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Dual on-the-move electrochemical immunoassays for the simultaneous determination of amyloid-β (1−42) and Tau in Alzheimer's patient samples

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ABSTRACT

Here, we report catalytic micromotors (MM)-based electrochemical immunoassays for the on-the-move dual and simultaneous determination of Amyloid-β (Aβ-42) and Tau protein (Tau) ($MM_{Aβ\text{-}42}$-MM_{Tau}) as relevant Alzheimer's disease biomarkers in brain tissue, cerebrospinal fluid, and plasma diagnosed samples. Combining the binding capacity of the antibody's functionalized polypyrrole (PPy) layer of MM with the self-propulsion from the PtNPs layer thanks to the decomposition of hydrogen peroxide, the approach yielded excellent detection limits ($LOD_{Aβ\text{-}42}$=0.04 ng/mL, LOD_{Tau}= 0.4 pg/mL) using low sample volumes (30 µL) and short analysis times (15 min) to detect both biomarkers. Quantitative analysis by $MM_{Aβ\text{-}42}$-MM_{Tau} was carried out without any clinical sample dilution (linear ranges are between 0.1 and 5 ng/mL for Aβ-42 and from 1 to 10^6 pg/mL in the case of Tau), highlighting the versatility of the approach to quantify Aβ-42 and Tau levels at different dynamic ranges. $MM_{Aβ\text{-}42}$-MM_{Tau} showed superior analytical capabilities to the single molecule counting technology (SMCx) during quantitative analysis in all sample classes tested, reporting a difference in quantitative levels for both biomarkers between healthy and diseased individuals and an increase in the levels with disease progression, except in plasma samples where no relationship between biomarker levels and disease progression was found.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide and the leading cause of dementia in the elderly [1]. AD impairs thinking skills, and cognition causing personality changes, memory loss, and language deterioration [2]. It's estimated that Alzheimer's disease and other dementias will affect over 55 million people worldwide in 2023 and will increase two times every 20 years implying billions of costs in total societal burden [3]. This disease presents risk factors such as old age, lifestyle choices [4], susceptibility genes, environmental factors [5], and other diseases [6]. Despite immense efforts, this disease is still incurable due to the unreliable diagnosis of AD in the pre-dementia stages, which results in late treatment, implying non-reduction of the possibilities of death, and continuing with the necessity of high societal costs that come from the development of new prevention modalities [7].

Currently, the diagnosis of AD in major cases still requires post-mortem verification. Besides, during patient life, sample/biopsies analysis technologies are very invasive in the extraction process, and are not specific enough, needing long times for their development [8,9]. The misunderstanding of the molecular events and biological pathways [10] leads to an unreliable diagnosis of AD in the pre-dementia stages, being Alzheimer's disease patients clinically diagnosed when symptoms appear and treatments is less effective [11]. Current AD diagnosis is based on analysis of cerebrospinal fluid (CSF) and imaging biomarkers such as Amyloid-β (Aβ) plaques, fibrillary Tau, and biomarkers of

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neuronal damage [9,12,13]. Analysing them requires expensive and highly invasive techniques, available only in specialized clinical centers. Strategies for identification of clinical biomarkers to provide supportive features for the diagnosis of AD in the early stage, before the onset of symptoms, is a challenging area to apply effective therapies, reducing the side effects of the disease, such as brain deterioration [14,15].

The principal hallmarks of AD are the intracellular accumulations caused by hyperphosphorylated forms of Tau in neurofibrillary tangles (NFTs) and the extracellular deposits caused by insoluble aggregates of Aβ peptide, causing chronic neuroinflammation, oxidative stress, synapse dysfunction, and neuronal death [16,17].

The 42-amino acid form of Aβ (Aβ-42) is produced by the amyloidogenic proteolysis of amyloid precursor protein (APP) by β- and γ-secretases and is the major component of amyloid beta plaques, which are formed by the accumulation of Aβ oligomers and insoluble fibrils in the walls of cerebral blood vessels. [18]. Even, some studies indicate Aβ biomarkers are useful for monitoring AD progression due to the early saturation produced by the Aβ accumulation [19,20].

Tau is a microtubule-binding protein necessary for the assembly and stabilization of microtubules and localized in the axons of nerve cells. In the healthy adult human brain, Tau presents six different isoforms, produced from a single gene by alternative mRNA spicing [21]. However, mutations and post-translational modifications of Tau lead to a change in their structure and the formation of aggregates [22].

The measurements of both Aβ and Tau allow an approach to a clinical diagnosis of AD due to their cost-effectiveness, and easier accessibility compared with other biomarkers [23]. Although these biomarkers are mainly used for Alzheimer's diagnosis through individual detection it does not provide an accurate diagnosis. In this frame, it is important to highlight that the simultaneous detection and quantification of clinical biomarkers at the earliest stages of AD is a challenging area. As stated above, some studies consider that Aβ biomarker is useful to monitor the disease progression because its accumulation in the brain stabilizes the level of Aβ-42 in CSF over time [19,20]. Besides, other studies consider that the multiplexed evaluation of two of these biomarkers (Aβ-42 and Tau) is more accurate than individual approaches because of the dementia pathology and the complexity of AD pathogenesis [24–26].

Moreover, the obtention of clinical samples can be challenging, even by professionals, due to the invasive extraction in some cases as CSF, or the difficulties in the extraction procedure in some patients with old age, obtaining a low volume of samples. Because of that, the necessity to implement a multiplexed detection with several biomarkers to ensure the diagnosis of the disease using the low volume as possible is a goal to reach.

The literature has reported different approaches for individual determination of Aβ-42 [27–32] and Tau [33–38] based on different approaches and techniques. However, these methods require sophisticated instrumentation, are highly complex, sample- and time-consuming, and costly. In addition, as mentioned above, samples from Alzheimer's patients are difficult to obtain, so the development of novel diagnostic tools with low sample requirements is also needed.

Song et al. reported an interesting label-free optical nanosensor for dual Aβ-42 and Tau detection in a mixture of diluted CSF samples with a limit of detection (LOD) of 7.8 pg/mL of Aβ-42 and 15.6 pg/mL of Tau in buffer and only requiring 1 μL [39]. The work of Song et al. obtained one of the lowest limits of detection (59.1 and 8.6 fg/mL for Tau and Aβ-42 respectively) for these proteins in a multiplexed format [40], allowing the detection of these biomarkers at the earliest stages when the treatment is more effective. However, most of the methods reported in the literature do not use real clinical samples [39,41–43], need long times of analysis (> 40 min) [40,41,44,45], or do not analyse different matrix samples, focusing individually only on studying CSF [46–49], or plasma [50,51]. The last aspect is a relevant parameter due to the difficulties in obtaining patient samples.

Micromotor (MM) technology become a disruptive approach to analytical biosensing [52]. Catalytic MM are microdevices with autonomous motion capabilities coming from a chemical reaction (commonly decomposition of hydrogen peroxide as fuel on platinum into water and oxygen bubbles), which allows the search of the analyte in low volume of sample with improved analytical performance due to the enhancement of fluid mixing produced by the oxygen gas microbubbles tail [53]. Among other protocols, MM can be prepared by template-based electrodeposition of polymers [54], metals [55], and carbon nanomaterials [56], and can be functionalized with several receptors, such as antibodies [57], aptamers [58], and enzymes [59].

Recently, we have developed MM-based immunoassays for the determination of Aβ-42 [29] and aptassays for the determination of its oligomer [60] with excellent analytical results, revealing the analytical potential of this technology for the diagnosis of AD too. Inspired by our previous works, here we are proposing for the first time a dual immunoassay approach using magneto-catalytic MM for the simultaneous determination of two hallmarks of Alzheimer's disease (Aβ-42 and Tau) in relevant clinical samples such as left prefrontal cortex brain tissue (BT), cerebrospinal fluid (CSF), and plasma. The analytical approach proposed was based on two independent individuals *on the move* electrochemical immunoassays sandwich-type for each biomarker and measured through a double screen-printed carbon electrode (dSPCEs). Due to the complexity of this analysis, the final goal of this work is to propose a screening tool for fast and reliable pre-assessment of the presence of both biomarkers, to discriminate the difference of levels between individuals and disease stages paving the way to a more reliable diagnostic.

2. Experimental

2.1. MM functionalization

Reagents, samples, and apparatus as well as MM electrosynthesis were described in Supporting Information. MM were previously functionalized to bind the specific capture antibodies. In the case of Tau recognizing MM, APBA was used to provide oriented immobilization of Tau-specific antibodies. In this sense, carboxyl-terminated groups of the MM surface (25 μL of MM solution, approximately the number of MM was 5000) were activated through the addition of 6.25 μL of a 100 mM EDC/NHSS solution prepared in 0.1 M MES buffer, pH 5, for 30 min at 25°C. Then, MM were washed 2 times with MES buffer, then added 12.5 μL of APBA 50 mM in 0.1 M MES buffer, pH 5.0, for 3 h at room temperature. Then, the functionalized MM were incubated with a solution of 12.5 μL of 25 μg/mL c-Ab_{Tau} for 30 min. Once the MM were washed with PBS buffer to remove the supernatants, they were resuspended in PBS and maintained at 4°C for up to 60 days.

The functionalization of Aβ-42 recognizing MM was performed following the conditions described in our previous work [29]. Briefly, 25 μL of MM solution, approximately 5000 MM were activated through the addition of 6.25 μL of a 100 mM EDC/NHSS solution prepared in MES buffer, pH 5, for 30 min at 25°C. After washing twice with MES buffer, 12.5 μL of streptavidin 400 μg/mL in MES buffer was added and incubated for 1 h at room temperature. After adequate washing steps with MES and PBS buffer, the streptavidin-modified MM was incubated in 12.5 μL of a solution containing 25 μg/mL c-AbAβ-42 in PBS for 30 min. Finally, MM were separated from the supernatant and resuspended in 25 μL of PBS for maintenance for up to 50 days at 4 °C.

2.2. MM Immunoassay strategy

Individual sandwich-type immunoassays for each analyte were previously performed in independent microcentrifuge tubes, while simultaneous electrochemical detection was carried out in a dual screen-printed carbon electrode.

Anti-Tau modified MM were added to a solution (2 μL of total volume) that contained the sample analyte (1.77 μL), d-Ab_{Tau} (1 μg/mL) dissolved in PBS-BSA and H_2O_2 (2 %) as MM fuel. The self-propulsion of

MM, due to the catalytic reaction between the inner Pt layer and H_2O_2, provides the biorecognition of Tau in the sample in 10 minutes, while the addition of 100 μL of PBS causes the MM to stop and, thanks to the intermediate magnetic layer and the aid of a magnet, the supernatant can be removed. After several washing steps with PBS, the MM wearing the immunocomplex on board can be translated to the surface of the screen-printed electrode for electrochemical measurements.

Aβ-42 was determined in a similar way as described in detail in our previous work [29]. In this case, 25 μL of total volume was used (23 μL of the sample), and 15 minutes were needed for on-the-move biorecognition of Aβ-42 in the sample.

2.3. Dual electrochemical measurements

MM-immunocomplexes of Tau and Aβ-42 were respectively reconstituted in 5 μL of 1 mM of hydroquinone (HQ) each and transferred to their corresponding working electrode (WE_1 for Aβ-42 and WE_2 for Tau), followed by the addition of hydroquinone to reach a final volume of 45 μL. To perform the (Chrono)amperometric measurements, a potential of −0.2 V was applied. After baseline stabilization, a solution of H_2O_2 (5 μL to obtain a 5 mM final concentration) was added, while the signal was obtained. The results were based on the difference between the steady-state and background currents at 200 s. Sigma Plot 15.0 software was used to fit the results to a four-parameter logistic equation, as shown in Eq. 1.

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

In the equation, i is the amperometric current, while i_{max} and i_{min} are the maximum and minimum values of the calibration plot, respectively. EC_{50} is the value of the analyte concentration corresponding to 50 % of i_{max}, and h is the hill slope. The limit of detection (LOD) was estimated using the criteria 3 S/m and the limit of quantification (LOQ) 10 S/m. S was calculated as the standard deviation (n=10) measuring the lowest biomarker concentration used in the calibration (0.1 ng/mL for Aβ-42 and 1.0 pg/mL for Tau), while m is the slope from the linear calibration plots.

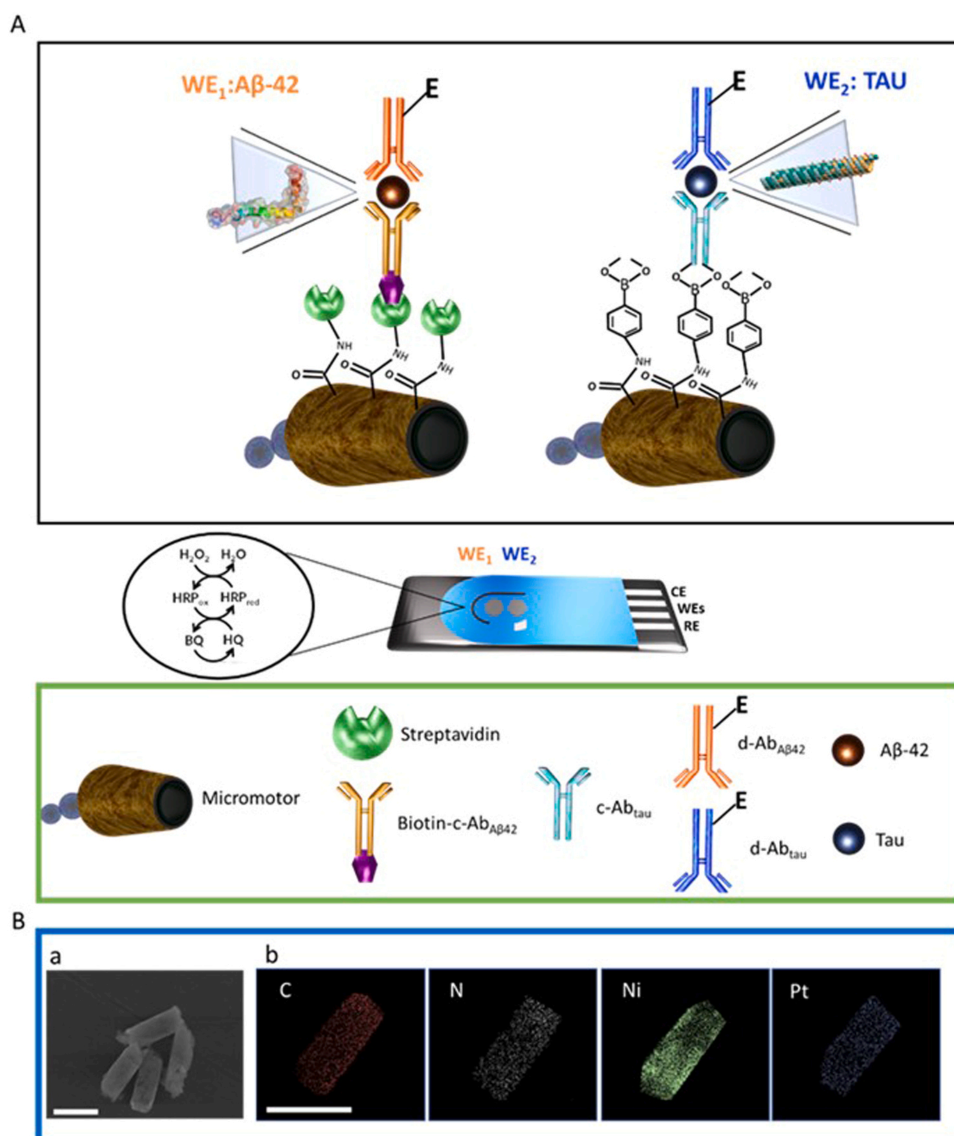

Fig. 1. Scheme for dual MM-based sandwich-type electrochemical immunoassays for Aβ-42 and Tau determination (A). SEM images (a), and EDX analysis (b) of PPy/Ni/PtNPs MM_{Tau} (B). Scale bar: 10 μm SEM and EDX images.

2.4. SMCxPRO immunoassays

SMCxPRO instrument (Merck) was used for the total Aβ-42 (03–0146–00) and Total Tau (03–0185–00) determination in BT protein extracts, CSF, and plasma samples. In the quantification of Aβ-42 or total Tau protein levels, CSF and plasma samples were 1/5 diluted, whereas 100 μg of each BT protein extract sample was analyzed.

3. Results and discussion

3.1. MM-based dual immunoassay approach: strategy, characterization, and optimization

Inspired by our previous work [29] on the development of MMs for the determination of Aβ-42, and due to the significance of the multiplexed determination of Alzheimer's disease biomarkers, here we propose an analytical approach based on PPy/Ni/PtNPs MMs for the dual determination of Aβ-42 and Tau ($MM_{A\beta\text{-}42}$-MM_{Tau}). Fig. 1 illustrates a scheme of the analytical strategy proposed. After specific on-the-move independent biorecognition of each protein biomarker, simultaneous electrochemical detection is performed onto dSPCEs improving the analytical information toward a more reliable diagnostic, while using low clinical sample volumes. The magnetic characteristics of MM through their intermediate Ni layer allow their retention onto each defined electrode, thanks to the use of a magnet positioned on the electrode back. Although both immunoassays make use of the same reporter enzyme (HRP), the spatial differentiation of each electrode avoids the crosstalk of the respective specific signals, as it will be demonstrated in the following sections.

As illustrated in Fig. 1A, pioneer MM_{Tau} were designed involving an APBA functionalization-based chemistry, and then, to improve the reliability of the AD diagnosis, the $MM_{A\beta\text{-}42}$-MM_{Tau} approach was evaluated toward the dual biomarker's determination using clinically diagnosed samples with Alzheimer's disease (left prefrontal cortex brain tissue, cerebrospinal fluid, and plasma).

Specifically, MM_{Tau} functionalization involving APBA instead of streptavidin was used to provide oriented immobilization of Tau-specific antibodies. Differences between functionalization approaches are mainly due to the commercial antibodies' characteristics. While Aβ-42 capture antibodies are biotin labelled, which allows their binding to streptavidin functionalized MM, commercially available Tau antibodies do not present any adequate labelling. In this sense, it is well-known that APBA leads to the formation of cyclic boronate esters at room temperature [61] by reaction with antibody sugars, allowing their oriented immobilization onto activated MM surface [62].

PPy/Ni/PtNPs MM_{Tau} characterization was carried out (Fig. 1B). The defined shape and dimensions (approx. 7 μm of width, 15 μm of height, see Fig. 1B-a), together with their homogeneous layer-by-layer composition (C and N from PPy as the sensing outer layer, Ni as the magnetic intermediate layer, and Pt as the catalytic inner layer) was also confirmed for MM_{Tau} as revealed in Fig. 1B-b.

In the first stage, on-the-move immunoassay optimization was performed for Tau individual determination (MM_{Tau}). Different variables related to the antibody immobilization onto the MM_{Tau} surface for reliable biosensing, and efficient propulsion were studied, and selected values were listed in Table 1.

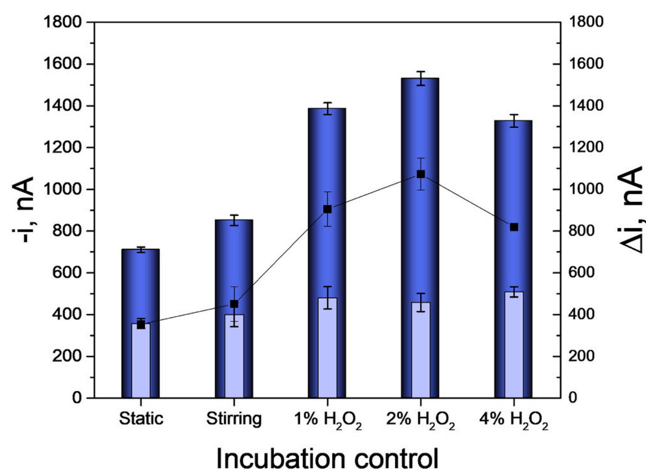

Fig. 2. Tau biosensing using stirring, static, and MM_{Tau} at different H_2O_2 levels. Conditions: 2 μL of standard Tau and 10 min of recognition time (other conditions see Table 1). Dark blue: *on-the-move* immunoassay (excess of Tau). Light blue (controls without Tau).

MM_{Tau} number is the first variable to be studied in the optimization of *on-the-move* biosensing. The optimal MM_{Tau} number for the Tau protein was 5000 (25 μL solution). This variable is critical to have enough binding sites for the immunocomplex's formation while avoiding their aggregation. The MM_{Tau} functionalization with capture antibodies (c-Ab_{Tau}) was carefully studied for both antibody concentration and incubation time. As depicted in Figures S1A and S1B the highest signal-to-noise ratio was reached at 25 μg/mL of c-Ab_{Tau} and 30 min., respectively. The reduction of nonspecific binding was accomplished by the addition of 5 % BSA.

Then, the detection antibody (d-Ab_{Tau}) concentration was tested in a range of 0.25–1.5 μg/mL using autonomous propulsion of MM_{Tau} in the absence and presence of Tau. As depicted in Figure S2A, 1 μg/mL produced the highest signal, being selected as the optimal concentration. Since available sample volumes, especially for CSF, are scarce and obtained through invasive procedures that can produce side effects, the necessary sample volume is a key parameter to study. As can be observed in Figure S2B, with only 2 μL of standard or sample (BT, CSF, or plasma) our approach can detect the Tau levels. The on-the-move affinity interaction time for Tau recognition was also evaluated. As can be observed in Figure S2C, 10 minutes are selected, since larger times do not produce significative signal improvement.

Fig. 2 compares MM_{Tau} bubble propulsion with different amounts of H_2O_2, with stirring and static conditions. As can be seen, the autonomous propulsion of MM obtains better biorecognition yields with 2 % H_2O_2 compared to external stirring and static conditions. These results

Table 1
Optimized parameters for *on-the-move* Tau and Aβ-42 dual determination.

Assay step	Parameter	Range studied MM_{Tau}	Selected Value	
			MM_{Tau}	$MM_{A\beta\text{-}42}$
Formation Inmunocomplex	**Micromotors number**	2400–10000	5000	5000
	[c-Ab] , μg/mL	5–50	25	25
	c-Ab time, min.	15–60	30	30
On-the-fly recognition	**[d-Ab] , μg/mL**	0.25–1.5	1	1
	Block step, [BSA] %	1–10	5	5
	Sample Volume, μL	2–25	2	25
	Recognition time, min.	5–45	10	15
	Fuel (H_2O_2), %	0–4	2	2

show that MM allows better mixing of fluids in low volumes increasing the efficiency of the immunoassay. On the other hand, a higher fuel concentration causes negative effects due to the increase in the number of bubbles generated, which makes the movement of the MM difficult, avoiding the free movement of them searching the analyte and also the inactivation of peroxidase enzyme due to the long-time presence of H_2O_2 needed for propulsion [63].

Finally, the potential interference of Aβ-42 on Tau determination was also studied, in the presence of BSA as blocking agent and a potential interferent as major protein constituent of CSF and plasma. As can be observed in Fig. 3 there are barely any differences between the signals obtained in the absence and presence of Aβ-42, demonstrating an excellent assay selectivity.

3.2. Analytical performance of $MM_{Aβ\text{-}42}$-MM_{Tau} approach for dual Aβ-42 and Tau determination

Then, the reliable dual determination of both biomarkers (Aβ-42 and Tau) was approached using a set of functionalized micromotors ($MM_{Aβ\text{-}42}$-MM_{Tau}).

Fig. 4 shows the control signals obtained at both working electrodes (WE_1 and WE_2) in the absence of one biomarker and the presence of the other at an elevated concentration, in each case. This experiment demonstrates that during all the measured time, the crosstalk by the diffusion of the enzymatic reaction product between both working electrodes was negligible.

Analytical features for the dual determination of Aβ-42 and Tau ($MM_{Aβ\text{-}42}$-MM_{Tau}) are listed in Table 2. Fig. 5 shows the sigmoidal curves and the calibration plots of Aβ-42 and Tau. For Aβ-42, the linear concentration ranged between 0.1 and 5 ng/mL, with a LOD and LOQ of 0.04 and 0.1 ng/mL, respectively. In the case of Tau, the linear working ranged from 1 to 10^6 pg/mL, with a LOD and LOQ of 0.4 and 1 pg/mL, respectively. In both cases, the LOQ and the linear response range allow the analyses without dilution of CSF, BT, and plasma samples of the clinically relevant concentrations in Alzheimer's disease. Excellent precision was also demonstrated toward repeatability (intra-day assays, RSD <4 % n=5) and intermediate precision (inter-day assays, RSD <5 % n=8), with independence of the biomarker/electrode channel used in the dual determination.

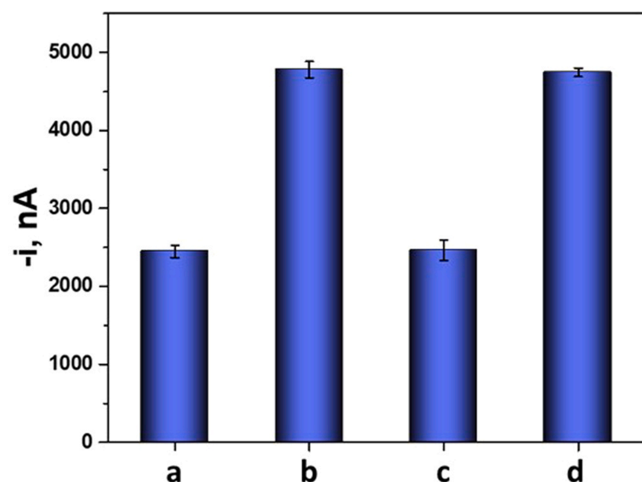

Fig. 3. Interference of Aβ-42 on individual immunoassay of Tau; a) 0 ng/mL Aβ-42 + 0 ng/mL Tau; b) 0 ng/mL Aβ-42 + 500 ng/mL Tau; c) 50 ng/mL Aβ-42 + 0 ng/mL Tau; d) 50 ng/mL Aβ-42 + 500 ng/mL Tau (in the presence of BSA as blocking agent and potential interferent as major protein constituent of CSF and plasma).

The stability of MM-c-Ab_{Tau} complexes was also studied for use as stock 'reagents". To this end, a battery of functionalized MM with the c-Ab_{Tau} was prepared and stored at 4 °C until use before the development of the MM_{Tau} immunoassays. The results demonstrated the stability of the MM-Ab_{Tau} complexes for at least 50 days (see Figure S3). Since stability was also shown for MM-$Ab_{Aβ\text{-}42}$ [29], excellent overall stability is consequently inferred for the $MM_{Aβ\text{-}42}$-MM_{Tau} approach. This is a valuable feature, as it reduces overall analysis times of the dual immunoassay.

3.3. Analysis of AD patients' samples from BT, CSF, and plasma

$MM_{Aβ\text{-}42}$-MM_{Tau} dual approach was applied for the analysis of three relevant clinical samples with different analytical challenges in the determination of biomarkers in them. Indeed, high selectivity is required for the analysis of the left prefrontal cortex BT as a high-significance *post-mortem* sample due to its inherent complexity. CSF is the gold clinical sample where the Alzheimer's disease biomarkers levels are at medium levels and fewer interferents can be found; however, it requires invasive techniques, and subsequently sample availability is hard to obtain. Plasma samples should be the most desirable one since a non-invasive technique is required, but a high sensitivity is needed due to the low concentration levels of biomarkers.

The quantitative results obtained by the MM-based dual immunoassay ($MM_{Aβ\text{-}42}$-MM_{Tau}) and the single molecule counting technology (SMCx) are listed in Table 3.

Different observations are inferred from the results shown, revealing the complexity of these analysis. First, for BT and CSF samples, a high agreement was obtained for the Aβ-42 levels obtained in both approaches (dual $MM_{Aβ\text{-}42}$-MM_{Tau} and SMCx), revealing an excellent accuracy for the determination of this biomarker in all examined samples as already demonstrated in its determination using a single approach [29]. This fact indicates that the determination of this biomarker in dual format is also reliable. It should be noted that this comparison was not possible for plasma samples due to the extremely low sample availability.

Secondly, the $MM_{Aβ\text{-}42}$-MM_{Tau} approach was able to quantify Tau levels in all three clinical samples analyzed and more importantly able to differentiate between healthy and Alzheimer's disease patients in post-mortem BT samples. Furthermore, the SMCx approach did not show analytical capabilities to differentiate between samples from diseased individuals and healthy individuals nor between Tau levels in all the different clinical samples used. Probably, the analysis of total Tau by SMCx was subjected to matrix effect problems associated with a high concentration of Tau in the samples, affecting SMCx measurements. However, these matrix effects were not identified in $MM_{Aβ\text{-}42}$-MM_{Tau} approach either, consequently, these results confirmed the great versatility of the $MM_{Aβ\text{-}42}$-MM_{Tau} approach to measure different biological matrices.

Additionally, the $MM_{Aβ\text{-}42}$-MM_{Tau} approach informed that in BT and CSF samples both Aβ-42 and Tau levels were observed to increase in parallel to the progression of the disease. It was interesting to notice the difference in the levels of both biomarkers in both samples, remarkably in Tau, between Braak stages, whereas plasma levels of both biomarkers were not found associated with the stage of the pathology. This agrees with the literature where during the analysis comparing eight groups

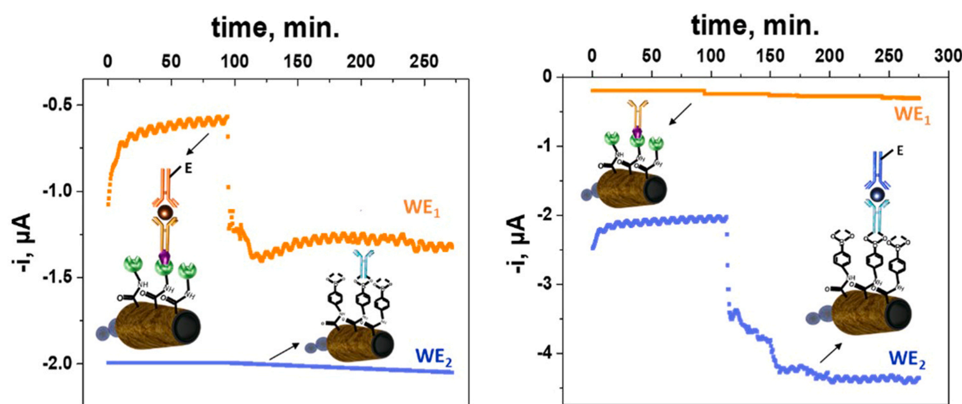

Fig. 4. Diffusional crosstalk between electrode assays: measurements of the signals produced by 50 ng/mL Aβ-42 (WE_1) + 0 ng/mL Tau (WE_2) (left); 0 ng/mL Aβ-42 (WE_1) + 500 ng/mL Tau (WE_2) (right). For other conditions see Table 2.

Table 2
Analytical characteristics for *on-the-move* Aβ-42 and Tau dual determination.

Analytical Characteristic	Aβ-42 assay (WE1)	Tau assay (WE2)
Linear range	0.1–5 ng/mL	1–10^6 pg/mL
r	0.994	0.992
LOD	0.04 ng/mL	0.4 pg/mL
LOQ	0.1 ng/mL	1 pg/mL
RSD intra-day (n=5)	4 %	2 %
RSD inter-day (n=8)	5 %	4 %

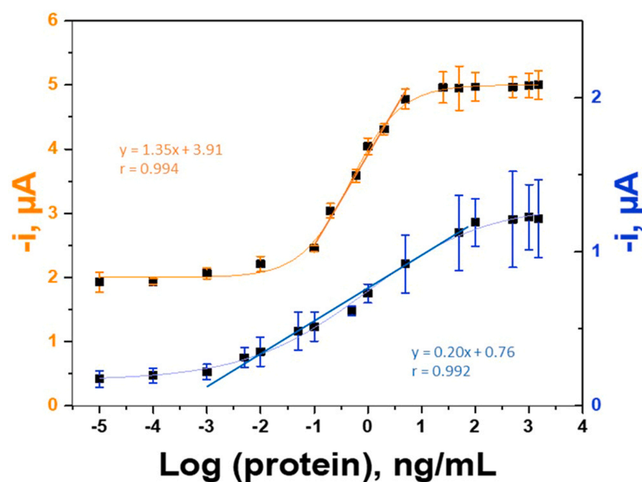

Fig. 5. Sigmoidal curves of Aβ-42 and Tau with their correspondence linear calibration plots (in PBS pH 7.5). For other conditions see Table 1.

defined by cognitive status and neuroimaging measures, there was a high overlap in plasma Tau levels. These results suggest that plasma Tau will not be useful as a stand-alone diagnostic biomarker in preclinical or prodromal AD [64]. Unfortunately, there is no evidence to date of a correlation between novel Alzheimer's biomarkers and disease stage progression in plasma samples.

Importantly, Tau values of the samples measured by $MM_{Aβ\text{-}42}$-MM_{Tau} were in the same order of magnitude between cases in comparison to controls, whereas for the BT protein extracts analysis, at least 100 times lower amount of total Tau was observed. These results also highlighted the versatility of these MM to detect Aβ-42 and Tau levels at different dynamic ranges. Noticeable, MM_{Tau} allow measuring total Tau, including the different variants of hyperphosphorylated Tau. Since increased hyperphosphorylated Tau deposits have been described in the advanced stages of the disease, the observed increase in Tau detection might be also associated with the measurement of hyperphosphorylated Tau, which is the main hallmark associated with neurofibrillary tangles in AD patients. Thus, the development of MM able to measure Aβ-42, total Tau, and the phosphorylated variants of Tau that have been described as more associated with the disease (phospho-(p)Tau181, pTau217, and pTau231) are guaranteed [65]. All these results confirmed the complexity of the analysis.

In this way, it is necessary to note that in these complex samples, the speed diminishes respecting buffer study from 102±20 μm/s to 58±5, 78±9, 84±7 μm/s in BT, CSF, and plasma, respectively (**video S1**). However, this reduction in the speed doesn't affect the function of our MM.

Supplementary material related to this article can be found online at doi:10.1016/j.snb.2024.136785.

Finally, compared with other reported works described in the literature for dual determination of Aβ-42 and Tau (see Table S1), the $MM_{Aβ\text{-}42}$-MM_{Tau} approach allowed the simultaneous detection of both biomarkers in shorter analysis times (<15 min) [40,44,45], and using diagnosed AD samples with high significance (BT, CSF, and plasma) in comparison with other works where mostly spiked samples and only a few cases dealing with just one type of clinical (diluted) samples assayed [39,40,45–51]. Although it has been reported that plasma Aβ-42 correlates with CSF levels and with Aβ-42 burden in the brain [66], for Tau it has been described a low correlation between Tau levels in CSF and plasma as indicated above [64]. Here, using unpaired BT, CSF and plasma samples, we observed, despite inter-individual variability, an increase in the levels of Aβ-42 in parallel to the progression of the disease in the samples analyzed. In contrast, while a correlation in BT and CSF samples was observed between Tau levels and disease progression too, this correlation was not found for plasma samples. The latter is also in agreement with a previous report, as discussed above, comparing eight groups of patients according to cognitive status and neuroimaging measurements, where a high overlap was observed in the plasma Tau levels among patients [64]. This makes this $MM_{Aβ\text{-}42}$-MM_{Tau} dual approach an interesting and competitive alternative in the field of biosensing of biomarkers of high significance in the search for an accurate diagnosis of Alzheimer's disease. On the other hand, the absence of a correlation between MM_{Tau} and SMCx prevented us from obtaining further inferences that should be explored in further reports.

Table 3
Analysis of BT, CSF, and plasma samples from Alzheimer's patients and healthy individuals.

Brain tissue (BT)					
Samples	B-Amyloid (WE_1)			Tau (WE_2)	
	$MM_{A\beta\text{-}42}$[a] (pg/mL)	SMCx[b] (pg/mL)	Er (%)	MM_{Tau}[a] (pg/mL)	SMCx[b] (pg/mL)
Control 1 Female, 61 years (Healthy)	287 ± 37	293	2	90 ± 40	36362
Patient 2 Female, 92 years (Braak IV)	683 ± 28	746	8	8000 ± 100	38471
Patient 5 Female, 86 years (Braak V)	2000 ± 81	1996	5	30000 ± 1000	34187
Cerebrospinal fluid (CSF)					
Samples	B-Amyloid (WE_1)			Tau (WE_2)	
	$MM_{A\beta-42}$[a] (pg/mL)	SMCx[b] (pg/mL)	Er (%)	MM_{Tau}[a] (pg/mL)	SMCx[b] (pg/mL)
Patient 7 Male, 86 years (Braak IV)	195 ± 24	184	6	26 ± 3	13489
Patient 8 Female, 93 years (Braak V)	824 ± 78	747	10	5000 ± 200	11341
Patient 10 Male, 90 years (Braak VI)	507 ± 23	559	9	94072	905
Plasma					
Samples	B-Amyloid (WE_1)			Tau (WE_2)	
	$MM_{A\beta-42}$[b] (pg/mL)	SMCx[c] (pg/mL)	Er (%)	MM_{Tau}[b] (pg/mL)	SMCx[c] (pg/mL)
Patient 12 Female, 75 years (Braak III)	120	ND	-	602101	ND
Patient 14 Female, 86 years (Braak IV)	82.6	ND	-	100000	ND
Patient 15 Male, 78 years (Braak IV)	177	ND	-	18724	ND
Patient 17 Male, 60 years (Braak VI)	196	ND	-	1176	ND

[a] Values are expressed and Mean values ± Standard Deviation (n=3).
[b] n = 1 due to the lack of sample availability.
[c] SMCxPRO immunoassay did not allow to measure of these samples (ND), probably because of the matrix effect or because Tau is present at a high concentration. Due to low sample availability, these plasma samples were not further measured by SMCx.

4. Conclusions

The first MM-based dual electrochemical *on-the-move* immunoassays for the rapid and reliable determination of Aβ-42 and Tau in high-assay clinical samples diagnosed with Alzheimer's disease have been successfully developed.

The $MM_{A\beta\text{-}42}$-MM_{Tau} approach allowed the reliable dual determination of both protein biomarkers simultaneously (Aβ-42 and Tau) in Alzheimer's disease-diagnosed clinical samples using a low volume of sample (30 µL) under only 15 min of the assay with high sensitivity demonstrating its applicability as an alternative pre-screening diagnostic tool, in the BT, CSF, and plasma samples without any dilution, reducing the complexity of the analysis. Although, as limitation of the study, the analyses were performed using Aβ-42 and Tau as surrogate AD markers to be measured and also as potential interferents of each other in the presence of albumin as blocking agent and potential interferent since it is the major constituent protein of CSF and plasma, the results presented here suggest the enormous value of MM in the field of Alzheimer's disease diagnosis, including as a potential tool for POCs or *bedside* devices with the advantages of minimal sample volume, low time of measurement and preparation. The high stability of MM-$Ab_{A\beta\text{-}42}$ and MM-Ab_{Tau} complexes to be used as stock "reagents" (n=50 days) without losing the efficiency of the assay is also seen as an added value. Therefore, it could be a very useful tool for early diagnosis, improving the treatment and therefore reducing the effects of the disease. This is a further step towards MM technology becoming established as a reliable and relevant alternative in the diagnosis of diseases, confirming its special relevance in those with low availability of clinical samples.

However, despite the promising results obtained, it has also to be highlighted the high complexity of the sample analysis from Alzheimer's disease patients. The quantitative results obtained for BT and CSF were consistent with both the differentiation of healthy from diseased individuals and an increase in biomarker levels with disease progression. In contrast, the analysis in plasma samples was not significant for Tau, whereas for Aβ-42 an increase in the Aβ-42 levels were observed despite of the inter-individual variability for patient #14 at Braak IV. Conversely, although different classes of clinical samples were analyzed, which gives undoubted value to our proposed MM-based approach, more analyses should be performed to obtain more representativeness, as we were limited by sample availability. However, these clinical sample limits give exceptional additional value to the MM technology because of its pertinent utility at low sample volumes.

Ethics

We further confirm that any aspect of the work covered in this manuscript that has involved human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Written informed consent was obtained from all individuals, following the ethical issues and brain bank's guidelines. The Institutional Ethical Review Board of the Spanish Research Center for Neurological Diseases Foundation (CIEN), the Instituto de Salud Carlos III and the Universidad de Alcalá approved this study on the analyses of biomarkers of Alzheimer's disease (CEID2021/4/108).

Intellectual property

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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CRediT authorship contribution statement

Rodrigo Barderas: Writing – review & editing, Supervision, Formal analysis, Data curation. **Miguel Ángel López:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **María Moreno-Guzmán:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ana Montero-Calle:** Writing – review & editing, Investigation, Formal analysis, Data curation. **José M. Gordón Pidal:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alberto Escarpa:** Writing – review & editing, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

No conflict of interest exists. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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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.snb.2024.136785.

Data availability

Data will be made available on request.

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