Supporting Information

Micromotor-based dual electrochemical immunoassays for reliable simultaneous determination of amyloid-β (1-42) and Tau in Alzheimer's diagnosed clinical samples.

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Experimental

- **Reagents and solutions**

β-Amyloid reagents as biotin anti-β-Amyloid 1-42 (c-$Ab_{Aβ-42}$), obtained from Biolegend, were prepared in a PBS solution. Human β-Amyloid Peptide 1-42 (Aβ-42) and HRP anti-β-Amyloid 1-16 antibody (d-$Ab_{Aβ-42}$) were obtained from Biolegend. Their corresponding dilutions were prepared in PBS-BSA 5% solution.

Tau reagents as purified anti-Tau 6-18 (c-Ab_{Tau}), recombinant human Tau-441 (2N4R), and HRP anti-Tau 210-230 (d-Ab_{Tau}) were obtained from BioLegend. They were prepared in phosphate-buffered saline (PBS), pH 7.5: 2.7 mM KCl (99%) and 0.1M Na_2HPO_4 (99%) from Sigma-Aldrich; 138 mM NaCl (99%) and 0.1 M NaH_2PO_4 from Panreac. In the case of Tau protein and d-Ab_{Tau}, BSA 5% was added (PBS-BSA 5% solution).

3-aminophenylboronic acid (APBA), Streptavidin, N-hydroxysulfosuccinimide (NHSS) and 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC) were prepared in 2-(N-Morpholino) ethane sulfonic acid (MES) solution 0.1 M pH 5, and purchased from Sigma-Aldrich (Madrid, Spain). Hydroquinone (HQ), purchased from Sigma-Aldrich, and hydrogen peroxide (H_2O_2) 30%, purchased from Fisher Chemical, were prepared in phosphate buffer (PB) 0.1 M, pH 7.

Pyrrole-3-carboxylic acid, potassium nitrate (99%), hexachloroplatinic (IV) acid (H_2PtCl_6), nickel (II) sulfamate tetrahydrate ($H_4N_2NiO_6S_2$), nickel (II) chloride hexahydrate (Cl_2Ni-$6H_2O$), dichloromethane, isopropanol and ethanol were purchased from Sigma-Aldrich. Boric acid (H_3BO_3) (99.5%) was purchased from Fluka. 5 µm diameter conical pores polycarbonate membranes (PC) were purchased from Whatman. MicroPolish Alumina (0.05 µm) was purchased from Buehler.

All chemicals used were analytical-grade reagents. Deionized water was obtained from a Millipore Milli-Q purification system (18.2 MΩ cm at 25 °C).

Single screen-printed electrode DRP-110 (3.4×1.0×0.05 cm, length x width x height) and 4 mm Ø were used. Dual scree-printed carbon electrodes (dSPCEs) (DRP-C1110, Dropsens), consisting of two elliptic carbon working electrodes (6.3 mm^2 each), a silver pseudoreference electrode, and a carbon counter electrode were used.

- **Samples**

Synthetic human serum from human male blood type AB was obtained from Sigma-Aldrich (Madrid, Spain) without any content of Aβ-42 neither Tau. BT, CSF, and plasma samples were obtained from the CIEN Foundation's Tissue Bank (BT-CIEN). 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). Protein extracts from the BT samples were obtained as previously described and stored at -20ºc until use [1,2].

For single Tau analysis, 6 BT samples were studied, 2 samples from healthy individuals, and 4 samples from AD patients (1 Braak IV sample, 2 Braak V samples, and 1 Braak VI sample). 3 plasma

samples, together with 2 CSF samples from AD patients (1 Braak V sample, and 1 Braak VI sample). All samples were measured with the MM and the SMCxPRO technology.

For the dual assay, 3 BT samples were analyzed, 1 sample from a healthy individual and 2 samples from AD patients (1 Braak IV sample and 1 Braak V sample). Additionally, 4 plasma samples were studied. Finally, 3 CSF samples from AD patients (1 Braak IV, 1 Braak V, and 1 Braak VI samples) were studied. All these samples were measured with the MM and the SMCxPRO technology.

The Institutional Ethical Review Board of the Spanish Research Center for Neurological Diseases Foundation (CIEN), the Instituto de Salud Carlos III, and the University of Alcalá de Henares approved this study on the analysis of biomarkers of Alzheimer's disease (CEID2021/4/108).

- **Apparatus**

For template-assisted electrochemical deposition of PPy/Ni/PtNPs and to carry out single amperometric measurements, an electrochemical station µ-Autolab Type III (Eco Chemie, Utrecht, Holland) was used. For dual determination, a multi potentiostat/galvanostat µSTAT 8000 from DropSens (Oviedo, Spain) was also used. Incubation stages were performed by an advanced VortexMixer-ZX3 from VWR and Thermosaker TS-100 C from Biosan. Besides, a Magnetic block DynaMag-2, obtained from Thermo Fisher Scientific, was used for handling magnetic PPy/Ni/PtNPs MM. Scanning electron microscopy (SEM) images were obtained with a JEOL JSM 6335 F instrument and X-ray analysis was performed through an EDX detector attached to a SEM instrument.

- **Electrochemical synthesis of tubular catalytic PPy/Ni/PtNPs MM**

PC membranes were treated with a sputtered thin gold film to provide conductive properties as working electrode (WE). Then, the membrane was assembled onto a Teflon-plated cell covered with aluminum foil to provide electrical contact to the WE. The first layer deposited (biosensing layer) was electropolymerized through a solution of 25 mM pyrrole-3-carboxylic acid (PPy) and 7.5 mM KNO_3, using a voltage of 0.8V until the current was stabilized. After that, an intermediate Ni layer (Magnetic layer) was plated inside the PPy layer using a galvanostatic method divided into two steps: i) 10 pulses of 20 mA for 0.1s for the generation of nucleation spots; ii) a constant current of -6 mA for 300 s was applied to grow the Ni layer from a solution of 1.2 M $H_4N_2NiO_6S_2$, 82 mM $Cl_2Ni\text{-}6H_2O$, and 464 mM boric acid. Finally, the catalytic layer was deposited by amperometry at -0.4V for 750 s from an aqueous solution containing 4 mM of H_2PtCL_6 and 0.5 M boric acid. After the electrodeposition, the sputtered gold layer was gently hand-polished with 0.05 µm alumina slurry, and the cleaned membrane was dissolved in CH_2Cl_2 for the release of MM (3 times, 15 min). Successive washing of the MM with isopropanol (3 times, 10 min), ethanol (2 times, 5 min), and water (1 time, 5 min) to get a neutral medium were also carried out. All this process was performed using a magnet-holding block. All MM were stored in ultrapure water at 4°C when not in use. The template preparation method resulted in reproducible MM with a similar shape and size.

Results and Discussion

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

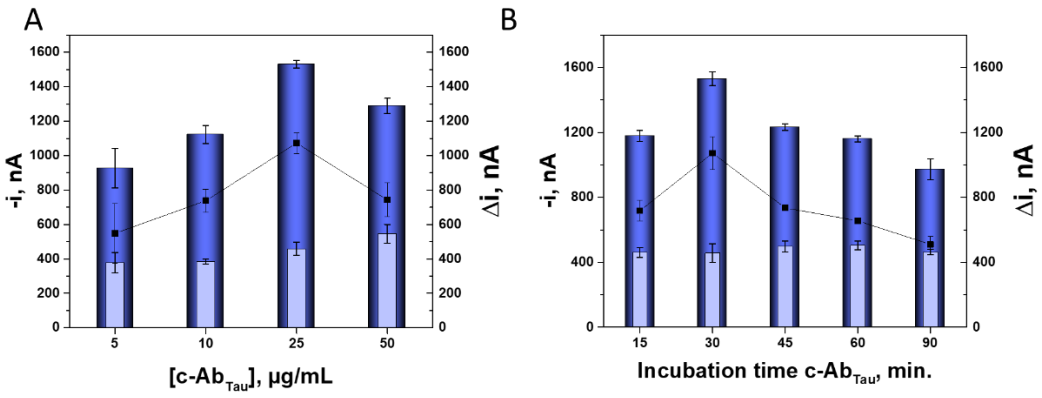

Figure S1. Optimization of the MM functionalized variables: c-Ab$_{Tau}$ concentration (A), c-Ab$_{Tau}$ incubation time (B). Dark blue: *on-the-fly* immunoassay (Tau, 500 ng/mL, d-Ab = 1 µg/mL). Light blue (controls without Tau, d-Ab = 1 µg/mL).

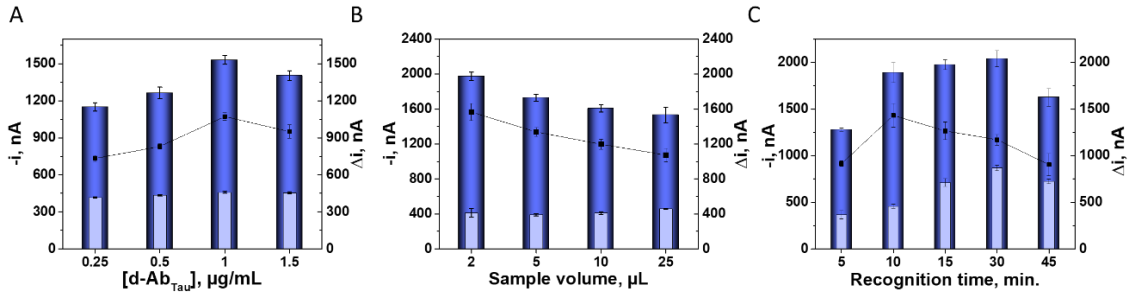

Figure S2. Optimization of on-the-fly immunoassay variables: d-Ab$_{Tau}$ concentration (A), sample volume (B), recognition time (C). Dark blue: *on-the-fly* immunoassay (Tau, 500 ng/mL, 15 min incubation time (B)). Light blue (controls without Tau).

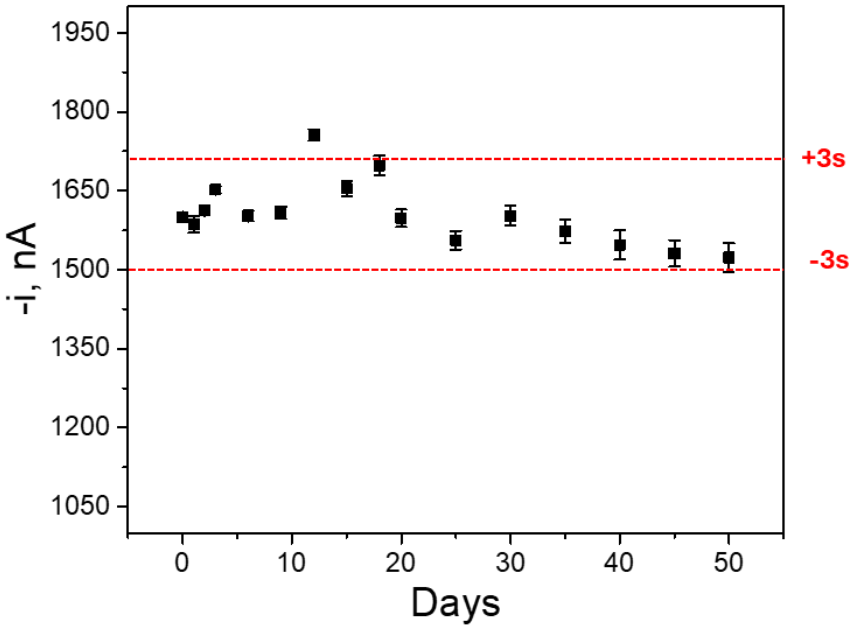

Figure S3. Amperometric currents of the MM_{Tau} immunoassays (n=16) during 50 days from the stored c-Ab_{Tau}. Control limits: red lines, calculated as i±3×s where s is calculated from mean values of three amperometric currents obtained on the preparation on the first day. Conditions: c-Ab = 25 µg/mL, Tau = 500 ng/mL. Other conditions are described in Table 1.

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

Table S1. Main analytical features of selected works for dual Aβ-42 and Tau determination for Alzheimer's disease.

Bioassay approach	Detection	WR	LOD	Analysis time	Sample volume	Sample	Ref
Microfluidic-Immunoassay	Optical reflection	31.25 to 500 pg/mL Aβ-42 125 to 2000pg/mL Tau	7.8 pg/mL Aβ-42 15.6 pg/mL Tau	20 min	1 µL	Spiked CSF	[39]
Microdroplet-Immunoassay	SWV	0.1 to 100 pg/mL	0.86 pg/mL Aβ-42	240 min	10 µL	Serum[a]	[40]

		(both biomarkers)	0.059 pg/mL Tau				
Microfluidic-Immunoassay	Fluorescence	100 pg/mL to 10ng/mL Aβ-42 10 pg/mL to 1 ng/mL Tau	100 pg/mL Aβ-42 10 pg/mL Tau	> 60 min	-	Spiked CSF and Serum	[41]
IME-Immunoassay	Impedance	0.01 to 100 pg/mL (both biomarkers)	9.9 fg/mL Aβ-42 9.6 pg/mL Tau	20 min	-	Plasma[a]	[42]
PA-Aptassay	SERS	0.45 ng/mL to 45 µg/mL Aβ-42 0.048 fg/mL to 0.144ng/mL Tau	0.167 ng/mL Aβ-42 0.020 fg/mL Tau	15 min	2.5 µL	Spiked CSF	[43]
Microarray-Aptassay	DPV	0.1 to 1000 pg/mL (both biomarkers)	0.125 pg/mL Aβ-42 0.142 pg/mL Tau	60 min	10 µL	Serum[a]	[45]
SIM-Immunoassay	Fluorescence	0 to 11.25 pg/mL Aβ-42 0 to 120 fg/mL Tau	104 fg/mL Aβ-42 0.67 fg/mL Tau	60 min	25 µL	Spiked CSF	[48]
AuNPs-Immunoassay	Localized surface plasmon	45 fg/mL to 450 ng/mL Aβ-42 0.48 to 4.8 ng/mL Tau	117 fg/mL Aβ-42 1.13 fg/mL Tau	> 60 min	-	Spiked Blood	[51]
PPy/Ni/PtNPs MM-Immunoassay	Amperometry	0.1 to 5 ng/mL Aβ-42	0.04 ng/mL Aβ-42	15 min Aβ-42	25 µL Aβ-42	BT CSF Plasma	This work

	1 to 10 pg/mL Tau	0.4 pg/mL Tau	10 min Tau	2 µL Tau

[a] Denotes sample analysis in clinical no spiked samples

Abbreviations used: AuNPs-Gold Nanoparticle; **BT**-Brain Tissue; **CSF**-Cerebrospinal fluid**; DPV**-Differential Pulse Voltammetry; **IME**-Interdigitated microelectrode; **MM**-Micromotor; **PA-Aptassay-**Polyadenine aptassay**; PPy/Ni/PtNPs**-Polypirrole/Nickel/Platinum nanoparticles; **SERS**-Surface-enhanced Raman scattering; **SIM-**Indolium-based turn-on fluorophore; **SWV**-Square wave voltammetry.

Video S1.

References

[1] M. Garranzo-Asensio, P.S. Segundo-Acosta, J. Martínez-Useros, A. Montero-Calle, M.J. Fernández-Aceñero, A. Häggmark-Månberg, A. Pelaez-Garcia, M. Villalba, A. Rabano, P. Nilsson, R. Barderas, Identification of prefrontal cortex protein alterations in Alzheimer's Disease, Oncotarget. 9 (2018) 10847–10867. https://doi.org/10.18632/oncotarget.24303.

[2] A. Montero-Calle, R. Coronel, M. Garranzo-Asensio, G. Solís-Fernández, A. Rábano, V. de los Ríos, M.J. Fernández-Aceñero, M.L. Mendes, J. Martínez-Useros, D. Megías, M.T. Moreno-Casbas, A. Peláez-García, I. Liste, R. Barderas, Proteomics analysis of prefrontal cortex of Alzheimer's disease patients revealed dysregulated proteins in the disease and novel proteins associated with amyloid-β pathology, Cell. Mol. Life Sci. 80 (2023) 141. https://doi.org/10.1007/s00018-023-04791-y.