Towards cerebrospinal fluid-free ultrasensitive Alzheimer's diagnostics using molecularly imprinted polymers

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***Abstract*—Neurofibrillary tangles (NFTs), vital in Alzheimer's disease (AD) diagnosis, consist of Tau-441, a Tau protein with 441 residues. Originating in the entorhinal cortex, these tangles spread as AD progresses. Combining the identification of tau proteins in plasma by Zetterberg and co-workers and the shared ectodermal origin of the brain and skin, suggests the presence of AD biomarkers in ISF and blood. This study presents the development of a molecularly imprinted polymer (MIP)-based sensor surface for sensitive Tau-441 detection from ISF. The biosensor surface was created by electropolymerizing aniline in the presence of the Tau-441 template using cyclic voltammetry. A non-molecularly imprinted polymer (NIP) was developed for comparison, using phosphate-buffered saline (PBS) solution at pH 7.4. Sensor performance studies of the developed MIP sensor involved detecting Tau-441 in PBS and ISF. The MIP showed a linear decrease in current with increasing concentration of Tau-441, indicating successful binding of Tau-441 to the polymeric backbone of the MIP. The sensor was able to detect Tau-441 in the range of 50 fg/mL to 500 ng/mL (1.22 fM/L to 12.2 nM/L) achieving a limit of detection (LOD) of 587 pg/mL (12.2 fM/L) in ISF. Future investigations will explore the use of MIP-modified electrodes and microneedle patches for Tau protein detection from blood and ISF, respectively.**

Keywords—Tau-441, Interstitial Fluid, molecularly imprinted polymer

I. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that primarily affects cognitive functions, including memory, thinking, and behaviour. It is the most common cause of dementia, with symptoms typically starting mildly and worsening over time. The hallmark characteristics of AD are the accumulation of abnormal protein deposits in the brain, namely tau tangles and beta amyloid plaques[1]. These deposits disrupt communication between brain cells, leading to their eventual death and the associated decline in cognitive abilities. Diagnosing AD can be challenging, especially in its early stages. Currently, diagnosis relies heavily on a combination of clinical assessments, medical history, physical examinations, and cognitive tests to evaluate memory, problem-solving, and other cognitive functions. Additionally, imaging techniques such as magnetic resonance imaging and positron emission tomography may be used to detect structural changes in the brain and assess the presence of beta-amyloid plaques[2].

Neurocognitive assessments are resource intensive whilst CSF extraction involves a lumbar puncture under general anaesthesia. Once extracted, analysis of the biomarkers including Tau proteins offer a comprehensive understanding of the intricate pathology associated with AD. The use of PET scans, which present multiple challenges like patient discomfort provide a three-dimensional visualization of Tau distribution to achieve diagnosis and monitoring of AD progression[3]. It is imperative for AD diagnostics to move towards minimally invasive, cost-effective, and patient friendly techniques like detection of biomarkers from blood and interstitial fluid (ISF) for better diagnostic outcomes and patient comfort. Blood and ISF-based diagnostics and prognostics that use electrochemical modalities offer promising alternatives to existing methods[4]. The two compartment and ectodermal origin theory substantiate the presence of the same neurological biomarkers in the brain, blood, and dermal ISF[5]. Over the last 10 years, the analysis of AD biomarkers using ultrasensitive minimally invasive methods has shown great potential due to its simplicity, diagnostic accuracy (95% - 97%), and cost effectiveness. Low concentrations of these biomarkers have been detected using composites of nanomaterials and bio-receptors[6, 7].

Molecularly imprinted polymers (MIPs) represent an innovative approach in biosensing and diagnostic technology, offering distinct advantages compared to antibodies. MIPs are synthetic polymers designed to recognize and bind to target molecules with high specificity. These are synthesized through a process called molecular imprinting, where template molecules are incorporated into a polymer matrix and subsequently removed, leaving behind cavities with shapes complementary to the template molecule. This allows MIPs to selectively bind to target molecules based on their molecular shape and functional groups, offering robustness, stability, and resistance to degradation compared to biological receptors[8]. Additionally, MIPs can be easily synthesized in large quantities, making them cost-effective and scalable for diagnostic applications. There are numerous electroconducting polymers that can be used such as the family of polyaminophenols However, polyaniline (PANI) and its derivates are amongst the most investigated. PANI possesses characteristics such as environmental stability, tuneable morphology, reversible redox chemistry and its electrical conductivity can be controlled[9]. Furthermore, PANI can be synthesized at low-cost, which is a very attractive characteristic for this research. Due to its superior electrochemical properties, PANI is very efficient in sensing and detecting conductance changes in response to various concentrations of target proteins. Additionally, as the backbone of PANI is amine enriched, it offers a plethora of possibilities for immobilizing and binding biomolecules [10]. Therefore, the electropolymerisation was carried out with the functional monomer aniline, which is polymerized

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to PANI to produce a thin, insulating film on the MIP based sensor.

Herein, we report on the development of a PANI-based MIP for the detection of Tau-441, which is an important biomarker for AD from artificial ISF. A comparison of the performance of the MIP sensor was made with a non-molecularly imprinted polymer (NIP). It was found that the NIP did not exhibit a linear response to the presence of Tau-441. The sensor performance was analysed using differential pulse voltammetry (DPV) with ferricyanide as an external redox probe. The performance of the sensor was observed both in phosphate buffer saline (PBS) and ISF and it was found that the sensor was able to detect concentrations of Tau-441 from 50 fg/mL to 500 ng/mL (1.22 fM/L to 12.2 nM/L) achieving a limit of detection (LOD) of 587 pg/mL (~ 12.2 fM/L) in ISF.

II. Materials and Methods

All chemicals used throughout this project were of analytical grade. The deionised water (resistivity = 18.2 MΩ/cm) was obtained using a Milli-Q water purification. Potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$) and potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]$) were purchased from Sigma Aldirich. Oxalic acid, which was used for template elution (0.5 M) was purchased from Afla Aesar (Harverhill, MA, USA) [56]. Aniline ≥99% was purchased from Avantor, VWR. Phosphate buffered saline (PBS, 0.01 M, pH 7.4), containing 0.0004% DTT and 25% glycerine, was made by diluting one tablet of PBS purchased from Fisher BioReagents (Pennsylvania, USA) in 200 mL of water (1.0 mmol L^{-1}), to yield 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl. A solution of Tau-441 protein, purchased from AbCAM, was prepared in the PBS buffer and stored at -20 °C. To monitor the response of the MIP based sensors in physiological conditions, artificial ISF was used. To prepare the ISF 2.5 mM of $CaCl_2$ (Merck) was mixed with 5.5 mM glucose (Alfa Aesar), 3.5 mM potassium chloride, KCl (Carlo Erba), 10 mM HEPES buffer (Sigma-Aldrich), 7.2 mM sucrose (Riedel-de Häen), 123 mM sodium chloride, NaCl (Normapur), 1.5 mM sodium dihydrogen phosphate dihydrate, NaH_2PO_4 (Scharlau), and 0.7 mM magnesium sulphate, Mg_2SO_4 (Pancreac). The pH of the solution was then adjusted to pH 7.4 [11] .

A. Biosensor Design

To design the biosensor, four main steps were carried out. These included the pre-treatment steps, electropolymerisation/imprinting step, template removal and rebinding of the protein to the template cavities created. The process for developing the MIP based sensors is summarized in the schematic in **Fig**. 1A, made with BioRender.

B. Pre-Treatment of G-SPEs

Cleaning of G-SPEs was carried out by rinsing with de-ionised water and ethanol following by drying with a stream of air.

C. Electropolymerisation/Imprinting

Imprinting was carried out using cyclic voltammetry (CV), after applying 70 µL of the MIP solution directly on to G-SPEs. The MIP solution was made of by mixing 0.2 M aniline and 0.02 mg/mL of the Tau-441 protein. The CV was carried out thrice from -0.2 V to +0.8 V with a scan time of 0.025 V/s. These CVs were observed to confirm the molecular imprinting process (not shown in manuscript). A decrease of peaks in the deposition CVs confirmed the deposition of polyaniline on the electrode surface.

The NIP was fabricated by using the same procedure as the MIP, however the Tau-441 was not included during the fabrication of the NIP.

D. Template Removal

To remove the template protein, the modified sensors were incubated overnight at -4 °C in the fridge with the addition of 70 µL oxalic acid to the working electrode. After incubation, the oxalic acid was removed by washing followed by drying in a stream of air. After removal of the template, the MIPs were stabilised in PBS. A ferricyanide (Fe^{2+}/Fe^{3+}) based redox probe was used for probing the electrochemical performance of the modified sensor.

E. Rebinding of Template

The rebinding of the template Tau-441 onto the electrode surface was performed by pipetting increasing concentrations of Tau-441 in PBS and ISF, respectively onto the electrodes and incubating for 30 min at room temperature.

III. Results and Discussion

Molecular imprinting of the Tau-441 was performed by electropolymerization of the polyaniline monomer followed by removal of the template by incubation in oxalic acid overnight (**Fig. 1A**). The different stages of modification for both the MIP and NIP were characterized using CVs in ferricyanide solution.

It can be observed from **Fig. 1B and 1C** that both MIP and NIP exhibited high electron transfer resistance. In the case of the MIP, this high electron transfer resistance was due to the presence of the template and the insulating nature of the PANI film, which impeded the redox reaction of the probe whereas in the case of the NIP this could be attributed to the insulating nature of the PANI film alone.

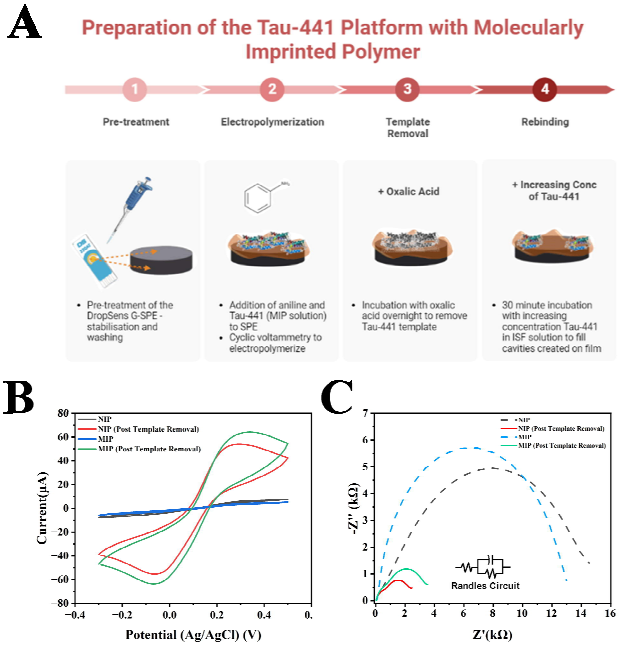

Fig 1: (A) Schematic to detail the process followed for preparing the Tau-441 Platform with MIP. Image Created in BioRender.com. (B) and (C) Cyclic Voltammograms (CVs)

and Impedance spectra of the modified electrodes containing NIP and MIP in 5 mM ferricyanide solution before and after template removal step.

However, post incubation with oxalic acid the currents for both the MIP and NIP were found to increase. For the MIP this could be attributed to the elution of the template by the oxalic acid[12]. In the case of the NIP the increase in the current values could be attributed to the interaction with oxalic acid, which makes the surface conducting[13]. A similar trend is observed in the case of the EIS also where the diameter of the semicircle, which indicates electron transfer resistance was found to reduce post oxalic acid treatment (**Fig. 1C**). The incubation with the oxalic acid led to an approximately 63% decrease in electron transfer resistance for the MIP while an 86% decrease for the NIP. The substantial decrease in the electron transfer resistance for the MIP highlights that the protein was successfully removed from the polymeric network, and that voids in the shape of the template protein were created.

The MIP and the NIP were then employed for the electrochemical detection of Tau-441 in 1 mM PBS (pH 7.4). It could be observed from the Nyquist plots that a linear response for the detection of Tau-441 (50 pg/mL and 5 ng/mL) could only be observed from the MIP (and not the NIP), which implied that the MIP was could detect increasing concentrations of Tau-441 from PBS (**Fig. 2A**). DPV analysis demonstrated the MIP sensor's linearity towards detecting Tau-441 from 50 fg/mL to 5 ng/mL (1.22 fM/L to 122 pM/L) in PBS (**Fig. 2B**).

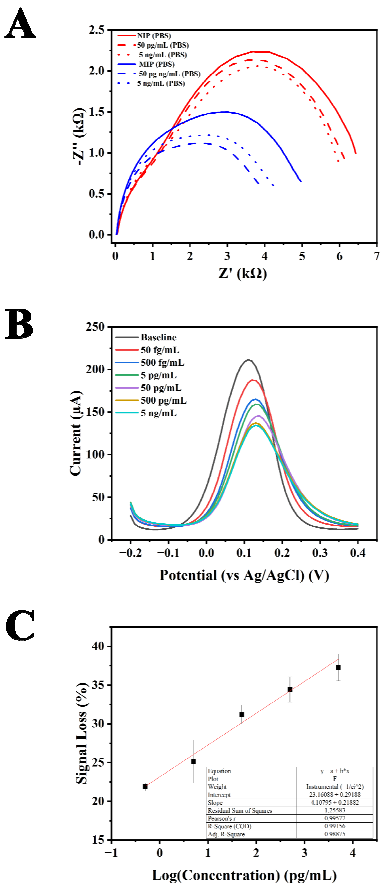

Fig 2: (A) Impedance spectra of the MIP (▬) and NIP (▬) in 0, 50 and 5000 pg/mL of Tau-441, (B) DPV of the MIP modified electrodes incubated with different concentrations of Tau-441, and (C) Calibration plots of the sensors showing linearity in sensor performance in 1 mM PBS (pH 7.4).

In the absence of Tau-441, a baseline current of ~200 µA was recorded, decreasing to ~132 µA with increasing Tau-441 concentrations (**Fig. 2B**). This reduction is attributed to the protein-induced blockage of MIP pores, also evidenced by a slight positive shift in peak potential. The linearity of the sensor was plotted as a function of logarithm of Tau-441 concentration, and it was found that the sensor was able to exhibit a limit of detection (LOD) of approximately 200 fg/mL (~6.1 fM/L) in 1 mM PBS (pH 7.4) (**Fig. 2C**).

Based on these findings, the performance of the sensor was evaluated in ISF. This method involved the binding of the protein followed by measurements in ISF. The sensor's performance in ISF revealed enhanced detection capabilities, successfully identifying Tau-441 across a significant concentration span (50 fg/mL to 500 ng/mL, equivalent to 1.22 fM/L to 12.2 nM/L), encompassing the clinically relevant tau protein range. The sensor achieved a LOD of 587 pg/mL (~12.2 fM/L), aligning with the threshold above which Tau protein concentrations (>4.3 pM/L) indicate AD symptoms.

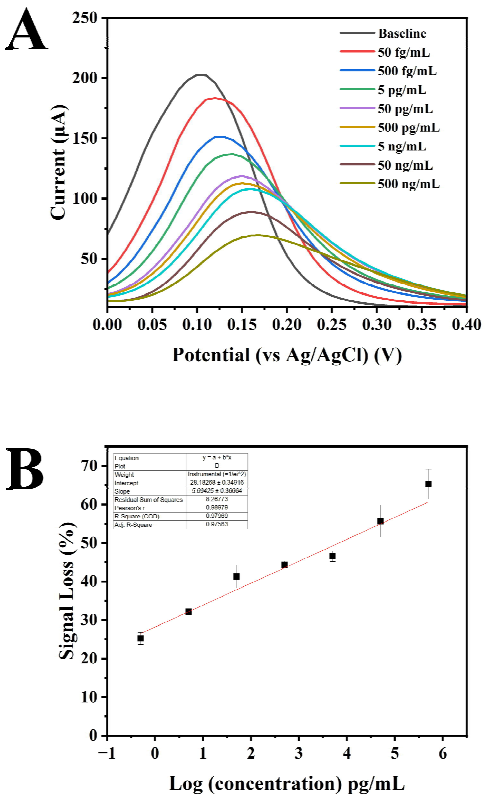

Fig 4: (A) DPV of the MIP modified sensor in different concentration of Tau-441 in ISF. (B) Graph showing linearity of the developed sensor.

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