or Tau proteins in blood due to the lower invasiveness, analysis time, cost-effectiveness, and easier accessibility compared to cerebrospinal fluid (CSF) or positron emission tomography (PET) biomarkers.[14] However, several studies limit the usefulness of Aβ biomarker for monitoring the disease progression and response to treatment because of early saturation due to Aβ accumulation in the brain.[15,16]

Tau protein levels appears as more effective than Aβ to reflect the clinical onset of AD although its clinical status is also lost with the neurodegeneration process.[17,18] This lack of accuracy in the diagnosis of AD during disease progression, especially in the preclinical phase, can be addressed by measuring Tau proteins pertaining to AD pathophysiology along with other neurodegeneration-related biomarkers to predict and monitor the onset caused by AD, and with specific biomarkers that play an important role in the development of common brain pathologies that may coexist with AD and induce its progression.[19–22]

Tau is a microtubule-associated protein that localizes mainly in the axon of neurons and comprises six human isoforms.[23] Tau protein phosphorylation is a physiological process that promotes the regulation of Tau protein production, although in pathological conditions the Tau protein is hyperphosphorylated causing significant conformational changes that reduces the affinity of Tau for microtubules and destabilizes them structurally, leading to disruption of axonal transport and resulting in the formation of hyperphosphorylated NFTs that characterized AD.[24–27] The longest isoform of Tau (Tau_{441}) has reduced phosphorylation and a stronger affinity for microtubule binding, so post-translational modifications (e.g., phosphorylation) of this protein could cause neurodegeneration and make it useful for AD detection.[28] Clinical studies have reported high levels of phosphorylated Tau protein isoform on threonine 181 ($p\text{-}Tau_{181}$) in preclinical and prodromal stages of the disease and is considered as a highly specific pathological biomarker of AD because it is maintained at normal levels in other neurodegenerative disorders.[29–33] Although Tau and p-Tau are well-established AD blood-biomarkers, their usefulness for early detecting and predicting progression of AD can be markedly improved if they were measured in combination with other neurodegenerative biomarkers. Among them, Neurofilament Light Chain (NfL) is the most abundant component of large myelinated axons and one of the main clinical biomarkers for cognitive impairment and neurodegeneration,[33–36] and TAR DNA-Binding Protein 43 (TDP-43) is a transcriptional repressor associated with increased brain atrophy, memory loss and cognitive impairment that may be present in co-pathologies in AD (up to 50% cases).[37–39] Thus, the simultaneous and complementary determination of these four biomarkers (Tau, p-Tau, NfL and TDP-43) can provide valuable information to detect AD at early stages, as well as to predict the course of neurodegeneration or variants presentations of AD, identify AD co-pathologies, characterize disease progression and monitor response to treatment.[20]

In recent years, the use of biosensing strategies have experience a high growth for medical applications, especially in the case of electrochemical biosensors for early-stage disease detection, patient monitoring and treatment response assessment in prevalent worldwide diseases,[40] including neurodegenerative disorders such as AD.[41,42] In fact, these devices have shown to be competitive in terms of sensitivity with ELISA methodologies commonly adopted in this field. So, quantification of NfL in plasma at $pg\,ml^{-1}$ level (concentrations not detectable with conventional ELISA) has been reported.[43] In addition, electrochemical biosensors exhibit clear advantages in terms of cost and point-of-care applicability compared to state-of-the-art methodologies such as the ultrasensitive single molecule array (Simoa™). In this work, we report the first multiplexed electrochemical bioplatform able to determine individually or simultaneously Tau, p-Tau, NfL and TDP-43 proteins in human plasma as a tool to contribute to the early and reliable diagnosis of AD. The strategy is based on the implementation of individual batches of MBs with sandwich immunocomplexes for each of the target biomarkers attached and their coupling on single or quadruple screen-printed carbon electrodes (SPCE or SP_4CE) to perform the amperometric measurements through the H_2O_2/hydroquinone (HQ) system.

Results and Discussion

This work reports the design and preparation of four immunoplatforms for the single determination of NfL, Tau, p-Tau and TDP-43, and their further integration in a multiplexed bioplatform to carry out the simultaneous detection of these target biomarkers in a single device. The strategies for preparing the different immunosensing platforms were inspired by a recent work in which we reported the first immunoplatform for the determination of NfL. This strategy involved the use of MBs as support for sandwich immunocomplexes formation and amperometric transduction on SPCEs.[43] The rationale of the methodology and the fundamentals of the amperometric transduction for the determination of the four target neurodegenerative biomarkers are displayed in Figure 1, being the immunoassay format employed in each case driven by the commercial availability of the corresponding immunoreagents. In brief, using magnetic immunoconjugates (CAb), specific detection antibodies (DAb), HRP-labelled secondary antibodies (HRP-Ab) and commercial enzyme polymers (Strep-HRP), batches of MBs carrying HRP-labelled sandwich immunoconjugates for each of the 4 biomarkers were prepared. Electrochemical measurements were performed by amperometry in the presence of H_2O_2/HQ after depositing the resulting MBs on the working electrode (WE) surface of screen-printed carbon electrodes (SPCE). In all cases, the variation of the cathodic current originating from the reduction of H_2O_2 by HRP and mediated by HQ was proportional to the concentration of the target biomarker.

The main experimental variables affecting the preparation of the bioplatforms for the single determination of the target biomarkers were tested. The amperometric responses recorded in the presence (signal, S) and in the absence (blank, B) of target biomarker were recorded and larger S/B ratios were adopted as the selection criterion of the checked variable. Both the working conditions for the determination of NfL[43] and those involved in the amperometric transduction (detection potential or composition of electrolytic cell and H_2O_2/HQ

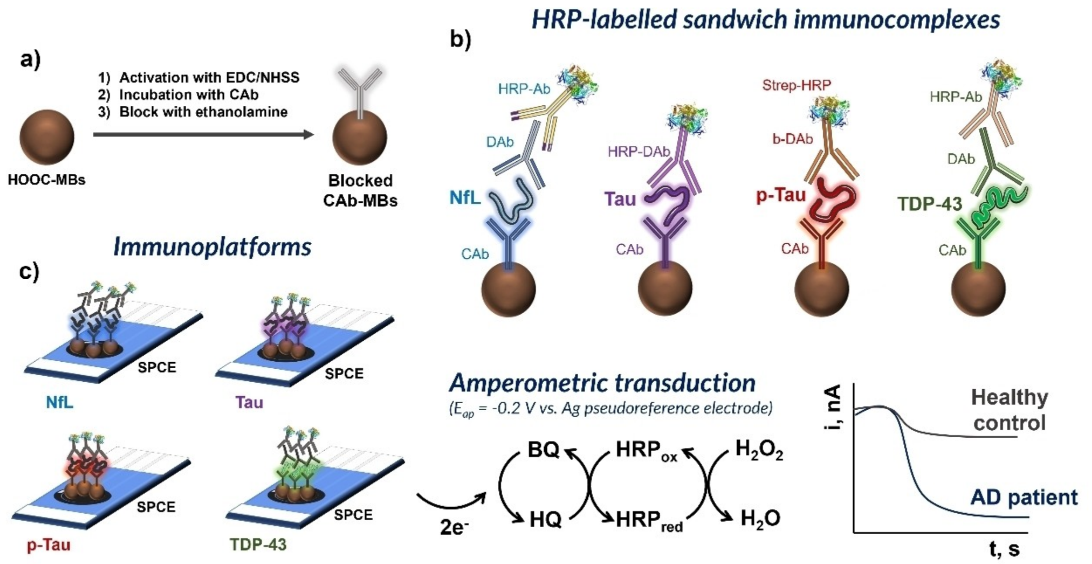

Figure 1. Schemes of the developed electrochemical immunoplatforms for the determination of NfL, Tau, p-Tau and TDP-43 involving the modification of MBs with CAbs (a), the formation of sandwich immunocomplexes labelled with HRP (b) and amperometric detection at SPCEs (c).

system) were taken from previous works.[44–46] Table 1 summarizes the obtained results whose detailed discussion is made in the Supporting Information (Figures S1–S3 and related text).

Analytical performance for the single determination of NfL, Tau, p-Tau and TDP-43

Under the selected experimental conditions summarized in Table 1, the developed bioplatforms provided the calibration graphs and the analytical characteristics shown in Figure 2 and Table 2, respectively. It should be noted that the results obtained with the NfL immunoplatform have already been reported previously[43] and are again presented in this paper only for comparative purposes.

Figure 2 shows as in all cases linear calibration plots between the variation in the measured cathodic current and the concentration of the respective neurodegenerative biomarker ($r > 0.99$) were obtained over a wide range of concentrations. It is important to highlight the very low detection limit (LOD) values achieved in assays lasting between 60 and 90 min counting from the CAb-MBs preparation (Table 2). The obtained sensitivity allows the determination of the target biomarkers in human serum or plasma, where the reported values for healthy individuals are close to 5–10 $pg\,ml^{-1}$ for NfL,[47,48] Tau,[29,49] and p-Tau.[32,50]

Table 1. Optimization of key experimental variables affecting the preparation of the developed immunoplatforms for the single amperometric determination of NfL, Tau, p-Tau and TDP-43.

Experimental variable	Selected value NfL	Tau	p-Tau	TDP-43
[CAb], $\mu g\,ml^{-1}$	25	25	25	25
Incubation time CAb, min	60	60	60	60
Number of incubation steps	2	2	2	2
[DAb_{NfL}], dil.	1/25	–	–	–
Incubation time NfL + DAb_{NfL} mixture solution, min	30	–	–	–
[$HRP\text{-}Ab_{NfL}$], dil.	1/10	–	–	–
Incubation time $HRP\text{-}Ab_{NfL}$, min	30	–	–	–
Incubation time Tau, min	–	45	–	–
[$HRP\text{-}DAb_{Tau}$], $\mu g\,ml^{-1}$	–	1	–	–
Incubation time $HRP\text{-}DAb_{Tau}$, min	–	15	–	–
[$b\text{-}DAb_{p\text{-}Tau}$], dil.	–	–	1/100	–
Incubation time p-Tau + $b\text{-}DAb_{p\text{-}Tau}$ mixture solution, min	–	–	60	–
[Strep-HRP], dil.	–	–	1/500	–
Incubation time Strep-HRP, min	–	–	30	–
[DAb_{TDP-43}], $\mu g\,ml^{-1}$	–	–	–	1
Incubation time TDP-43 + DAb_{TDP-43} mixture solution, min	–	–	–	60
[$HRP\text{-}Ab_{TDP-43}$], dil.	–	–	–	1/500
Incubation time $HRP\text{-}Ab_{TDP-43}$, min	–	–	–	30

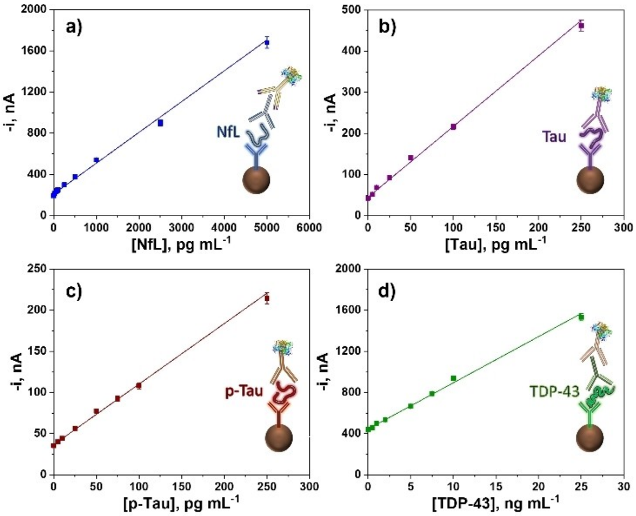

Figure 2. Calibration plots provided by the proposed immunoplatforms for the single amperometric determination of NfL (a), Tau (b), p-Tau (c) and TDP-43 (d) standards.

TDP-43 levels in plasma are not clearly defined for subjects without dementia. However, elevated TDP-43 concentrations in plasma[51–53] and plasma neuronal-derived exosomes[54] have been reported for patients diagnosed with AD, suggesting that TDP-43 levels in human plasma would be in the ng ml^{-1} range. Therefore, the bioplatform developed for TDP-43 would be suitable for its determination. Moreover, the four bioplatforms exhibited a great reproducibility in their measurements with relative standard deviation (RSD) values between 2.3–3.3 % calculated from the amperometric responses measured with 10 different bioplatforms prepared in a similar manner on the same day. The stability of the CAb-MBs (kept after their preparation resuspended in 100 mM PBS filtered at pH 7.4 at 4 °C) (see Figure S4 in the Supporting Information) allow us to conclude that no significant loss of sensitivity of the prepared bioplatforms was apparent for at least 20 days.

In addition, the selectivity of the immunoplatforms was assessed by performing amperometric measurements for 0 and 500 pg ml^{-1} NfL, 100 pg ml^{-1} Tau, 50 pg ml^{-1} p-Tau, and 10 ng ml^{-1} TDP-43 standards prepared in the absence and in the presence of other proteins and non-target neurodegenerative biomarkers coexisting in human plasma at the concentration levels expected in healthy controls (HC, Figure S5 in the Supporting Information). None of the tested substances provoked a significant interference for the determination of NfL and Tau (Figures S5a and S5b, respectively), while the determination of p-Tau is affected by the presence of human serum albumin (HSA) (Figure S5c) and that of TDP-43 by the presence of HSA and human immunoglobulin G (hIgG) (Figure S5d). The interference of these two biomolecules has already been reported for other immunosensors and may be attributed to the coexistence of human anti-animal antibodies (HAAAs) in commercial hIgG and non-overly purified HSA.[55,56] However, as Figures S5c and d show, such interferences became insignificant when their concentration is 5-fold reduced, which makes it possible to rule out their influence if the analyzed plasma samples are at least 5-times diluted.

The analytical performance of the developed electrochemical bioplatforms has been compared with that claimed for other methodologies reported so far for the single determination of these neurodegenerative biomarkers (whose characteristic features are summarized in Table S1 in the Supporting Information). Several biosensors (electrochemical, optical, etc.) have been reported. For instance, Özgür et al., designed an NfL impedimetric immunosensor providing a LOD value (5.21 pg ml^{-1}) similar to that achieved with the developed bioplatform but with a methodology taking a much longer preparation time (14 hours).[57] Ye et al.[58] have recently developed a single-layer exfoliated reduced graphene oxide Tau immunosensor with a high sensitivity, similarly to that achieved by Yu et al.[59] with a SERS-based immunoassay involving tannin-capped silver nanoparticles and magnetic graphene oxide. Manoccio et al.[60] prepared an optical chip-based metamaterial, combining 3D chiral geometry with a functional core-shell nanoarchitecture, for the determination of TDP-43. Despite the high sensitivity achieved, these approaches imply the use of complex home-made materials and require multiple synthesis steps and long preparation times for the biosensor set-up

Table 2. Analytical characteristics offered by the electrochemical bioplatforms for the single determination of NfL, Tau, p-Tau (in pg ml^{-1}) and TDP-43 (in ng ml^{-1}).

Parameters	Individual bioplatforms NfL	Tau	p-Tau	TDP-43
Linear range	10–5,000	4.6–250	5.3–250	0.85–25
R^2	0.985	0.986	0.996	0.994
Slope	0.30 ± 0.01 nA ml pg^{-1}	1.72 ± 0.09 nA ml pg^{-1}	0.74 ± 0.01 nA ml pg^{-1}	45 ± 1 nA ml ng^{-1}
Intercept, nA	210 ± 5	45 ± 2	37 ± 1	445 ± 7
LOD[a]	3.0	1.4	1.6	0.26
LQ[b]	10.0	4.6	5.3	0.85
Assay time, min	60	60	90	90
RSD (n = 10), %[c]	3.3 (500)	3.0 (100)	3.3 (50)	2.3 (10)
Stability, days	22	20	26	23

[a] LOD = 3 × s_b/slope; [b] LQ = 10 × s_b/slope (s_b = standard deviation of 10 measurements in the absence of target protein); [c] The concentration of the standard at which the RSD was evaluated is indicated in parentheses.

(more than 10 hours reported by Ye et al.[58]), which does not make them compatible with application in point-of-care (POC) devices.

Commercial kits and state-of-the-art methodologies using specific antibodies are widely utilized in clinical laboratories worldwide, mainly ELISA or SIMOA assays. However, the low sensitivity (LODs close to 10 pg ml^{-1} for NfL, Tau and p-Tau) of the commercial ELISA kits make these methodologies not useful for the determinations in certain samples such as plasma or serum where the concentrations of these biomarkers in healthy individuals are close to their detectability (Table S1 in the Supporting Information). In fact, they have been developed and their use recommended for the analysis of cerebrospinal fluid (CSF), tissue homogenates, buffered solutions, and cell culture supernatants.

On the other hand, SIMOA (Single Molecule Array) commercialized by Quanterix has established itself as the "gold standard" for the determination of many neurodegenerative biomarkers due to their ultra-sensitivity, in the fg ml^{-1} range for serum or plasma analysis (see Table S1 in the Supporting Information), and automated simple protocols.[61] However, the expensive cost of the equipment, the need for skilled personnel, and the large amount of sample per determination (e.g., 152 μl of diluted fluid sample in SIMOA® Tau #101552) restrict its use to high-resource centralized settings. In this context, the developed immunoplatforms are advantageous because their sensitivity allows the determination of AD-related biomarkers in plasma using small sample volumes (5 μl of plasma per measurement), short assay times, simple protocols, and lower cost of instrumentation.

In fact, these remarkable advantages in combination with the compatibility of multiplexed electrochemical detection led us to transfer the developed electrochemical immunoplatforms to a multiplexed platforms to perform the simultaneous determination of the multi-AD-related biomarkers, with the advantages this entails for more reliable diagnoses. Recently, Kim et al., designed a multiplexed fluorescence biosensing platform using densely aligned single-walled carbon nanotubes (CNTs) for the simultaneous determination of Aβ_{40}, Aβ_{42}, t-Tau and p-Tau$_{181}$ in human plasma.[62] This sensor array achieved femtomolar LOD level and allowed a clear discrimination of AD patients from healthy individuals. However, the CNT-based sensor required multiple steps for the preparation and functionalization of the densely aligned CNT film, prolonging the fabrication for more than 24 hours. The simplicity of the preparation protocols and short assay times provided by electrochemical biosensors involving MBs would make the immunoplatforms presented in this work ideal candidates for multidetermination. Therefore, we report here the first multiplexed electrochemical bioplatform for the simultaneous determination of NfL, Tau, p-Tau and TDP-43.

Multiplexed immunoplatform for the determination of NfL, Tau, p-Tau and TDP-43 AD-related biomarkers

Figure 3a compares the amperometric responses using magnetic bioconjugate replicates prepared in the absence and presence of each biomarker, and also the corresponding S/B ratio values, recorded at SPCEs and SP$_4$CE. As expected, smaller signals were obtained at the SP$_4$CE due to the smaller surface

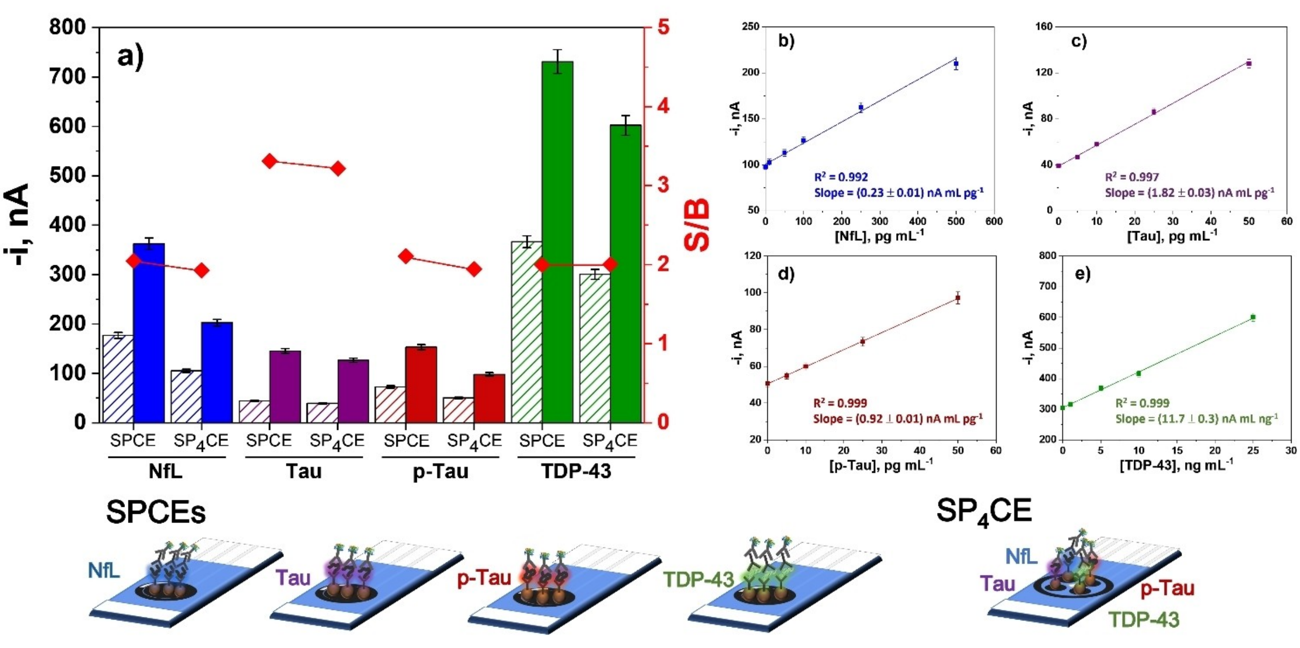

Figure 3. Comparison of the amperometric signals achieved at the multiplexed electrochemical bioscaffolds (SP$_4$CE) and the single ones (SPCEs) in the absence (stripped bars, B) and in the presence (solid bars, S) of 500 pg ml^{-1} NfL, 50 pg ml^{-1} Tau, 50 pg ml^{-1} p-Tau and 25 ng ml^{-1} TDP-43 standards, and the respective S/B ratios (a). Calibration plots constructed with the multiplexed immunoplatforms for the amperometric determination of NfL (b), Tau (c), p-Tau (d) and TDP-43 (e) standards.

area of 4-WE compared with that of the WE in the SPCE (ϕ_{WE} = 4 mm in SPCE vs. 2.95 mm in SP_4CE). However, quite similar S/B ratios were found for the single and multiplexed platforms, and, therefore, no apparent cross-reactivity between adjacent working electrodes was observed thus allowing the simultaneous determination of NfL, Tau, p-Tau and TDP-43 in a single device. Figures 3b–e displays the calibration plots recorded with the multiplexed platform.

The multiplex immunoplatform was faced to the analysis of plasma samples from AD patients and HC. Following the procedure previously reported for the determination of NfL in human plasma,[43] the samples were 5-fold diluted with BB (5 µl of plasma in a total incubation volume of 25 µl per replicate), and quantification was performed by applying the standard addition method with NfL increasing concentrations (10–100 pg ml^{-1}), Tau (10–50 pg ml^{-1}), p-Tau (10–50 pg ml^{-1}) and TDP-43 (2.5–10 ng ml^{-1}) standard solutions. The results obtained are summarized in Table 3 and shown in Figure 4.

As shown in Figure 4, all AD patients exhibited increased expression of the four target neurodegenerative biomarkers compared to HC (p-value < 0.000001 in all cases). Regarding NfL, larger concentrations were found in patients diagnosed with AD (mean value of 33 ± 2 pg ml^{-1}), allowing a clear discrimination with HC (mean value of 7 ± 1 pg ml^{-1}). These results agree with previous reports on NfL expression[47,63,64] and support their validation as a clinical biomarker of AD. The estimated average concentrations of Tau (12 ± 1 pg ml^{-1}) and p-Tau (20 ± 2 pg ml^{-1}) in plasma from AD patients were 3.5 and 3.8 times larger than those for HC, respectively, and the found levels are comparable to reported clinical values.[65] Moreover, TDP-43 levels from AD patients (mean value of 7.0 ± 0.6 ng ml^{-1}) were 4.3 times larger than for HC, thus confirming the potential of this plasma biomarker to contribute to the diagnosis of AD patients.

As it has been already mentioned, the limited sensitivity of conventional ELISA methodologies for NfL, Tau and p-Tau does not allow to contrast the accuracy of the results provided by the multiplexed immunoplatforms. However, interestingly, the results obtained for NfL match well with those reported

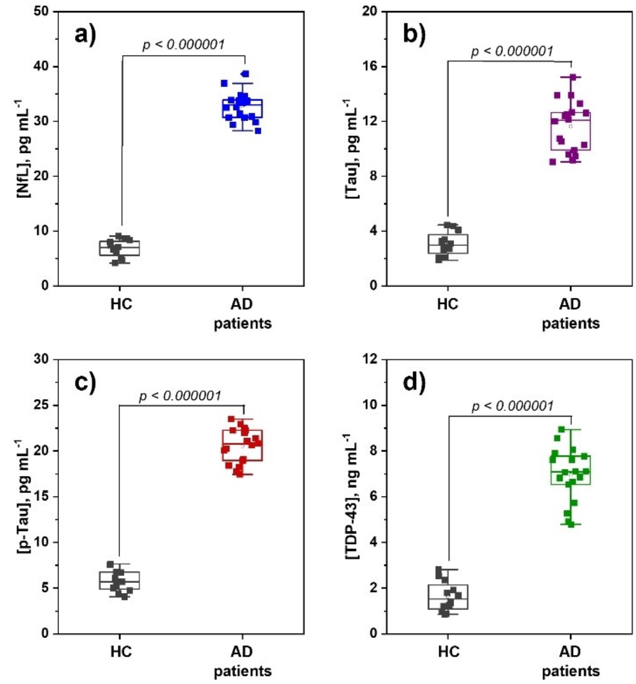

Figure 4. NfL (a), Tau (b), p-Tau (c) and TDP-43 (d) concentrations determined with the multiplexed immunoplatform in human plasma from 4 HC and 6 AD patients (three replicates per determination). Range, median and interquartile range (IQR) and p-value obtained from a t-test statistical analysis are shown in boxplots.

previously for the analysis of the same samples with the individual immunoplatform (see Table 3).[43]

Moreover, the ROC curves shown in Figure S6 (in the Supporting Information) confirmed the diagnostic potential of this signature of biomarkers (AUC, sensitivity, and specificity values of 100% when considering both individually and jointly the 4 biomarkers). This allows the establishment of the following cut-off values for the four biomarkers in human plasma: NfL (19.35 pg ml^{-1}), Tau (6.75 pg ml^{-1}), p-Tau (12.5 pg ml^{-1}) and TDP-43 (3.8 ng ml^{-1}).

It is worth emphasizing that the parameters of the ROC curves displayed in Figure S6 seem to indicate that even

Table 3. Concentrations in plasma of NfL, Tau, p-Tau (in pg ml^{-1}) and TDP-43 (in ng ml^{-1}) determined with the multiplexed electrochemical immunoplatform in HC and AD patients. RSD values in % for n = 3 are given into parentheses. For comparative purposes, the NfL concentrations measured with the immunoplatform previously reported for its single determination[43] are also given in the Table.

Plasma sample	Age[a]	Multiplexed immunoplatform NfL	Tau	p-Tau	TDP-43	NfL concentration found with the single immunoplatform[43]
HC 1	72 ± 2	5 ± 1 (9.9)	2.8 ± 0.6 (8.4)	6 ± 1 (8.2)	1.3 ± 0.2 (6.0)	4 ± 1
HC 2		7 ± 1 (7.0)	3.2 ± 0.7 (9.2)	5.3 ± 0.9 (6.5)	0.9 ± 0.2 (7.6)	7 ± 2
HC 3		7 ± 1 (7.5)	2.0 ± 0.3 (5.3)	7 ± 1 (6.1)	2.6 ± 0.6 (9.2)	5 ± 2
HC 4		8.7 ± 0.9 (4.2)	4.3 ± 0.5 (4.5)	4.4 ± 0.8 (7.7)	1.8 ± 0.3 (6.2)	9 ± 2
AD 1	85 ± 3	32 ± 4 (4.4)	13 ± 1 (3.6)	19 ± 4 (7.7)	7 ± 1 (5.4)	31 ± 5
AD 2		30 ± 3 (4.7)	14 ± 3 (9.4)	20 ± 5 (8.9)	8 ± 2 (9.4)	26 ± 3
AD 3		36 ± 6 (7.1)	10 ± 1 (5.8)	23 ± 2 (2.7)	6 ± 1 (8.9)	30 ± 3
AD 4		33 ± 3 (3.8)	13 ± 2 (7.1)	18 ± 2 (4.2)	5.0 ± 0.6 (5.0)	38 ± 4
AD 5		35 ± 4 (4.7)	9.2 ± 0.5 (2.2)	21 ± 3 (5.9)	7 ± 1 (6.8)	41 ± 6
AD 6		31 ± 4 (5.5)	11 ± 2 (8.5)	21 ± 2 (4.7)	8 ± 2 (7.8)	32 ± 5

[a]Mean value ± SEM (standard error of the mean). Biomarker concentrations given as mean value ± $ts/\sqrt{n}$ (n = 3; α = 0.05).

analyzing a limited cohort of samples (4 healthy subjects and 6 patients diagnosed with AD) the single determination of the 4 selected biomarkers already provides a full discrimination between healthy individuals and patients diagnosed with AD. Although this may lead to think that quadruple screening would not be necessary, the multi-determination would be advantageous with respect to single screening in other cohorts of patients such as, for example, healthy individuals and patients diagnosed in early stages of Alzheimer's disease, as well as to discriminate between AD patients in proximal Braak stages or to improve reliability in longitudinal studies of patients where the variations of the 4 target biomarkers were smaller. It is also important to note that since protocols involved to perform single or quadruple detection are the same and that the only difference lies in performing amperometric transduction by capturing the magnetic immunoconjugates batches on disposable platforms for single or quadruple transduction, multiplexed detection does not really represent a significant complication.

Moreover, being fully aware that more studies are needed to analyze a larger number of patients diagnosed at different stages of AD, to assess the potential for disease staging, and to perform longitudinal studies of disease progression to monitor the neurodegenerative process and possible response to treatment, we consider these results very promising to offer AD patients a minimally invasive and more reliable diagnosis considering the heterogeneity and complexity of AD pathology.

Conclusion

This work reports the first multiplexed electrochemical immunoplatform for the simultaneous determination of four AD-candidate biomarkers (NfL, Tau, p-Tau and TDP-43). The methodology involves the formation of sandwich-type immunocomplexes labelled with HRP for each of the target biomarker and their amperometric transduction on platforms for quadruple detection using the H_2O_2/HQ system. The excellent analytical performance in terms of sensitivity and selectivity allows the sensitive and precise determination of NfL, Tau, p-Tau and TDP-43 in human plasma using only 5 μl of sample per determination and in an assay-time comprised between 60 and 90 min. The analysis of the obtained results by means of ROC curves demonstrates the potential of the immunoplatform to open a new avenue for the minimally invasive, reliable, and clinically actionable diagnosis of patients with AD. It is also important to bring up that the simplicity, compatibility with miniaturization and the affordable cost (both by determination and the instrumentation required) make the immunoplatform suitable to be employed by any user and both in hospitals and other decentralized and/or low-resource environments.

Experimental Section

Apparatus and electrodes, reagents and solutions, and the followed procedures (preparation of the bioplatforms, amperometric measurements and analysis of plasma) are described in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Alzheimer's disease · Candidate biomarkers · Electrochemical multiplexing immunoplatform · Magnetic microbeads • Plasma

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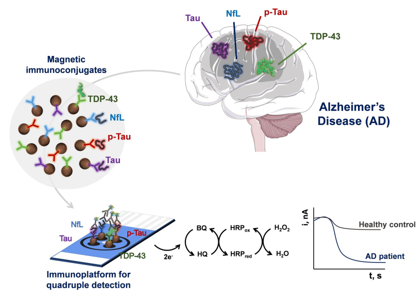
RESEARCH ARTICLE

A quadruple immunoplatform for the amperometric determination of AD-candidate protein blood biomarkers (neurofilament light chain, NfL, Tau, phosphorylated Tau, p-Tau and TAR DNA-Binding Protein 43, TDP-43) to assist in the reliable and minimally invasive Alzheimer's disease diagnosis was developed.



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Paving the Way for Reliable Alzheimer's Disease Blood Diagnosis by Quadruple Electrochemical Immunosensing

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