



Electrochemical immunosensors for detection of neurodegenerative diseases biomarkers

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“Ever tried. Ever failed. No matter. Try again. Fail again. Fail better.”

(Samuel Beckett)

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Abstract

The prevalence of neurodegenerative diseases is expected to rise with the increasing life expectancy in most countries. This event will impose a high financial burden on society as these degenerative conditions can span for decades with increasing limitations.

Alzheimer's disease (AD) is the most common neurodegenerative disease being associated with most cases of dementia while Parkinson's disease (PD) is clinically defined as a progressive movement disorder. Currently, there are no simple and reliable diagnostic tests for both diseases. It remains essentially a clinical diagnosis, subject to variations in patient presentation and physician assessment. This can lead to errors as several dementias and movement disorders often present overlapping clinical symptoms making their differential diagnosis, particularly in the early stages, extremely challenging. In addition, with the emergence of specific therapeutic interventions, there is an ever-increasing demand for the development of simple and accurate diagnostic tools.

Recognizing the challenges and limitations associated with current diagnostic techniques, the goal of this work was to develop novel electrochemical immunosensors capable of specifically detect core biomarkers of AD and PD. Throughout this work, different immunosensors design and sensing strategies were addressed in order to develop simple and low-cost biosensors with fast detection processes, while maintaining a sensitive analytical behavior. To attain these goals, the integration of nanomaterials in the biosensors design revealed to be essential to increase the electrochemical behavior of the transducers while providing an effective immobilization matrix for the biological recognition events.

Therefore, in a first study a sensitive and specific label-free electrochemical immunosensor was developed for the detection and quantification of PD main biomarker, α -synuclein. The detection process was based on the electrochemical signal of a redox indicator, which decreased upon analyte recognition. Here, the immunosensor was developed using two different electrochemical transducers, glassy carbon electrode and screen-printed carbon electrode, being their electrochemical and analytical behavior compared. The nanostructuration of the transducers was extensively characterized through cyclic voltammetry, electrochemical impedance spectroscopy, square-wave voltammetry and scanning electron microscopy techniques. Modification

with single-walled carbon nanotubes and gold nanoparticles produced the best electrochemical behavior, being further modified with thiolated monoclonal antibodies for the biorecognition events. Using the proposed immunosensor, excellent analytical behavior was attained being able to detect and quantify low levels of this biomarker in serum. In addition, the immunosensor based on screen-printed carbon electrodes exhibited the best analytical behavior while only requiring small volumes to conduct the whole experimental procedure, important characteristic for integration into point-of-care devices.

In a different detection strategy, an electrochemical immunosensor based on a sandwich detection scheme was developed towards detection of amyloid β 42 (A β 42), one of AD main biomarkers. In this study, an antifouling matrix capable of resisting non-specific binding of proteins was developed in order to improve the immunosensors analytical behavior in biological samples. In addition, a simple and low-cost immunosensing probe for signal amplification was developed in order to avoid the use of more costly and time consuming amplification strategies based on enzymatic reactions. In this work, two different antifouling coatings were evaluated being that the most effective and stable coating resulted from the formation of a polyethylene glycol layer on the surface of a screen-printed carbon electrode modified with carboxylated single-walled carbon nanotubes. The developed immunosensing probe was based on colloidal gold nanoparticles labeled with Pb^{2+} ions and functionalized with monoclonal antibodies, which produced an electrochemical signal specifically related to A β 42 concentration. Under optimized conditions it was possible to detect and quantify clinically relevant levels of this biomarker. Nonetheless, this immunosensing probe requires further studies as it revealed reproducibility and stability problems.

The work developed in this thesis enabled the development of simple and sensitive electrochemical immunosensors, which exhibited good indicators for point-of-care application, appearing as alternative diagnostic tools to more traditional methods.

Keywords: Alzheimer's disease, Parkinson's disease, Biomarker, Amyloid β 42, α -synuclein, Biosensor, Electrochemistry, Voltammetry, Electrochemical immunosensor, Screen-printed carbon electrode, Glassy carbon electrode, Nanomaterials, Gold nanoparticles, Carbon nanotubes, Antifouling

Resumo

À medida que a esperança média de vida aumenta na maioria dos países, é expectável que a prevalência de doenças neurodegenerativas também aumente. Prevê-se que este facto venha a ter um impacto financeiro significativo na sociedade, uma vez que estas doenças envolvem processos degenerativos lentos, que podem durar décadas, com limitações crescentes para os indivíduos afetados.

A doença de Alzheimer (DA) é a doença neurodegenerativa mais comum, sendo responsável por grande parte dos casos de demência. Por outro lado, a doença de Parkinson (DP) é clinicamente definida como uma doença degenerativa que afeta o sistema motor. Atualmente, não existem testes de diagnóstico simples e eficientes para estas doenças. O seu diagnóstico é essencialmente um exercício clínico, sujeito a variações na apresentação clínica do paciente e na avaliação do médico. Esta situação pode conduzir a erros de diagnóstico, pois este tipo de doenças apresentam sintomas clínicos semelhantes, tornando seu diagnóstico diferencial extremamente complicado, principalmente nas fases iniciais das doenças. Além disso, com o aparecimento de intervenções terapêuticas mais direcionadas, há uma necessidade cada vez maior para o desenvolvimento de ferramentas de diagnóstico mais simples e precisas.

Tendo em consideração os desafios e limitações das técnicas de diagnóstico atuais, o objetivo deste trabalho consistiu no desenvolvimento de imunossensores eletroquímicos capazes de detectar especificamente os principais biomarcadores da DA e DP. Foram desenvolvidos biossensores com diferentes estratégias de deteção de forma a garantir biossensores simples, de baixo custo e com um comportamento analítico exato e preciso. Neste sentido, a integração de nanomateriais no desenvolvimento dos mesmos desempenhou um papel fundamental para aumentar o comportamento eletroquímico dos transdutores, fornecendo ao mesmo tempo uma matriz eficaz para a imobilização dos elementos de reconhecimento biológico.

No primeiro trabalho experimental apresentado, foi desenvolvido um imunossensor eletroquímico para a deteção e quantificação da α -sinucleína, um dos principais biomarcadores da DP. O processo de deteção foi baseado no estudo do sinal eletroquímico de um agente redox, cujo sinal reduziu após reconhecimento do analito pelo imunossensor. Neste trabalho, o imunossensor foi desenvolvido usando dois transdutores eletroquímicos diferentes, um eléctrodo de carbono vítreo e um eléctrodo de carbono serigrafado, sendo feita a comparação do seu comportamento eletroquímico e

analítico. A nanoestruturação dos transdutores foi extensivamente caracterizada por voltametria cíclica, voltametria de onda-quadrada, impedância eletroquímica de espectroscopia e microscopia eletrônica de varrimento. A modificação dos transdutores com nanotubos de carbono de parede simples e nanopartículas de ouro demonstrou o melhor comportamento eletroquímico, sendo que anticorpos monoclonais foram imobilizados na sua superfície. Os imunossensores desenvolvidos exibiram um excelente comportamento analítico capaz de detetar e quantificar baixas concentrações de α -sinucleína em soro, sendo que o melhor comportamento analítico foi obtido com os elétrodos de carbono serigrafados. Inclusivamente, este tipo de elétrodos requerem pequenos volumes, tanto de reagentes como de amostras, para completar todo o processo experimental, características importantes para integração em dispositivos de deteção rápida.

Numa diferente estratégia, um imunossensor eletroquímico com deteção tipo sanduíche foi desenvolvido para a deteção da β -amilóide 42, um dos principais biomarcadores da DA. Em primeiro lugar, neste estudo, foram testadas diferentes camadas capazes de resisitir à adsorção de proteínas, com o objetivo de melhorar o comportamento analítico do imunossensor após exposição a amostras biológicas. Além disso, nanopartículas de ouro foram desenvolvidas para a amplificação do sinal eletroquímico, de forma a substituir o uso de estratégias de amplificação mais caras e morosas com base em deteção enzimática. Neste trabalho, duas camadas com propriedades resistentes à adsorção não específica de proteínas foram estudadas, sendo que a modificação mais eficaz e estável resultou da formação de uma camada de polietilenoglicol na superfície de um elétrodo de carbono serigrafado modificado com nanotubos de carbono funcionalizados com grupos carboxílicos. As nanopartículas de ouro foram marcadas com iões Pb^{2+} e modificadas com anticorpos específicos para a β -amilóide 42, produzindo um sinal eletroquímico especificamente relacionado com as concentrações do biomarcador. Em condições otimizadas, o imunossensor foi capaz de detetar e quantificar concentrações clinicamente relevantes. No entanto, as nanopartículas de ouro desenvolvidas revelaram problemas significativos de reprodutibilidade e estabilidade, sendo necessário realizar mais estudos para ultrapassar estas limitações.

O trabalho desta tese permitiu o desenvolvimento de imunossensores eletroquímicos para os principais biomarcadores da DA e DP, apresentando bons

indicadores para integração em dispositivos de detecção rápida e surgindo como ferramentas de diagnóstico alternativas aos métodos mais tradicionais.

Palavras-chave: Doença de Alzheimer, Doença de Parkinson, Biomarcador, β -amilóide 42, α -sinucleína, Biossensor, Eletroquímica, Voltametria, Imunossensor eletroquímico, Elétrodo serigrafado de carbono, Elétrodo de carbono vítreo, Nanomateriais, Nanopartículas de ouro, Nanotubos de carbono, *Antifouling*

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List of Abbreviations

2-MU - 2-Mercaptoethanol

3-MPOH - 3-Mercapto-1-propanol

5CB - 4-cyano-4'-pentylbiphenyl

AAO - Anodic aluminum oxide

ABTS - 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate);

ABTS-PDDA/SWCNTs - 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)-poly(diallyldimethylammonium chloride)-bi functionalized single-walled carbon nanotubes composite

aCSF - Artificial cerebrospinal fluid

AD - Alzheimer's disease

AgNPs - Silver nanoparticles

ALIDEs - Surface modified aluminum interdigitated electrode

ALP - Alkaline phosphatase

APMES - 3-(ethoxydimethylsilyl) propylamine

ApoE - Apolipoprotein E

APP - Amyloid precursor protein

APTES - 3-aminopropyltriethoxysilane

Arg - Arginine

AuE - Gold electrode

AuNPs - Gold nanoparticles

A β - Amyloid beta peptide

BMIMBF₄ - 1-butyl-3-methyl-imidazolium tetrafluoroborate

CA - Chronoamperometry

CNFs - Carbon nanofibers

CNTs - Carbon nanotubes

CS - Cysteamine

CSF - Cerebrospinal fluid

CTAB - hexadecyltrimethylammonium bromide

CV - Cyclic voltammetry

DDA - 4,4 -dithiodibutyric acid

DMF - N,N-dimethylformamide

DMSO - Dimethyl sulfoxide;

Cys - Cysteine

DLB - Dementia with Lewy bodies

DLS – Dynamic Light Scattering

DNA - Deoxyribonucleic acid

DPV - Differential pulse voltammetry

DTSP - Dithiobis (succinimidyl propionate)

DTSSP - 3,3'-dithiobis(sulfosuccinimidyl propionate)

EC - Electrochemistry

ECL - Electrochemiluminescence

EDC - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

EDTA - Ethylenediaminetetraacetic acid

EGOFET - Electrolyte-Gated Organic Field-Effect Transistor

EIS - Electrochemical impedance spectroscopy

ELISA - Enzyme-linked immunosorbent assays

FBS - Fetal bovine serum

FITC - Fluorescein isothiocyanate

GCE - Glassy carbon electrode

GO - Graphene oxide

GOx - Glucose oxidase

HAc/NaAc - Sodium acetate/acetic acid buffer

HRP - Horseradish peroxidase

IDE - Interdigitated electrode

IGZO - Amorphous indium gallium zinc oxide

ITO - Indium tin oxide

LOD - Limit of detection

LOQ - Limit of quantification

LSPR - Localized surface plasmon resonance

Mal-PEG-NH₂ - Polyethylene glycol functionalized with amine and maleimide groups

MES - 2-(N-Morpholino) ethanesulfonic acid

MHDA - 16-mercaptohexadecanoic acid

MNH - 9-mercapto-1-nonanol

MNPs - Magnetic nanoparticles

MOFs - Metal organic frameworks

MPA - Mercaptopropionic acid

MSA - Multiple system atrophy

MU - 11-mercapto-1-undecanol

MUA - 11-mercaptoundecanoic acid

MUD - 11-mercaptoundecanol

MWCNTs - Multi-walled carbon nanotubes

NDDs - Neurodegenerative diseases

NHS - N-hydroxysuccinimide

NHSS - N-hydroxysulfosuccinimide

NKB - Neurokinin B

NR - Non referred

ODT - 1,8-octanedithiol

***p*-AP** - *p*-aminophenol

***p*-APP** - *p*-aminophenyl phosphate

PBS - Phosphate buffered saline

PD - Parkinson's disease

PDDA - poly(diallyldimethylammonium chloride)

PDG - poly (D-glucosamine)

PEC - Photoelectrochemical

PEG - Polyethylene glycol

PET - Polyethylene terephthalate

PGA - Polyglutamic acid

PMCA - Protein-misfolding cyclic amplification

POC – Point-of-care

poly(DMA-co-NAS-co-MAPS) - Ter-copolymer made with an optimised composition of dimethylacrylamide (DMA), 3-(trimethoxysilyl)propyl methacrylate (MAPS) and N-Acryloyloxy succinimide ester (NAS)

POPA - Co-polymer of polytyr- amine/3-(4-hydroxyphenyl) propionic acid

***p*SC₄** - Sulfonatocalix[4]arene

***p*-Tau** - Phosphorylated tau

QCM - Quartz crystal balance

QD - Quantum dots

RT – Room temperature

RT-QuIC - Seeding aggregation assays such as real-time quaking-induced conversion

SA-ALP - Streptavidin- modified alkaline phosphatase

SAM - Self-assembled monolayer
SF - Silk fibroin
SPCE - Screen-printed carbon electrode
SPGE - Screen-printed gold electrode
SPR - Surface plasmon resonance
STM - Scanning tunneling microscopy
SWV - Square-wave voltammetry
t-A β - Total A β
TBAPy - 1,3,6,8-tetrakis(p-benzoic acid)pyrene
TCEP - Tris(2-carboxyethyl)phosphine
TiPNPs - Titanium phosphate nanoparticles
TsDNA - Triple-stranded DNA
t-Tau - Total tau
ZIF - Zeolitic imidazolate framework

Chapter 1. Preface

1.1. Motivation

Neurodegenerative diseases are highly debilitating conditions that can affect people of different ages causing a progressive degeneration and eventual death of neurons to which there is no current effective treatment. Today, these diseases pose one of the biggest medical and socioeconomic challenges since their progressive degenerative nature leads to increasing limitations that become more severe over time. Patients may start experiencing small problems related with motor or cognitive function that in more advanced stages of disease may lead to a total inability to perform any type of daily activity. For this reason, the caregivers and family members who accompany the day-to-day lives of these patients also end up being invisible victims of the disease. It is easy to imagine the strong negative effect that an illness of this type has on the family nucleus with tremendous professional, social and financial impact.

Alzheimer's (AD) and Parkinson's disease (PD) are the most common human neurodegenerative diseases (NDDs). Dementia manifests as AD major symptom while PD is characterized by motor dysfunction, and together they affect around 50 million people worldwide [1-3]. Since their incidence increases with age, this number is expected to increase exponentially in the upcoming decades, as a result of the continuous aging of the world population. AD and PD are considered multifactorial conditions mainly characterized by the aggregation of a small number of proteins in the brain [1-3]. Diagnosis of both these diseases is based on the assessment of clinical symptoms, which can lead to misdiagnosis or diagnosis made in advanced stages of the pathology [4, 5]. In fact, clinical symptoms become more evident and easier to be identified in more advanced stages of disease, commonly when patients have already lost more than 50% of their neurons. Consequently, as with most diseases, diagnosis performed in these later stages severely limits the efficacy of the available treatment options.

Therefore, it is of utmost importance to develop analytical tools that can contribute to the identification and determination of biomarkers for both AD and PD. In neurodegenerative diseases the clinical need is not only for prognostic assessment, but also for biomarker-based diagnosis, since the clinical presentations of diseases such as AD, PD and frontotemporal degeneration are insidious and often overlap. This will facilitate the diagnostic process and identification of patients at earlier stages, as well as monitor biochemical effects of treatments. Moreover, with the implementation of new

and more efficient diagnostic techniques, it will be possible to provide patients a healthier and more autonomous life by slowing or preventing the disease progression in its early stages.

1.2. Objectives

The research question that supports this thesis evaluates the possibility of developing novel and low-cost electrochemical immunosensors for the highly sensitive and selective detection of clinically relevant levels of the main biomarkers of AD (amyloid β 42) and PD (α -synuclein). To achieve this goal several intermediate stages had to be accomplished in order to overcome some of the main challenges associated with biosensors design:

- Literature review concerning the biosensors developed for the detection of AD and PD main biomarkers;
- Optimization of distinct nanomaterials to improve the transducers electrochemical and analytical behavior;
- Development of an antifouling platform to improve the immunosensors stability over time and maintain their functionality after exposure to biological fluids;
- Study of different detection mechanisms with the purpose of attaining the most sensitive analytical procedures while also enabling the adaptation of these platforms towards multiplexed detection.
- Validation and evaluation of the developed immunosensors for detection of the biomarkers of interest in serum.

1.3. Thesis Layout

The presented thesis is divided in five chapters based on original work.

Chapter 1 | Preface

In this first chapter, the thesis is introduced describing the motivation, proposed objectives and thesis layout.

Chapter 2 | General Introduction

The main theoretical topics of this work are addressed in this chapter from biosensors to electrochemical techniques as well as integration of nanomaterials in biosensors design.

Chapter 3 | Parkinson's disease Biosensors

Chapter 3 covers the subject of biosensors for Parkinson's disease from the state of the art to experimental work. This section of the thesis starts with a subchapter concerning the state of the art on this topic from electrochemical to optical biosensors. Then, the developed experimental work is presented in the following secondary chapters where a nanostructured label-free immunosensor was developed for α -synuclein detection. Here, two different electrochemical transducers were studied and compared under similar conditions in order to attain the best analytical behavior towards detection of low levels of this biomarker.

Chapter 4 | Alzheimer's disease Biosensors

Chapter 4 follows the same principle of the previous chapter but directed towards biosensors for Alzheimer's disease. It starts with a subchapter focusing on the state of the art, followed by presentation of developed experimental work. In this case, a study to improve and characterize the antifouling properties of the developed platforms was conducted. It was later followed by the adaptation of the optimized platform towards detection of one of Alzheimer's disease main biomarkers (amyloid β 42). This was made through the development of an immunosensor based on a sandwich detection approach.

Chapter 5 | Conclusions

Finally, chapter 5 summarizes the lessons learned, most relevant achievements, major drawbacks and future directions towards development of biosensing platforms capable of contributing with valuable information towards the early diagnosis of neurodegenerative diseases.

Chapter 2. General Introduction

*Adapted from original publications: (1) Carneiro, P, Morais S and Pereira MC, Biosensors on the road to early diagnostic and surveillance of Alzheimer's disease. *Talanta* 211 (2020) 120700. (2) Carneiro, P, Morais S and Pereira MC, Nanomaterials towards biosensing of Alzheimer's disease biomarkers. *Nanomaterials* 9 (2019) 1663 and from a submitted review to *Trac: Trends in Analytical Chemistry*: Carneiro P, Morais S and Pereira MC, Biosensors for α -synuclein detection: Towards an improved diagnosis of Parkinson's disease.

2.1 Biosensors

A biosensor is defined by the International Union of Pure and Applied Chemistry (IUPAC) as an integrated device capable of providing quantitative or semi-quantitative information about target analytes, in which biological recognition elements are in direct spatial contact with the transducer [6, 7]. Biosensors can be classified in terms of the biological recognition element, type of transducer used or both [8]. The biological recognition element is the molecular species responsible for detection and identification of the chemical or biological analyte in order to perform a selective and accurate diagnosis, thus becoming the key component of a biosensor [9, 10]. Based on the type of biorecognition element, biosensors can be classified as immunosensors (antibody-antigen), nucleic acid, cell/tissue and enzymatic biosensors, which can be further divided in two broad categories according to the biorecognition principle [8, 9, 11, 12]. Catalytic biosensors are typically characterized by the use of enzymes for the biological recognition events while affinity biosensors, termed as a result of the high affinity of biological events and species used for biorecognition, englobe the use of antibodies and DNA [12]. Moreover, biosensors can be further categorized as optical, thermal, piezoelectric and electrochemical, depending on the type of signal studied [8, 9, 13, 14]. Biosensors performance is usually experimentally evaluated based on its sensitivity, limit of detection (LOD) and quantification (LOQ), linear and dynamic ranges, precision of the response, selectivity and response to interferences and, finally, its stability over time.

When developing biosensors, one of the main goals is to develop an analytical platform that is both simple to use and sensitive. The simplest methods towards detection of relevant species are usually based on optical detection, as for example colorimetric evaluation where the output signal can be visible at naked eye. Despite the simplicity of these procedures, biosensors based on this type of transducers are likely to fall short in terms of sensitivity, particularly when the clinically relevant levels of the biomarkers of interest tend to be low. More sensitive biosensors have been accomplished through the use of electrochemical and optical (plasmonic techniques) transducers, attaining LODs in the femtomolar and picomolar ranges.

To sum up, biosensors are devices capable of converting biological activity into a measurable electronic signal, which can be detected through several techniques each having its own strengths and weaknesses. The relatively low cost, rapid response and

possibility of miniaturization of biosensors make them useful in a variety of fields including healthcare, biological analysis, environmental monitoring and food quality control, among others [7, 15-17], being one of the most active areas in the field of analytical chemistry. Various types of biosensors are currently being developed and many are commercially available [18]. Generally there are only two readout methods employed, optical or electrical. Biosensor developments contained herein are electrochemical based using antibodies as the biorecognition element and therefore a brief overview of electrochemical techniques and electrochemical immunosensors follows.

2.2. Electrochemical techniques

Electrochemistry is a powerful tool to probe reactions involving electron transfer, evaluating the relationship between the measurements of electrical quantities and chemistry. This way, electroanalytical techniques are able to quantify the current, charge, phase, potential and frequency signal outputs associated with chemical, biological or biochemical reactions. This field of chemistry encompasses all heterogeneous chemical reactions involving electron transfer, homogeneous reactions that influence reactions at electrode surfaces, properties of the interfaces (the double layer) and bulk properties of electrolytes [19].

Electrochemical techniques can be divided in static (potentiometric) and dynamic techniques (voltammetric, amperometric or impedimetric) [12, 20]. In static techniques, the potential of an electrode is measured relative to the potential of a reference electrode, drawing negligible current [20-22]. On the other hand, dynamic techniques that measure processes at electrode/electrolyte interfaces can be subdivided in controlled potential and controlled current techniques. In controlled potential techniques, the applied potential is controlled in a desirable manner, keeping it constant at a certain value (amperometric) or varying within a programmed range (voltammetric). The current is then measured as the answer of the system to the applied potential [20-23]. The objective of a controlled-potential electroanalytical experiment is to obtain a current response that is related to the concentration of the target analyte [23]. Voltammetric techniques, such as cyclic voltammetry (CV) and square-wave voltammetry (SWV), are the main techniques used throughout this study. In addition, an impedimetric analysis is frequently used to further support and electrochemically

characterize the changes occurring on the transducers surface. These techniques usually require an electrochemical cell with a three electrode configuration (working, reference and counter electrode) and an electrolyte [21, 23-25]. The working electrode can be considered the most important element of this electrochemical cell since it is the transducer where all of the relevant reactions are going to occur. These reactions can result from the direct electrochemical reduction/oxidation of the analyte or can be indicative of biomolecular interactions such as antibody-antigen binding events or the complementary binding of DNA strands. The reference electrode is a stable electrode that has a well-defined and stable equilibrium potential. It is used as the reference point against which the potential of other electrodes can be measured in an electrochemical cell. The most common reference electrode is silver/silver chloride (Ag/AgCl), since it is easy to construct, inexpensive and non-toxic. Finally, the purpose of a counter electrode is to close the electrical circuit. When a potential is applied to the working electrode, so that a reduction or oxidation process can start, a current begins to flow, being that this current is measured as a result of the flow of electrons between the working and counter electrode. This electrode is usually composed of highly stable and inert materials such as platinum or carbon and presents a large surface area in order to handle any potential variation that may damage the sensitive surface of the working electrode.

Different types of electrical signal used for quantification reflect the differences between voltammetric techniques [21, 22, 26]. Such electroanalytical measurements have been found to have a vast range of applications, where it could be highlighted the biomedical analysis.

2.2.1. Cyclic voltammetry

Cyclic voltammetry (CV) is the most used technique to obtain qualitative information about electrochemical reactions and it is often the first applied technique for the electrochemical study of a compound, a biological material, or an electrode surface [21, 23, 24, 27]. CV is commonly employed to study the reduction and oxidation processes of molecular species while also being invaluable to study electron transfer-initiated chemical reactions, including catalysis. This technique enables the electrode potential to be rapidly scanned in search of redox couples enabling its characterization

from the potentials of peaks on the cyclic voltammogram and from changes caused by variation of the scan rate [21, 23, 27].

CV consists of cycling the potential of an electrode and measuring the resulting current [21, 23, 28]. The excitation signal for CV is a linear potential scan with a triangular waveform (Figure 1A) [28, 29]. The potential excitation signal sweeps the potential of the electrode between two values, designed the switching potentials [28]. In Figure 1B it is possible to see an example of a cyclic voltammogram of a known redox pair as $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. A positive-scanning potential is chosen for the first half of the cycle, starting from a potential value where no reaction occurs. When a solution of $\text{Fe}(\text{CN})_6^{4-}$ is scanned to positive potentials, $\text{Fe}(\text{CN})_6^{4-}$ is oxidized to $\text{Fe}(\text{CN})_6^{3-}$ locally at the working electrode, resulting in the measurement of a current and depletion of $\text{Fe}(\text{CN})_6^{4-}$ at the electrode surface. In the beginning when the potential is not sufficient to oxidize the species $\text{Fe}(\text{CN})_6^{4-}$, the potential starts to be forward scanned (positive direction) leading to the generation of a small anodic current. The anodic current increases rapidly quickly consuming $\text{Fe}(\text{CN})_6^{4-}$ in the diffuse region near the working electrode. When the anodic peak is achieved the current is dictated by the delivery of additional $\text{Fe}(\text{CN})_6^{4-}$ via diffusion from the bulk solution. The volume of the solution at the surface of the electrode containing oxidized $\text{Fe}(\text{CN})_6^{3-}$, called diffusion layer, continued to grow throughout the scan slowing the mass transport of $\text{Fe}(\text{CN})_6^{4-}$ to the electrode, which results in a signal decrease as the signal is scanned into more positive potentials. When the switching potential is reached, the scan direction is reversed and the potential is scanned in a negative (cathodic) direction. While the concentration of $\text{Fe}(\text{CN})_6^{4-}$ at the electrodes surface was depleted, the concentration of $\text{Fe}(\text{CN})_6^{3-}$ increased. This way, as the reverse scan moves into more negative potentials, $\text{Fe}(\text{CN})_6^{3-}$ starts to be reduced back into $\text{Fe}(\text{CN})_6^{4-}$. The cathodic current increases rapidly, quickly consuming and depleting $\text{Fe}(\text{CN})_6^{3-}$ in the diffuse region near the working electrode. The anodic current then decreases consuming the remaining $\text{Fe}(\text{CN})_6^{3-}$ in the diffuse layer (Figure 1B) [30].

The wide application of CV results from its capability to rapidly detect the redox behavior over a wide potential range and to provide information on kinetics of heterogeneous electron-transfer reaction, coupled chemical reactions or adsorption processes [21, 23, 24, 27].

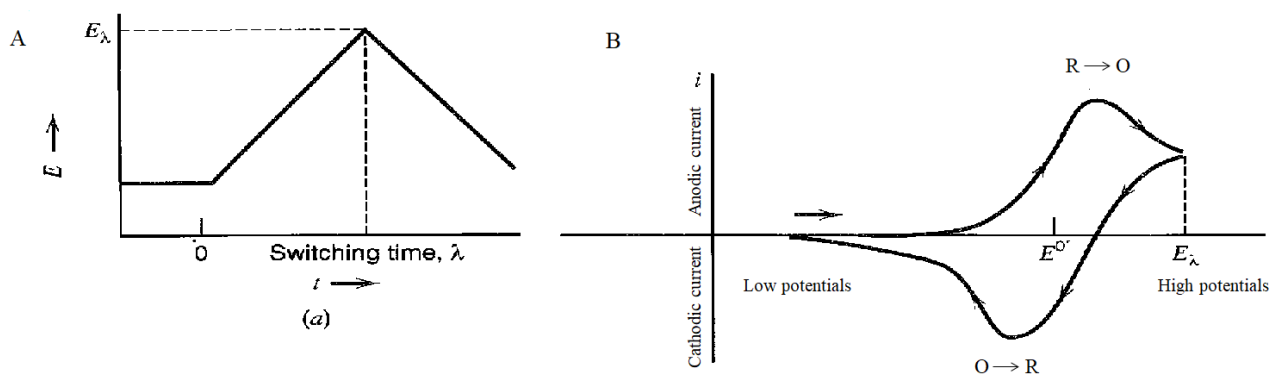


Figure 1 – A) Typical excitation signal for CV - a triangular potential waveform; B) Resulting cyclic voltammogram [26].

2.2.2. Square-wave voltammetry

Square-wave voltammetry (SWV) is a further improvement of staircase voltammetry, which is itself a derivative of linear sweep voltammetry [21, 23, 24, 31]. In linear sweep voltammetry the current at a working electrode is measured while the potential between the working electrode and a reference electrode is swept linearly in time. SWV is a large amplitude differential technique in which a waveform is composed by symmetrical square waves [21, 23, 24]. The excitation signal in SWV (Figure 2) consists of a symmetrical square-wave pulse of amplitude superimposed on a staircase waveform, where the forward pulse of the square wave coincides with the staircase step [21, 23, 24]. The current is sampled twice during each square-wave cycle, once at the end of the forward pulse and once at the end of the reverse pulse. The current is measured at the end of each potential change so that the contribution to the current signal from the capacitive charging current is minimized [23, 31]. The net current is obtained by taking the difference between the forward and reverse currents and is centered on the redox potential [23]. The peak height is directly proportional to the concentration of the electroactive species and direct detection limits as low as 10^{-8} mol L^{-1} are possible [21, 23]. The core principle behind pulse techniques resides in the difference in the decay rates of the charging and faradaic currents. The charging current decays much more rapidly than the faradaic current being that at the end of each pulse the capacitive current is negligible compared to that of the faradaic current. The increased ratio of faradaic to non-faradaic current allows for lower detection limits as well as higher sensitivity, and is therefore ideal for analytical purposes [23, 27, 32].

SWV has several advantages, namely excellent sensitivity and the minimization of background currents as a result of a large net current compared to either forward or backward current [21, 23, 24, 27]. The peak currents are four times higher than differential pulse voltammetry (DPV) response, while its major advantage is its speed [27]. The effective scan rate is of the order of 500 mV/s and as a result, the analysis time is drastically reduced being that a complete voltammogram can be recorded within seconds as opposed to the 2-3 min required with DPV [27]. Another advantage of this technique is the minimization of oxygen interference. The detection limits of this technique can be compared to those of chromatographic and spectroscopy techniques [21, 23, 31].

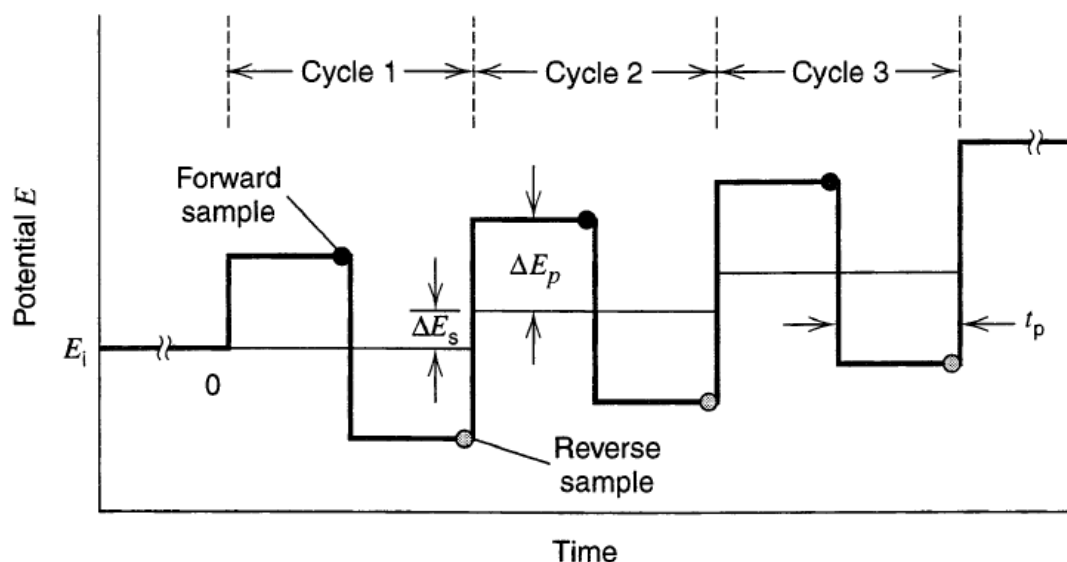


Figure 2 - Schematic waveform for square-wave voltammetry [26].

2.2.3. Electrochemical Impedance Spectroscopy

Electrochemical Impedance Spectroscopy (EIS) has seen tremendous increase in popularity in recent years [33]. Initially applied to the determination of the double layer capacitance, they are now applied to the characterization of electrode processes and complex interfaces [19, 33]. EIS studies the system response to the application of a periodic small amplitude AC signal. These measurements are carried out at different AC frequencies and analysis of the system response contains information about the interface, its structure and reactions taking place there [33]. By varying the excitation

frequency of the applied potential over a range of frequencies, one can calculate the complex impedance, sum of the real and imaginary impedance components, of the system as a function of the frequency (*i.e.* angular frequency, ω) [17]. Therefore, EIS combines the analysis of both real and imaginary components of impedance, namely the electrical resistance and reactance [17, 33, 34].

EIS possesses the ability to study any intrinsic material property or specific processes that could influence the conductivity/resistivity or capacity of an electrochemical system. Therefore, EIS is a useful tool in the development and analysis of materials for biosensor transduction, such as the study of polymer degradation [17, 19]. However it is a complementary technique and other methods must also be used to elucidate the interfacial processes [17, 33].

The electric equivalent circuit first proposed by Randles, shown in Figure 3, is commonly used in EIS for interpretation of impedance spectra [34]. It includes a solution resistance (R_s), a double layer capacitor (C_d) and a charge transfer (R_{ct}) or polarization resistance (R_p). When the charge transfer takes place at the interface, the mass transports of the reactant and product take on roles in determining the rate of electron transfer, which depends on the consumption of the oxidants and the production of the reductant near the electrode surface [34]. The mass transport of the reactants and the products provides another class of impedance, Warburg impedance (Z_w), which can be exploited by electroanalytical chemists because it shows up in the form of a peak current in a voltammogram or a current plateau in a polarogram [34].

In addition, to be a useful model in its own right, the Randles model is the starting point for other more complex models [34]. The double layer capacity is parallel with the impedance due to the charge transfer reaction [34]. Figure 3 shows an example of a Nyquist plot for a Randles cell. The solution resistance can be found by reading the real axis value at the high frequency intercept, which is the intercept near the origin of the plot [34]. The value at the right side of the real axis (low frequency region) is the sum of the charge transfer resistance and the solution resistance [34]. The intermediate-frequency component (*circle*) arising from the R_p and C_d is located in between [34]. In a simple situation, the Warburg element manifests itself in EIS spectra by a line with an angle of 45 degrees in the low frequency region [34].

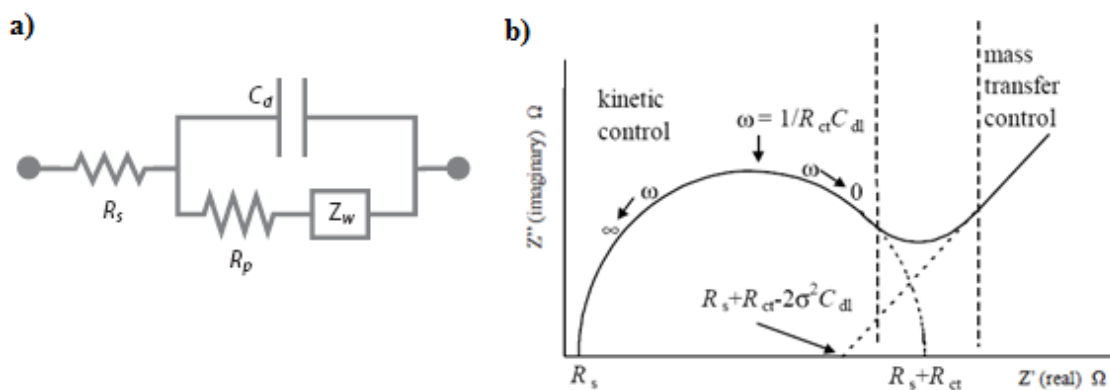


Figure 3 - a) A schematic diagram of an idealized Randles electrical equivalent circuit [34]; b) Nyquist plot showing the high and low frequency components [33].

For electrochemical sensing, impedance techniques are useful to monitor changes in electrical properties arising from biorecognition events at the surfaces of modified electrodes. For example, changes in the conductivity of the electrode can be measured as a result of protein immobilization and antibody-antigen reactions on the electrode surface [17, 19].

EIS has become a mature and well understood technique. It is now possible to acquire, validate and quantitatively interpret the experimental impedances. However, the most difficult problem in EIS is modeling of the electrode processes. There is almost an infinite variety of different reactions and interfaces that can be studied (corrosion, coating, conducting polymers, batteries and fuel cells, etc.) and the main effort is now applied to understand and analyze these processes [17, 19].

2.3. Electrochemical immunosensors

Immunosensors are affinity ligand-based biosensing devices that couple immunochemical reactions to appropriate transducers [35]. Due to the combination of specific antibody-antigen interactions with the high sensitivity of electrochemical transducers, electrochemical immunosensors have been the focus of considerable interest exhibiting intrinsic advantages that include remarkable sensitivity, operational simplicity, low-cost instrumentation, inherent miniaturization and potential of automation. The merits of immunosensors are related to selectivity and affinity of the antibody-antigen reaction [36]. High specificity is achieved by the molecular recognition of target analytes by antibodies to form a stable complex on the surface of an immunosensor [35, 37-40]. In fact, the affinity between an antigen and antibody is a

measure of the strength of the binding forces resultant from the antigen-antibody complex, where values up to 10^{15} have been reported for the association constant under normal pH, temperature and buffer solution [15]. Nevertheless, minor chemical modifications to the molecular structure of the antigen can drastically affect the antibodies affinity [15].

On the other hand, sensitivity depends on several factors including the use of high affinity analyte-specific antibodies, their orientation after being immobilized on the transducers surface and the appropriate detection system for measuring the analytical signal [39, 40]. This recognition reaction defines the high selectivity and sensitivity of the transducer device [35]. The electronic part is used to amplify and digitalize the physicochemical output signal from the transducer devices such as electrochemical (potentiometric, conductometric, capacitive, impedance, amperometric), optical (fluorescence, luminescence, refractive index) and microgravimetric devices [35].

Studies have shown that electrochemical methods are robust and can accurately detect biomarkers in complex unpurified heterogeneous biological samples, making electrochemical immunosensors highly applicable for detection of clinically relevant proteins, from cancer, infectious disease to neurodegenerative diseases, highlighting their applicability for the future of medical diagnosis.

2.3.1. Immunoassays

Immunoassay is the predominant analytical technique for quantitative measurements, being used over a wide range of concentrations, in many different biological matrices and in a range of delivery formats. Immunoassays are the quantitative methods of analysis where antibodies are the primary binding agents for the antigen of interest [35, 36, 39, 41].

All immunoassays depend on measuring the fractional occupancy of the recognition sites. Usually, immunoassays are heterogeneous, which means that either the antibody or the antigen is immobilized on a solid carrier and an immunocomplex is formed upon contact with a solution containing the other immunoagent. The evaluation of the immunoassay relies on either the measurement of occupied sites or, indirectly, on measuring unoccupied sites. This leads to the development of a competitive, non-competitive or direct immunoassay format [36, 39].

In a competitive immunoassay, unlabeled analyte in the test sample is measured by its ability to compete with the labeled antigen for a limited number of antibody-binding sites [36, 39]. In electrochemical immunoassays, an enzyme label or an electroactive label is commonly used. Quantitative analysis can be achieved by determining the amount of labeled analyte that interacted at the binding sites [12, 39]. Therefore, with a fixed number of antibody sites, a smaller signal is expected when the ratio between the quantities of sample to labeled analyte is large [39]. In contrast, a larger signal is obtained when there is a small quantity ratio. Therefore, the signal produced by the bound labeled analyte is usually inversely proportional to the amount of sample analyte [36, 39].

Non-competitive immunoassays (also known as a “sandwich” immunoassay) give the highest level of sensitivity and specificity because of the use of a couple of match antibodies. In this format the sample analyte is captured by an excess of a capture antibody, separating it from the bulk sample [12, 39]. The captured analyte is then exposed to an excess of second signal antibody (detection antibody), which will only bind to the existing capture antibody-analyte complex [39]. Enzyme-labeled antibodies are often used as detection antibodies that result in amplification of the measurement signal [36, 39]. In an ideal non-competitive immunoassay, no signal would be produced in the absence of any analyte because there are no appropriate sites available for binding to the signal antibody [39]. However, in practice, this is not the case due to nonspecific interactions between the signal antibody and other components of the immunoassay [39]. Therefore, it is always desirable to use a blocking reagent to reduce these nonspecific interactions. Nonspecific adsorption also needs to be considered when determining the quantity of signal antibody for use in a system [39]. Although this immunoassay format often offers superior specificity, it can only be used for the quantification of analytes with two antigenic determinants that can be simultaneously recognized [36, 39].

Finally, an immunoassay can also be performed with direct and label-free detection of the analyte in a one-step process, which allows real-time monitoring [13]. The simplicity, speed and inexpensiveness of this approach are its major advantages and are among the driving forces for the development of label-free transduction principles. However, the performance in the detection of low concentrations or small molecules (or both) may be limited. Direct detection without labeling can be performed by cyclic voltammetry, chronoamperometry, impedimetry, and by measuring the current during

potential pulses (pulsed detection). These methods are able to detect a change in capacitance and/or resistance of the electrode induced by binding of protein [13, 42]. These immunosensors have been developed using various substrates [15, 41, 42].

2.4. Working electrodes and nanomaterials

The electrode material and fabrication specifications are crucial aspects of the development of a biosensor as they will directly impact the device performance, particularly the manner in which the electrodes surface is going to be modified and coated [12, 15, 43, 44]. The choice of an electrode material depends on a great extent on the useful potential range of the electrode in the particular solvent employed and the purity of the materials [21-23, 26]. The usable potential range is limited by one or more of the following factors: decomposition of the supporting electrolyte, electrode dissolution and formation of a layer of an insulating or conducting substance on its surface [21]. Additionally, solid electrodes can be adversely affected by poisoning through contact with solutions containing contaminants [21, 22, 26].

The most frequently used working electrodes involve solid materials composed of platinum, carbon and gold. Owing to its physical and chemical properties, glassy carbon electrodes (GCE) are also widely used in electrochemistry [45]. GCE presents a microstructure composed of discrete fragments of curved carbon planes, like imperfect fullerene-related nanoparticles. The network of randomly and tangled carbon planes forms a dense carbon structure [23, 25, 45]. Due to low reactivity, high hardness, impermeability, high temperature resistance and good electrical conductivity, GCE is commonly employed as an electrode material for electroanalysis [23, 25, 45]. In addition, one of the crucial points when working with solid materials is that the analysis is dependent of the surface state of the electrodes and solid electrodes have the disadvantage of possible contamination via contact with different solutions [21]. This way, the surface of these electrodes needs to be regenerated before each use through a cleaning process involving mechanical polishing and potential cycling, consuming time and reagents. In addition, due their large size these type electrodes require the use of larger volumes for the biorecognition events and electrochemical studies.

Screen-printed electrodes (SPEs) have been receiving a lot of attention in recent years with applications in a wide range of fields due to their operation simplicity, reliability, potential portability and small instrumental setups as they comprise the

working, reference and auxiliary electrodes in the same low-size platform [12, 44, 46-49]. In fact, their small size enables to conduct electrochemical studies through the use of very small volumes (few microliters), which is ideal for the development of immunological procedures [50]. In addition, the decrease in the diffusion distances for the analytes to reach their surface immobilized receptor allows for shorter incubation periods and, therefore, faster assays [50]. Moreover, the disposable feature of SPEs make them reliable platforms for the direct analysis of several samples of different origins preventing electrode poisoning from recurrent use [47-49].

In recent decades, novel modified transducers have been developed based on the unique properties of nanoscale materials. In fact, the stimulating breakthroughs observed in the past decades in the development and synthesis of nanomaterials had a tremendous impact in the growth of biosensing technology as nanomaterials with different sizes, shapes and unique physical and chemical properties have been showing a crucial role in designing highly sensitive and selective biosensors [16, 46, 51-57]. Nanomaterials exhibit remarkable optical and electrical properties increasing the transducers conductivity, catalytic activity and plasmonic properties, which combined with their excellent biocompatibility and stellar physical properties such as high surface area and high surface-to-volume ratio, make them ideal materials to intermediate biorecognition events with different transducers [58, 59].

The most frequently applied nanomaterials in the development of immunosensors are carbon materials such as graphene and carbon nanotubes (CNTs) and gold nanoparticles (AuNPs), all having unique and specific properties applicable in the development of novel transduction schemes. Other nanomaterials such as silver (AgNPs) and magnetic nanoparticles, dendrimers and quantum dots (QDs) are also used, but to a lesser extent. These nanomaterials have most commonly been reported for the development of biosensors performing detection via sandwich immunoassays/DNA assays or through direct detection.

AuNPs are a very versatile nanomaterial with unique optical and electronic properties which combined to their high surface-to-volume ratio and excellent biocompatibility make them exceptional scaffolds for interfacing biorecognition events into electrochemical or optical signals [58, 60]. In addition, AuNPs can be synthesized in a very straightforward manner and with a high level of stability being their size, shape, charge and surrounding chemical environment easily tuned [60]. This way, by applying AuNPs with different properties, novel sensing platforms with improved

sensitivity, stability and selectivity have been developed either by using them as solid support providing a suitable microenvironment for biomolecule immobilization or by using them as labels for signal augmentation.

Carbon nanomaterials, such as graphene and CNTs, possess excellent mechanical, electrical and optical properties which in conjunction with their remarkable chemical stability, biocompatibility and ease of functionalization offered new possibilities to develop biosensors with improved analytical behavior. Graphene has attracted a lot of attention as a result of its mechanical strength, chemical stability, electrical conductivity, tunable optical properties and high surface area [61, 62]. Graphene can be chemically modified producing highly functional versions each presenting unique properties that broad the range of applications of this nanomaterial. The ideal graphene analogues for biosensing applications are graphene oxide (GO), a highly oxygenated graphene layer, and reduced graphene oxide (rGO) that results from the chemical reduction of graphene oxide [61]. The attractiveness of these materials result from their tunable conductivity and functional groups rich surface, which makes them highly attractive to conjugate with other nanomaterials or biomolecules [61, 62].

On the other hand, CNTs are well-ordered, hollow graphitic nanomaterials that consist of rolled-up sheets of single-layer carbon atoms and present two types of configuration being classified as single walled (SWCNTs) or multi-walled (MWCNTs) [62, 63]. These materials present excellent properties that contribute to the development of high performance biosensors such as high surface-to-volume ratio, exceptional electronic and optical properties, presence of edge-plane-like defects, ease of functionalization and excellent chemical and thermal stability [62, 63]. As with graphene, CNTs can be chemically modified with direct impact in their physicochemical properties [61]. Thus far, as a result of their high stability and ease of functionalization, these carbon nanomaterials have been frequently used to improve the physical and chemical properties of the transducers, in addition to contribute to the increase of their optical and electrical behavior, stability and life time.

Functionalization of nanomaterials has contributed to the development of highly sensitive and selective bioassays and biosensors for nucleic acids and proteins by integrating the biological recognition elements with the components of various transduction mechanisms [16, 52, 57, 64]. In this regard, considerable attention has been devoted to the immobilization of biological recognition elements as this aspect will impact the detection sensitivity, reproducibility and robustness, among other analytical

parameters [54, 57, 65, 66]. The performance of a biosensor is predominantly dependent on the binding affinity and specificity of biorecognition elements, their coating density onto the transducers surface and, finally and most importantly, their orientation after immobilization. These immobilization procedures should retain the biorecognition elements full biological activity by ensuring that its binding sections remain intact and accessible while also providing an effective electronic connection between the redox active sites in the biomolecules and the transducers surface [52, 55, 59, 67, 68].

Immobilization of biological components on the surface of transducers can involve varying levels of difficulty from simple and nonspecific methods, which often results in randomly oriented proteins, to highly structured bioconjugates with oriented immobilization, higher stability and reproducibility [59]. The simplest methods that have been used are based on physical immobilization, which can be promoted mainly via electrostatic force, ionic bonds and hydrophobic interactions [55, 59, 68, 69]. In addition, entrapment in (bio)polymer matrices has also been used to promote better attachment of biomolecules [59, 68]. Nonetheless, physical immobilization often results in random orientation and weak attachment and even in the cases of entrapment, limited recognition efficiency is still a problem due to burying of recognition sites [68]. A more robust, stable and reproducible way to immobilize bioreceptors, that results in higher sensitivity, is through covalent bonding involving the coupling of reactive chemical groups present on the protein and on the transducer surface [59, 68, 69]. Covalent links can be formed by amine, carboxyl and thiol coupling reactions, cross-linking reagents, such as glutaraldehyde, thiol derivatives and polymers, or click chemistry [55, 59, 68, 69]. Despite its crucial role in protein immobilization, covalent bonding has still one major drawback related with uncontrolled anchoring of biomolecules, which inevitably affects the domain responsible for the recognition event [68]. This way, techniques that promote site specific immobilization have also been explored. Non-covalent approaches for site-specific immobilization are based on bioaffinity interactions. The most widely applied is the biotin-strept(avidin) interaction where biotinylated biomolecules can be attached to substrates via avidin (or streptavidin) bridges [55, 68]. Another commonly used affinity based immobilization tag is through binding of biorecognition elements to protein G or A. Site-specific immobilization can also be achieved via covalent bonding through the introduction of unique chemical groups or sequences, such as azides or alkynes, at a specific location that will covalently bind the protein to the complementary functionalized surface, without affecting the binding domain [55, 67, 68]. In addition,

there is still the possibility of protein engineering, as for example through cleavage of the antibody at the hinge region in order to take advantage of their native thiol groups for immobilization on gold surfaces [68, 70, 71].

To conclude, the synergy between nanomaterials, biological recognition elements and different transduction techniques has been contributing for the development of biosensors that meet the required analytical parameters. In fact, the outstanding advances in nanotechnology, namely in the synthesis and production of a vast number of nanomaterials, has led to striking developments in the biosensing field. Biosensing platforms coupled with nanostructures not only exhibit a better electrochemical and optical behavior as they also provide an appropriate and biocompatible environment for the immobilization of recognition elements, which is a crucial stage in the development of immuno- and DNA- biosensors.

Chapter 3. Parkinson's disease biosensors

*Adapted from original publication: Carneiro, P Loureiro JA, Morais S and Pereira MC, Nanostructured label-free electrochemical immunosensor for detection of a Parkinson's disease biomarker. *Talanta* 252 (2023) 123838. Adapted from Carneiro P, Morais S and Pereira MC, Biosensors for α -synuclein detection: Towards an improved diagnosis of Parkinson's disease. *Trac: Trends in Analytical Chemistry* (In revision).

Abstract

Diagnosis of Parkinson's disease (PD) remains a clinical exercise based on the assessment of motor symptoms combined with neuroimaging evaluation, which often results in diagnosis performed in more advanced stages of the disease. Therefore, it is essential to develop biosensing platforms capable of performing accurate measurements of biomarkers closely reflecting its pathophysiology. A timely and accurate etiologic diagnosis of this condition will improve patient management as well as open the possibility of their enrollment in clinical trials of disease modifying treatments. In this regard, this chapter addresses the topic of biosensors towards detection of PD biomarkers, from state of the art to experimental work. This way, this chapter is organized in two sections. In the first part, a comprehensive review of the biosensors developed during the last decade towards detection of α -synuclein and its related species is presented, focusing on the advances, challenges and recent trends of PD biosensing. In the second part of this chapter, the development of a nanostructured label-free electrochemical immunosensor for detection of low levels of α -synuclein in simple and complex samples is presented. The proposed immunosensor was based on different electrochemical transducers modified in a layer-by-layer approach, which through extensive characterization led to their successful nanostructuration, through the drop-cast of single-walled carbon nanotubes suspension followed by electrodeposition of gold nanoparticles. The immunosensor was developed with two different electrochemical transducers namely, glassy carbon electrode and screen-printed carbon electrode, being their analytical behavior studied and compared as well as their suitability for clinical application into point-of-care devices. The nanostructured immunosensor provided a great interface for electronic transduction and biological recognition events, which enabled fast, sensitive and specific detection of α -synuclein while being based on a simple and inexpensive technology requiring small sample volumes.

3.1. State of the art

Parkinson's disease (PD) is a slow progressive nervous system disorder that arises from a combination of genetic and environmental factors and affects multiple neuroanatomical areas resulting in clinical heterogeneity with varied patterns of disease progression [3, 72].

Currently, PD is estimated to affect 6.2 million individuals worldwide being that its prevalence more than doubled from 1990 to 2015 [73-75]. Since its incidence sharply increases with age, its numbers are expected to double in the next two to three decades as a result of the continuous aging of the population, which can become a burden on the healthcare and socioeconomic systems [75-77]. In compliance with other degenerative diseases, the disease duration can span for decades [78].

PD is clinically defined as a progressive movement disorder, being particularly complex in its clinical assessment, diagnosis and treatment [3, 74, 76]. It is characterized by the early loss of dopaminergic neurons in the substantia nigra pars compacta, which leads to the depletion of dopamine in the basal ganglia and, consequently, the appearance of the classical parkinsonian symptoms [3, 73].

Diagnosis of PD relies mostly on clinical criteria which presents some limitations as classical PD symptoms such as motor dysfunction that manifests as resting tremor, bradykinesia, rigidity and postural instability only appear when there has been a substantial and irreversible loss of dopaminergic neurons, approximately 50% [78-81]. On the other hand, non-motor symptoms may arise years prior to the onset of motor symptoms but they are not sufficiently specific to detect and differentiate PD [80]. This way, in routine clinical practice, diagnosis are often made in advanced stages of the pathology or can result in misdiagnosis, with misclassifications errors ranging between 15 and 24%, as a result of clinical overlap with other parkinsonian disorders [74, 78, 79]. In fact, a definitive diagnosis of PD can only be established through identification of neuropathological changes in the post-mortem brain resulting from the presence of substantia nigra pars compacta degeneration along with Lewy pathology, which are neural inclusions composed of misfolded and aggregated α -synuclein [3, 78]. Therefore, the development of analytical tools to contribute to the identification of diagnostic and prognostic biomarkers reflecting the pathophysiology, progression and severity of PD is of paramount importance as it would significantly improve the diagnostic process of PD

as well as the design of clinical trials with potential disease-modifying drugs capable of preventing or delaying disease progression [81, 82].

This chapter focuses on the advances made for the development and application of biosensors for detection of PD potential biomarkers, α -synuclein and its related species, in simple and complex samples. These approaches, based on electrochemical and optical transducers, will eventually contribute to overcome the limitations of current diagnostic methods with higher emphasis being given to the innovation of the transducers functionalization and signal amplification strategies, responsible for the biosensors exceptional sensitivity. Finally, it brings into focus future perspectives on the biosensing of PD.

3.1.1. Parkinson’s disease biomarkers

In recent years, progress has been made to optimize the diagnostic process of PD, particularly through the identification of fluid biomarkers. A key pathological hallmark of PD is defined by the aggregation of α -synuclein leading to the development of Lewy pathology [3]. Although, the normal function of this protein is not completely understood, its location in the pre-synaptic nerve terminals suggests that α -synuclein might have an important function in vesicle trafficking in nerve terminals, lipid metabolism and synaptic plasticity [73, 83]. In a misfolded state, α -synuclein becomes insoluble and aggregates to form intracellular inclusions within the cell body (Lewy bodies) and processes (Lewy neurites) of neurons [3, 84]. Lewy pathology can also be found in the spinal cord and peripheral nervous system [3, 84-86]. The central role of α -synuclein in PD development is further supported by the fact that genetic mutations occurring in the gene encoding α -synuclein (SNCA) as well as post-translational modifications of this protein, such as phosphorylation, ubiquitination, nitration, truncation and oxidation, might influence protein misfolding and consequent aggregation and neurotoxicity [79, 84, 87, 88]. In addition, this pathological hallmark is also accompanied by a crowded environment of membranes, including vesicular structures and dysmorphic organelles [3, 72, 78].

Meta-analysis studies have evaluated the levels of α -synuclein species (total, oligomeric and phosphorylated α -synuclein) in cerebrospinal fluid (CSF) and despite lack of concordance between studies, most have verified a reduction of α -synuclein levels in PD patients when compared with healthy controls as opposed to an increase in

the oligomeric and phosphorylated species [79, 83, 89, 90]. The discrepancy reported on the levels of α -synuclein may result from a lack of standardized methods for α -synuclein determination as well as evaluation of PD patients with different characteristics such as phenotype, age, disease stage and therapeutics [79, 88, 90]. In these studies, immunoassays such as enzyme linked immunosorbent assay (ELISA) or electrochemiluminescence and xMAP technology were frequently used to analyze CSF α -synuclein [79].

Another significant problem in PD diagnosis resides in the overlap of α -synuclein levels with other synucleinopathies such as dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). Seeding aggregation assays such as real-time quaking-induced conversion (RT-QuIC) and protein-misfolding cyclic amplification (PMCA) have been developed, which can detect minute amounts of misfolded protein in CSF and explore their prion-like behavior [73, 79]. Since α -synuclein filaments may differ between synucleinopathies, CSF based α -synuclein seeding assays can be further optimized to differentiate between these diseases [73, 88].

Efforts have been made to establish a diagnostic and prognostic model to improve the diagnostic accuracy of PD, especially in its early stages. The success of this model may reside in the combination of multiple biomarkers to address several pathological mechanisms related with PD development, including evaluation of α -synuclein species, lysosomal enzyme activities, neurofilament light chain as well as Alzheimer’s disease (AD) related biomarkers like β -amyloid and tau protein [79, 83, 88, 91]. In addition, it has been reported that evaluating the ratio between oligomeric or phosphorylated α -synuclein and total α -synuclein can improve diagnostic accuracy [79, 88, 91].

This way, biosensors appear as the most promising alternative to solve the aforementioned problems by providing a sensitive, specific, and cost-effective analytical tool for the identification and determination of multiple analytes, which could significantly contribute to more precise and timely diagnosis of this debilitating disease.

3.1.2. Parkinson’s disease biosensors

Electrochemical and optical biosensors have been developed for the detection of α -synuclein [92-106] and its related species, including α -synuclein autoantibodies [107-109], phosphorylated α -synuclein [110], nitrated α -synuclein [111, 112] and α -synuclein oligomers [113-121], in simple and complex samples with higher emphasis being given

to its determination in serum. Detailed information about the type of biomarker, biosensors configuration and analytical information is described in Table 1. Most of these studies were able to detect and quantify very low levels of α -synuclein related biomarkers, attaining LODs exceptionally lower than commercially available products such as ELISA. This dynamic makes them excellent candidates for the development of diagnostic tools capable of performing accurate determination of this biomarker in more accessible biological fluids, such as blood, where the levels of this biomarker tend to be ten to one hundred times lower than the levels found in CSF [122]. In fact, despite CSF remaining the gold standard for the determination of biomarkers levels, its sampling method is highly invasive and with constraints for patients, making its repeated measurements very challenging.

Most affinity based biosensors require a label attached either to the antigen or the antibody to produce an amplified output signal in which the amount of labels is detected and assumed to be proportional to the analytes concentration [36]. Unexpectedly, most of the reported works for detection of these biomarkers were based on direct label-free strategies, in contrast to the trend observed in the works described for determination of AD biomarkers [123, 124]. In fact, from Table 1, it is possible to conclude that only 5 works developed sandwich assays [99, 103, 111-113]. Despite the signal amplification achieved during these analytical procedures, labelling a biomolecule might affect the biological recognition element-analyte binding efficiency and the yield of the labelling reaction is highly variable [42]. This way, the advantages and simplicity of direct label-free biosensors have been increasingly explored as it can be seen from Table 1. On the other hand, in these strategies without amplification of the analytical signal it is crucial to promote the immobilization of a large number of biorecognition elements or ensure their proper orientation upon immobilization, which in combination with effective approaches to minimize non-specific interactions can contribute for the development of biosensors with a high level of sensitivity and specificity [42]. This way, a common strategy applied for the modification of the transducers surface was through the formation of self-assembled monolayers (SAMs). SAMs are simple and organized chemical coatings that can have a profound effect on the electrode-solution interface. This way, they can be used to increase the antifouling properties of the transducers or offer molecular binding sites for the immobilization of biomolecules or for combination with different nanomaterials leading to the formation of supramolecular assemblies [125].

Table 1 - Biosensors for the detection of α -synuclein related biomarkers.

Transducer	Modification	Detection Method	Detection Technique	Sample	Detection range (nM)	LOD (nM)	Ref.
α-synuclein							
Electrochemical	SPCE modified with SWCNTs, AuNPs and monoclonal antibodies	Direct assay	SWV	Buffer and FBS	7.1×10^{-4} – 7.1×10^{-1} *	2.9×10^{-4} *	[92]
	GCE modified with a GO/AuNPs composite and antibodies	Direct assay	DPV and EIS	Buffer and human plasma	0.29 – 9.1*	0.29*	[93]
	Silver IDE modified with SWCNTs and gold nanourchin/antibody complex	Direct assay	Voltammetry	Buffer	1.0×10^{-6} – 1.0×10^{-3}	1.0×10^{-6}	[94]
	Fluorine-doped tin oxide electrode modified with a	Direct assay	DPV and EIS	Buffer and	0.710 – 71.4*	DPV - 0.26*	[95]

cystamine SAM and antibodies			human serum	EIS - 0.081*		
Gold electrode modified with a mixed SAM of 3-MPA and 2-MU and further functionalized with antibodies	Direct assay	EIS	Buffer and exosomes from patient serum	$7.1 \times 10^{-4} - 7.1$	2.1×10^{-5}	[96]
ITO coated PET modified with AuNPs, PGA and antibodies	Direct assay	EIS and SWV	Buffer and CSF	$2.9 \times 10^{-4} - 0.14 *$	$9.6 \times 10^{-6} *$	[97]
GCE modified via covalent binding with G4-polyamidoamine dendrimer-encapsulated AuNPs (PAMAM-Au nanocomposite), α -synuclein and antibodies	Indirect assay with AuNPs and detection antibodies labeled with HRP	Amperometry	Buffer	$1.43 \times 10^{-3} - 14.3 *$	$1.0 \times 10^{-3} *$	[98]

TiO ₂ nanotube array electrode functionalized with AuNPs and antibodies	Sandwich assay with AuNPs and GOx	PEC	Buffer	$3.6 \times 10^{-3} - 7.1 *$	$2.4 \times 10^{-3} *$	[99]
ALIDEs modified with zinc oxide and Au nanorod composite, further functionalized with antibodies	Direct assay	Voltammetry	Buffer and human serum	$1.0 \times 10^{-9} - 1.0 \times 10^{-3}$	1.0×10^{-9}	[100]
EGOFET with a gold gate electrode functionalized with a monoclonal antibody: Approach I: Au wire functionalized with a SAM; Approach II: Au wire functionalized with recombinant His-tag protein G	Direct assay	Conductance	Buffer	Approach I: $2.5 \times 10^{-4} - 1.0$ Approach II: $2.5 \times 10^{-4} - 250$	2.5×10^{-4}	[101]
IGZO-based electrolyte-gated field-effect transistor	Direct assay	Conductance	Buffer	$7.1 \times 10^{-7} -$	NR	[102]

	functionalized through amino-silanization with APTES and further modified with antibodies				$7.1 \times 10^{-2} *$		
Optical	Glass slides covered with Au film modified with a 3-MPOH and 11-MUA SAM, Fe_3O_4 nanoparticle functionalized with streptavidin and biotinylated antibodies	Sandwich assay through detection of a secondary monoclonal antibody	SPR	Buffer and human serum	$7.14 \times 10^{-7} - 7.14 \times 10^{-3} *$	$4.0 \times 10^{-7} *$	[103]
	U-shaped fiber optic probe immobilized with AuNPs and functionalized using a film of chitosan as bioreceptor	Direct assay	LSPR	Buffer	70 – 700	70	[104]
	5CB liquid crystal dispensed on a gold substrate followed by addition of CTAB surfactant mixed with DNA	Direct assay	Polarized Microscope	Buffer	0.010 – 0.030	0.010	[105]

aptamer and α -synuclein								
	DNA aptamer anchored to gold coated glass followed by formation of a mixed SAM, DNA aptamer and addition of 5CB liquid crystal	Direct assay	Polarized Microscope	Buffer	50 – 400	50	[106]	
α-synuclein autoantibody								
Electrochemical	Gold microelectrode arrays modified with cysteamine, GO and α -synuclein	Direct assay	EIS	Buffer and human serum	$5.0 \times 10^{-3} - 1.0$	1.2×10^{-3}	[107]	
	AuE modified with PEG thiol, α -synuclein	Direct assay	EIS	Buffer and human serum	0.050 – 1.0	0.021	[108]	
	AuE modified with PEG thiol, α -synuclein	Direct assay	EIS	Buffer and blood	0.50 – 10	0.055	[109]	

							serum
Phosphorylated α-synuclein							
Optical	Glass slides covered with Au film modified with a 11-MUA SAM and antibodies followed by α -synuclein detection and addition of TiPNPs	Direct assay - Signal amplification with TiPNPs for phosphorylated α -synuclein detection	SPR	Buffer, aCSF and CSF samples	Total α -synuclein: 7.14×10^{-5} – 1.43×10^{-3} *	Total α -synuclein: 5.0×10^{-6} *	[110]
					Phosphorylated α -synuclein: 7.14×10^{-6} – 7.14×10^{-4} *	Phosphorylated α -synuclein: 2.3×10^{-6} *	
Nitrated α-synuclein							
Optical	Gold chip modified with a 11-MUA SAM and antibodies	Sandwich assay with AuNPs functionalized	LSPR	Buffer and serum	0.50 – 17.1 *	0.13 *	[111]

		with pSC ₄ and detection antibodies		samples			
Electrochemical	Gold electrode modified with pSC ₄ , AuNPs functionalized with pSC ₄ , paraquat and antibodies	Sandwich assay with detection antibodies conjugated to MNPs	EIS	Buffer and serum samples	0.0714 – 71.4*	0.022*	[112]
α-synuclein oligomers							
Electrochemical	Dual mode immunosensor: 96 well plates modified with antibodies for the biorecognition event; ITO modified with TBAPy/AgNPs	Sandwich assay with detection antibodies conjugated to glucose-loaded liposomes	PEC and DPV	Buffer and human serum	PEC: 1.0 fg mL ⁻¹ – 10 ng mL ^{-1#} DPV: 1.0 fg mL ⁻¹ –1.0 ng mL ^{-1#}	PEC: 0.43 fg mL ^{-1#} 3.1×10 ⁻⁸ EC: 0.65 fg mL ^{-1#}	[113]

nanocomposite

GCE modified with PDG, AuNPs, MWCNTs, rGO and antibodies	Direct assay	SWV	Buffer and Plasma	$5.0 \times 10^{-8} - 5.0 \times 10^{-4}$	3.0×10^{-8}	[114]
Printed circuit board platform with a nanoporous alumina membrane and a silicone micro fluidic chamber. The surface was amine functionalized using APTES, DTSSP and antibodies	Direct assay	EIS	Buffer and CSF	$1.0 \text{ pg mL}^{-1} - 1.0 \text{ } \mu\text{g mL}^{-1\#}$	$1.0 \text{ pg mL}^{-1\#}$	[115]

Gold electrode modified with graphdiyne, AuNPs and TsDNA followed by WSe ₂ nanoflower signal enhancement	Indirect assay	PEC	Buffer and Serum	$1.0 \times 10^{-8} - 1.0$	3.3×10^{-9}	[116]
AuE modified with 1D/2D structural nanohybrid of CoMn-based ZIF nanosheets vertically grown around CNFs and functionalized with DNA aptamers	Direct assay	EIS	Buffer and Human Serum	$5.3 \times 10^{-5} - 0.10$	4.6×10^{-5}	[117]
GCE modified with methylene blue-tagged DNA aptamer adsorbed on rGO	Direct assay	DPV	Buffer and Human Serum	$1.0 \times 10^{-6} - 1.0$	6.4×10^{-7}	[118]
Biosensor 1: ITO glass modified with AuNPs/MOFs composite functionalized with	Direct assay	ECL	Buffer and Serum	Biosensor 1: $2.4 \times 10^{-6} - 4.9 \times 10^{-4}$	Biosensor 1 $- 4.2 \times 10^{-7}$	[119]

DNA aptamers

Biosensor 2: ITO glass modified with MOFs composite and functionalized with DNA aptamers

Biosensor 2: 1.4×10^{-6} – 2.4×10^{-4}
Biosensor 2: -3.8×10^{-7}

SPGE based on methylene blue, exonuclease I as an amplifying element of sensitivity, non-target induced increase size of DNA aptamer and its complementary strands triggered by terminal deoxynucleotidyl transferase

Indirect assay

DPV

Diluted Serum

0.060 – 150

0.010

[120]

Electrochemical and optical

Gold surface modified with DNA aptamers

Direct assay

Colorimetry, SPR and EIS

Buffer and Serum

Colorimetry: 20 – 3000

Colorimetry – 10

[121]

SPR: 0.1 – 500	SPR – 8.0×10 ⁻³
EIS: NR	EIS – 1.0×10 ⁻³

*Value was expressed in $\mu\text{g mL}^{-1}/\text{ng mL}^{-1}/\text{pg mL}^{-1}$ and converted to nM.

Values were not converted to nM since α -synuclein oligomers are heterogeneous structures with different molecular weights.

2-MU, 2-Mercaptoethanol; 3-MPA, 3-Mercaptopropionic acid; 3-MPOH, 3-Mercapto-1-propanol; 11-MUA, 11-Mercaptoundecanoid; 5CB, 4-cyano-4'-pentylbiphenyl; aCSF, Artificial Cerebrospinal fluid; AgNPs, Silver nanoparticles; ALIDEs, Surface modified aluminum interdigitated electrode; APTES, (3-aminopropyl)-triethoxysilane; AuE, Gold electrode; AuNPs, Gold nanoparticles; CNFs, Carbon nanofibers; CSF, Cerebrospinal fluid; CTAB, hexadecyltrimethylammonium bromide; DNA, Deoxyribonucleic acid; DPV, Differential pulse voltammetry; DTSSP, 3,3'-dithiobis(sulfosuccinimidyl propionate); EC, Electrochemistry; ECL, Electrochemiluminescence; EGOFET, Electrolyte-Gated Organic Field-Effect Transistor; EIS, Electrochemical impedance spectroscopy; FBS, Fetal bovine serum; GCE, Glassy carbon electrode; GO, Graphene oxide; GOx, Glucose oxidase; HRP, Horseradish peroxidase; IDE, Interdigitated electrode; IGZO, Amorphous indium gallium zinc oxide; ITO, Indium tin oxide; LSPR, Localized surface plasmon resonance; MNPs, Magnetic nanoparticles; MOFs, Metal organic frameworks; MWCNTs, Multi-walled carbon nanotubes; NR, Non referred; PAMAM, Amine-terminated polyamidoamine; PDG, poly (D-glucosamine); PEC, Photoelectrochemical; PEG, Polyethylene glycol; PET, Polyethylene terephthalate; PGA, Polyglutamic acid; *p*SC₄, Sulfonatocalix[4]arene, rGO, Reduced graphene oxide; SAM, Self-assembled monolayer; SPCE, Screen-printed carbon electrode; SPGE, Screen-printed gold electrode; SPR, Surface plasmon resonance; SWCNTs, Single-walled carbon nanotubes; SWV, Square-wave voltammetry; TBAPy, 1,3,6,8-tetrakis(p-benzoic acid)pyrene; TiPNPs, Titanium phosphate nanoparticles; TsDNA, Triple-stranded DNA; ZIF, Zeolitic imidazolate framework

3.1.2.1 Biosensors for α -synuclein

Promising biosensors are being designed for α -synuclein quantification (Table 1; n=15). Electrochemical techniques were the most reported for determination of α -synuclein [93-102], also presenting the most sensitive platforms. Moreover, from Table 1, it is possible to observe a superior analytical behavior from the works that used nanomaterials to improve the transducers electrochemical behavior, highlighting the frequent application of AuNPs.

Biosensors that presented a great possibility to be integrated into point-of-care devices (POC) were based on the miniaturization of semiconductors into the nanoscale producing highly sensitive field effect transistors (FET) with short turn-around time. FET takes advantage of the changes in the surface charge occurring at FET gate of dielectric layer to modulate the conductance of the semiconductor channel [126]. This way, Ricci S. et al. [101] developed an electrolyte-gate organic field-effect transistor (EGOFET) integrated with microfluidics to ensure the stable and sensitive detection of α -synuclein. The gate electrode (Au wire) was functionalized with monoclonal antibodies proving the advantages of an oriented antibody immobilization through His-tagged recombinant protein G over random covalent immobilization. In fact, both strategies presented similar sensitivity attaining a LOD of 0.25 pM, while the approach with oriented immobilization presented a wider linear range where the upper limit was two orders of magnitude higher, 0.25 pM to 250 nM. In this biosensing platform, the microfluidic device was essential to avoid cross-contamination between the organic semiconductor and the gate electrode [101]. In a different work concerning the application of FET technology to detect α -synuclein, an amorphous indium gallium zinc oxide (IGZO)-based electrolyte-gated field-effect transistor (IGZO-EGFET) was developed, but in this case the biological modifications occurred on the surface of the semiconducting material [102]. The IGZO surface was modified through amino-silanization with APTES, which enhanced the pH sensitivity of the surface while ensuring a platform for the covalent immobilization of monoclonal antibodies through surface activation with EDC/NHS, exhibiting a sensitivity 2 fold higher than the bare IGZO-EGFET. A sensitive detection of α -synuclein was attained with a linear detection range between 0.71 fM and 71 pM. From these two works, it is possible to conclude that a biological coating assembled onto the gate surface or on the semiconducting material can drastically affect the electrical performance of the transistor operated in aqueous

environments, where most biological events occur [102]. Actually, both works achieved excellent sensitivity although with slight differences. Both biosensors operated in a concentration range that is clinically relevant for determination of α -synuclein in more accessible biological fluids, where its concentration is lower, making them interesting tools to be integrated into POC devices. On the other hand, despite the fact that the work developed by Chae MS. et al. [102] was able to detect lower concentrations, its signal was affected by the ionic strength of the analyte solution being forced to operate with lower concentrations of phosphate buffered saline (PBS). In the work developed by Ricci S. et al. [101], some reproducibility problems were observed with some values of the calibration curve presenting large errors. In addition, despite the excellent sensitivity of both biosensors, other parameters such as reproducibility, specificity, stability and performance in complex samples should be further assessed to validate the practical applicability of these platforms.

Another electrochemical transducer that reported excellent sensitivities was based on the development of interdigitated electrodes [94, 100]. These studies demonstrated that by scaling the electrode gap size from μm to nm and through the integration of gold nanomaterials it was possible to develop extremely sensitive platforms capable of detecting α -synuclein in the attomolar to picomolar range. The nanostructuration of the interdigitated electrodes with gold nanorods [100] and gold nanourchins [94] was vital to achieve a better understanding of the charge transfer occurring on thin films while retaining the antibodies biological activity. Nonetheless, future studies of these works should focus on the evaluation of biosensors analytical behavior in complex samples.

In voltammetric or impedimetric analysis, label-free detection of analytes can be performed in two ways, through oxidation of redox-active residues on the analytes structure or through the use of an artificial electroactive agent capable of reflecting the electrical properties of the transducers surface [124]. Therefore, label-free electrochemical immunosensors frequently resorted to the study of ferro/ferricyanide ($\text{Fe}(\text{CN})_6^{3-/4-}$) redox behavior to characterize the transducers surface after every modification, eventually building the respective calibration curves relating the variations in electrochemical signal with increasing α -synuclein concentrations. This way, Karaboga M. et al. [97] reported the development of a voltammetric/impedimetric immunosensor based on an indium tin oxide sheet modified with electrodeposited AuNPs and electropolymerized glutamic acid monomer. The electrochemical deposition and electropolymerization are a simple and effective alternative to produce each layer

directly on a substrate in a single-step. The carboxylic acid groups of glutamic acid were activated through EDC/NHS to promote the successful covalent interaction with the antibodies amino groups. This platform was further blocked with bovine serum albumin (BSA) to ensure that the final modifications in the electrochemical signal resulted from the specific interaction between the immobilized antibodies and α -synuclein. The detection procedure lasted 1 hour at room temperature attaining a sensitive analytical behavior with a linear range of 0.29 to 140 pM and a LOD of 9.6 fM. The biosensor was analyzed through EIS and SWV attaining identical analytical behavior. Nevertheless, SWV is a faster detection technique since voltammetric scans can be completed within seconds, while in EIS requires the implementation of impedimetric tests in a frequency range, which means a period of serious analysis and effort. This biosensor was further tested in CSF samples through the standard addition method, being able to detect α -synuclein in the CSF of both healthy individuals and PD patients with high sensitivity, presenting an efficient and simple analytical tool for application in clinical diagnosis.

CSF remains the most relevant biological fluid for the evaluation of α -synuclein levels. However, the invasiveness of procedure required to extract this fluid fuels the search for simpler and more accessible biological fluids that enable the levels of this biomarker to be ascertained with similar significance. This way, Fu Y. et al. [96] developed a different approach for the evaluation of this biomarker levels in exosomes (Figure 4). This work presented a simple impedimetric biosensing architecture towards α -synuclein detection, where magnetic beads (MBs) were used to collect exosomes. The electrochemical transducer was composed of a gold electrode modified with a mixed SAM of 3-mercaptopropionic acid and 6-mercaptoundecanoic which played a dual role of minimizing non-specific interactions and promoting antibody immobilization through EDC/NHS activation. In addition, the surface was further blocked with fetal bovine serum to deactivate any residual carboxylic groups. With this fairly simple biosensor architecture an extremely sensitive analytical behavior was attained with a dynamic range of 0.71 to 7100 pM, attaining a LOD of 21 fM, being the calibration curve developed with α -synuclein concentrations prepared in 10% human serum. The main novelty of this study resides in the application of this biosensor for α -synuclein determination in exosomes. Exosomes are extracellular vesicles from endosomal origin within a size range of 40-160 nm that are secreted by all eukaryotic cells, including normal and disease cells, carrying enrichment proteins and nucleic acids [96, 127]. The

function of these vesicles remains largely unknown, although it has been hypothesized they can be involved in cell homeostasis or in the regulation of intercellular communication [127]. In addition, since they have been found in all biological fluids, they can be easily accessible through liquid biopsies constituting a novel tool for extensive screening of individuals, capable of providing insight into disease progression and response to therapy [127]. Therefore, taking advantage of the diagnostic potential of exosomes, Fu Y. et al. [96] developed MBs to isolate them from serum samples. For this purpose, the MBs were modified with a zwitterionic polymer, poly(carboxybetaine methacrylamide) (pCBMA), via a reversible addition–fragmentation chain transfer process. These polymers present opposite charged groups, anionic and cationic groups, along their length or side chain, which makes them extremely hydrophilic and improves their antifouling properties, making the modified particles more resistant to protein adsorption, bacterial adhesion and biofilm formation [96, 128]. Furthermore, these particles were functionalized with antibodies against L1 cell adhesion molecule through EDC/NHS activation in order to isolate the exosomes, which are typically separated through a time consuming and labor intensive procedure of differential ultra-centrifugation. After the isolation step, the exosomes were lysed and the products found inside were exposed to the developed biosensor achieving a successful differentiation between PD patients and healthy controls.

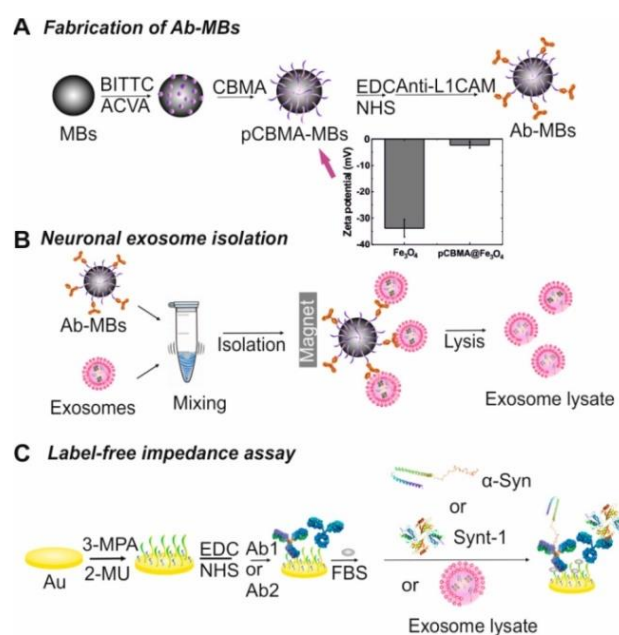


Figure 4 - Schematic representation of the MBs functionalization (A); Neuronal exosome magnetic isolation (B) and respective impedimetric assay (C) [96].

Whereas remarkable sensitivity was achieved through label-free detection, other studies required the use of a second biorecognition element or a label for signal amplification [98, 99, 103]. This way, Mandala S. et al. [103] developed a SPR biosensor where iron oxide (Fe_3O_4) nanoparticles were deposited on the gold surface being further functionalized with monoclonal antibodies (Figure 5). The gold surface of the SPR sensor chips was modified with a mixed SAM of 3-mercaptopropyl (3-MPH) and 11-mercaptoundecanoic acid (11-MUA), activated through EDC/NHS and further functionalized with Fe_3O_4 labeled with streptavidin. Finally, biotinylated antibodies were added to the functionalized Au surface via the avidin-biotin interaction, ensuring the antibodies proper orientation. The sandwich assay procedure was carried out during 40 minutes in PBST, attaining an excellent sensitivity with a wide analytical range of 0.71 fM to 7.1 pM and a LOD of 0.4 fM, while demonstrating the superior analytical behavior of rabbit antibodies over mouse antibodies. In addition, the developed biosensor exhibited a good analytical behavior for α -synuclein detection in diluted serum samples, emphasizing the vital role of a secondary antibody in the biosensors sensitivity, through a clear reduction of the background effect on the SPR signal [103]. Furthermore, the developed platform also presented good selectivity when exposed to BSA and IgG proteins spiked in human serum. To conclude, the developed biosensor presented excellent analytical behavior towards detection and quantification of α -synuclein as a result of the application of Fe_3O_4 nanoparticles on the gold surface, which combined with the oriented immobilization of antibodies, improved the biosensors sensitivity by 8 times when compared with the behavior of conventional gold films. Nevertheless, the biosensor presented some limitations towards its application in clinical samples, being that recovery and specificity studies were performed in serum diluted 1000 times.

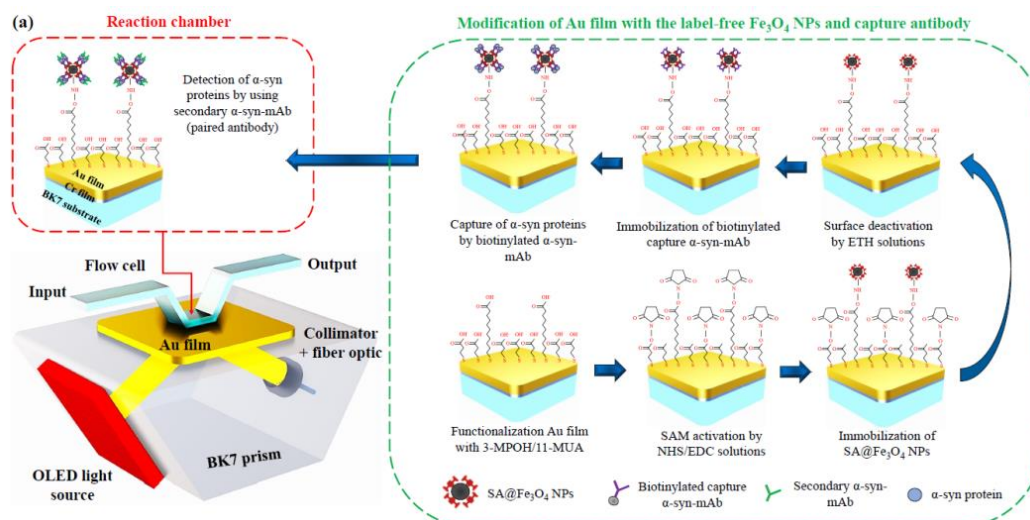


Figure 5 - Schematic representation of the SPR biosensor based on a sandwich detection assay for α -synuclein determination [103].

3.1.2.2. Biosensors for α -synuclein auto-antibodies

PD is a complex disorder with a multifactorial pathogenesis that can trigger a response from the immune system, responsible for the surveillance against pathogens and cell damage. This response results in the production of antibodies, referred to as autoantibodies, which are important for the homeostasis of the immune system [129]. Since α -synuclein is widely expressed in the brain during the early stages of PD, several studies have reported the presence of naturally occurring antibodies against α -synuclein in the serum of both healthy and PD patients. In fact, it has been suggested that autoimmunity towards α -synuclein and its aggregated species may play a protective role in PD, particularly in its initial stages [129]. This way, the potential role of this naturally occurring component has been investigated although to considerably less extent (Table 1) as the role of α -synuclein autoantibodies remains scarcely studied.

The group led by Davis J. has developed three different biosensors directed towards detection of α -synuclein autoantibodies based on label-free impedimetric strategies [107-109]. This type of techniques can sensitively monitor changes in the conductivity/resistivity or in the charge capacitance of the electrochemical interface while retaining the biosensors selectivity, even when analyzed in complex samples such as CSF, blood or serum. This way, the first two studies developed by this group were based on gold disks modified with a polyethylene glycol (PEG) SAM, which was further activated with EDC/NHS for the immobilization of α -synuclein [108, 109]. The modification of the gold surface with PEG SAM acted as double agent to increase the

antifouling properties of the transducer, while taking advantage of its biocompatibility to provide an active surface to promote the successful immobilization of the analyte. These studies presented similar analytical behavior although in the work developed by Xu Q. et al. [108] a slightly more sensitive biosensor was developed with the limits of the calibration curve being 10 times inferior in comparison with the previous study [109]. In this work [108], the use of ethanolamine or any other component to quench the residual active carboxylic was not reported, which might have helped to improve the analytical behavior, although could make the biosensing surface more susceptible to antifouling effects, such as the non-specific adsorption of biomolecules present in biological fluids. Furthermore, both works evaluated different cohorts of PD patients and healthy controls being able to quantify α -synuclein autoantibodies in complex samples. In the work developed by Bryan T. [109] et al. the authors were able to perform the quantification of the analyte in undiluted blood serum samples. On the other hand, in the work of Xu Q. et al, the serum samples had to be diluted 10 times in PBS in order to bring the autoantibody levels into the linear assay range. Nevertheless, both studies were able to successfully discriminate between PD patients and healthy controls being that PD patients presented higher levels of α -synuclein autoantibodies, particularly in its early stages. To sum up, two fairly simple electrochemical biosensors were developed through the PEGylation of gold electrodes, exhibiting high sensitivity and specificity. In fact, the successful performance of these biosensors in complex samples may result from the excellent interfacial selectivity attributed to the quality and density of PEG films. Furthermore, through the analysis of serum samples it was possible to distinguish PD patients from controls with statistical significance, underlining the clinical relevance of this biomarker. Nonetheless, through the evaluation of clinical samples it was not possible to effectively distinguish different stages of the disease.

Finally, in the last work of this group towards detection of α -synuclein autoantibodies, the most sensitive biosensor was developed by Xu Q. et al. [107]. In this work, a gold microelectrode was modified with a cysteamine SAM followed by incubation with graphene oxide and subsequent activation through EDC/NHS for α -synuclein immobilization. The modified surface was further blocked with ethanolamine to quench any residual active sites. Applying the developed biosensor it was possible to detect and quantify autoantibodies in the range of 0.0005 to 1 nM with a 0.12 pM LOD. In addition, the biosensor presented excellent interfacial selectivity when exposed to

human serum and BSA, revealing no response or negligible changes in signal. This way, given the sensitivity and selectivity of this biosensor, a similar analytical behavior was attained when evaluating different concentrations of the analyte in PBS and undiluted serum. In compliance with the previous chapter, the integration of nanomaterials in the structure of biosensors significantly increases their analytical performance, both in terms of sensitivity and specificity. In this case, the inclusion of graphene oxide increased the area of the electrochemical transducer, essential for the immobilization of a larger amount of α -synuclein while its hydrophilic interface and rich surface in hydroxyl, carboxylic acid and epoxy functional groups explains some of its antifouling properties [107]. Nevertheless, despite its exceptional analytical behavior, the performance of this biosensor was not evaluated in samples from PD patients.

To conclude, simple, sensitive and specific strategies were presented by using small sample volumes and having a fast turnaround time, essential characteristics for being integrated into point-of-care devices. In addition, with these three studies, the group was able highlight the clinical relevance of α -synuclein autoantibodies to distinguish between PD patients and healthy controls. Nonetheless, this biomarker was less effective to discriminate between different stages of the disease.

3.1.2.3. Biosensors for α -synuclein related species

Since several studies have raised questions about the validity of α -synuclein as an effective tool for application in clinical practice [90], various studies have focused their attention on aggregated and posttranslational forms as evidence suggested an association between these species and α -synuclein pathology [88]. This way, in recent years, there has been an increasing interest of the biosensing field towards detection of these α -synuclein variants through electrochemical or optical techniques (Table1). In compliance with previous chapters, biosensors towards detection of α -synuclein species were predominantly based on electrochemical techniques while optical biosensors based on plasmon resonance determination presented more expression with excellent analytical behavior [110, 111, 121].

Despite the fact that a combined biomarker approach has been proposed for the successful differential diagnosis of PD, only one work from Table 1 designed the biosensor towards detection of multiple biomarkers. This way, Yin Z. et al. [110]

developed an optical biosensor based on SPR direct detection for the quantification of phosphorylated α -synuclein and total α -synuclein in artificial CSF samples. The proposed biosensor presented a simple design where a gold chip was modified with a 11-decylundecanoic acid SAM that was further activated with EDC/NHS to promote the covalent immobilization of antibodies. Ethanolamine was further added to the surface to block any remaining active site and therefore reduce non-specific adsorption. In this work, only one type of antibody was used to recognize both α -synuclein and phosphorylated α -synuclein as a result of the close affinity to both analytes. Nevertheless, the differentiation between both analytes was performed in two steps. Firstly, after recognition of the analytes by the immobilized antibodies, a dip shift could be observed corresponding to the total α -synuclein, enabling its quantification. Then, titanium phosphate nanoparticles were introduced on the biosensor surface which bonded to phosphorylated α -synuclein as a result of the strong electrostatic interaction between titanium and phosphate groups. This way, the detection and quantification of both biomarkers can be performed in one step, using one sample and with only one biological recognition element. In addition, by performing the biorecognition events on a single interface, it avoids uncertainties related with different interfaces as well as changes in microenvironment associated with traditional dual channel detection. All of these factors contribute to minimize systemic errors and therefore contribute to increase the accuracy of the biosensor. With this biosensor design, the authors were able to quantify total α -synuclein and phosphorylated α -synuclein in the ranges of 0.0714-1.43 pM and 0.00714-0.714 pM with LODs of 5.0 and 2.3 fM, respectively. Remarkable sensitivity was achieved with this simple and easy-to-use biosensor being that lower levels of phosphorylated α -synuclein were able to be quantified through amplification of the SPR signal via integration of titanium phosphate nanoparticles. The developed immunosensor was further evaluated in terms of selectivity by being exposed to AD related biomarkers that can be found in clinical samples, to which the biosensor revealed negligible response. Finally, the performance of the biosensor was evaluated in artificial CSF to further evaluate its applicability in clinical samples. The artificial CSF was spiked with different concentrations of α -synuclein and phosphorylated α -synuclein obtaining recoveries between 90 and 95%, displaying a high correlation with commercial available immunoassays as ELISA.

Concerning the determination of nitrated α -synuclein two similar works were developed based on sandwich detection schemes although with different transducing

mechanisms being that the electrochemical biosensor presented higher sensitivity with a LOD one order of magnitude lower while exhibiting a wider linear range. On one hand, a platform based on LSPR detection was developed through the evaluation of the mass effect of a nanozyme with peroxidase activity applied to catalytic deposition for SPR signal amplification. In this sense, the nanozyme was synthesized through self-assembly of AuNPs with metalloporphyrin via 4-Sulfonatocalix[4] arene hydrate (pSC₄), being the biosensors sensitivity limited by the nanozymes catalytic activity [111]. In a similar approach but this time through impedimetric detection, a biosensor with excellent sensitivity was developed through the conjugation of AuNPs and magnetic nanoparticles (MNPs) (Figure 6) [112]. In this work, a composite was formed through self-assembly of AuNPs in which they were functionalized with pSC₄ and further conjugated with paraquat, leading to the formation of a supramolecular assembly. This nanocomposite presents a large specific area, strong conductivity and various active sites. Therefore, the modification of the electrochemical transducer (gold electrode) with this nanocomposite provided a faster electron transfer rate and a large specific surface area for the immobilization of antibodies. The antibodies were immobilized through non-covalent interactions and BSA was further added to minimize non-specific interactions. Finally, after the primary recognition event by the immobilized antibodies, the MNPs completed the sandwich assay with excellent sensitivity attaining a LOD of 22 pM and a calibration curve within a linear range of 0.071 pM to 71 nM. These MNPs were carboxylated and eventually activated with EDC/NHS to promote the covalent immobilization of the detection antibodies. Their application in the sandwich assay not only provided hydrophilic abilities, which can reduce the nonspecific binding, but also the alignment of MNPs in a specific direction induced by the electric field promotes the flow of electrons, resulting in a significant decrease in the EIS signal. Finally, the developed electrochemical immunosensor was tested in serum samples from PD patients, which had to be diluted 10 fold, exhibiting an excellent behavior by being able to effectively discriminate PD patients from healthy controls with statistical significance. To conclude, through the nanostructuration of an electrochemical transducer with different nanomaterials with exceptional electrical properties it was possible to develop a simple, fast and promising diagnostic tool for application in clinical practice.

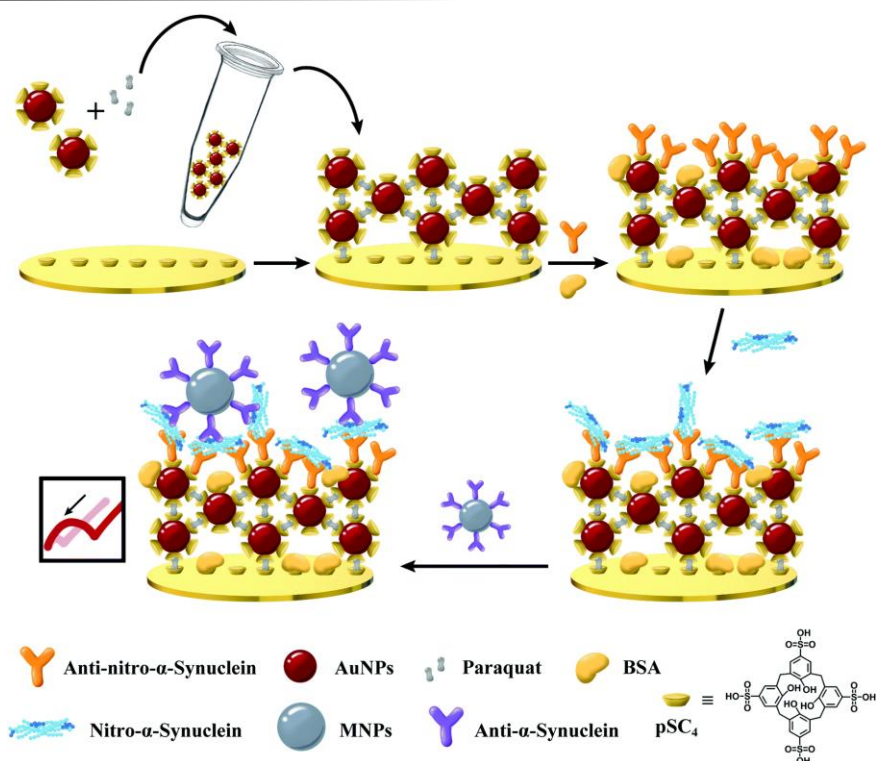


Figure 6 - Schematic representation of the electrochemical immunosensor based on a sandwich assay with MNPs signal amplification for nitrated α -synuclein determination [112].

Despite recent focus on different α -synuclein species resulting from the phosphorylation, ubiquitination, nitration, truncation and oxidation of this protein, the α -synuclein species that have gathered more interest during the last decade were α -synuclein oligomers as a result of their toxicity and impact in PD pathogenesis (Table 1). As with previous biomarkers, most of the works developed towards α -synuclein oligomers detection were based in electrochemical transduction with a higher emphasis being given to PEC determination as a result of their exceptional sensitivity (Table 1). PEC analyses are based on the exploration of a light energy to excite a photoactive transducer converting the biorecognition events into electrical signals. As a result of the complete separation between input signal, an excitation source such as light, and the output detection signal in the form of current, its background signals are greatly decrease eventually presenting higher sensitivities than conventional electrochemical techniques. Gao Y. et al. [113] developed a dual type immunosensor with sensitive electrochemical and PEC determination, being that PEC analysis presented a slightly lower LOD and a wider linear range. Despite its remarkable sensitivity, this biosensing mechanism was labor intensive and developed in two steps using two different platforms (Figure 7A). In the first place, the biorecognition event was based on a

sandwich assay developed in a 96 well plate, where detection antibodies were coupled to liposomes loaded with glucose. After the biorecognition event the liposomes were lysed followed by an enzymatic reaction with glucose oxidase resulting in the production of H_2O_2 and gluconic acid. This sub-product of the α -synuclein oligomers recognition was transferred to an electrochemical transducer based on an ITO surface modified with an electrostatic self-assembled nanocomposite of pyrene and silver nanoparticles (AgNPs), which induced AgNPs etching. Applying this sensing design it was possible to quantify α -synuclein oligomers within a wide linear range, 1 fg mL^{-1} to 10 ng mL^{-1} with an extremely low LOD of 0.43 fg mL^{-1} . Nonetheless, the configuration of the sensor compromises the biosensor definition in which the biological recognition element should be in direct spatial contact with the transducer. In addition, the use of two separate platforms for the quantification of synuclein oligomers involves more expensive and time-consuming procedures.

In a different PEC biosensor, a combined dual-signal amplification strategy for determination of α -synuclein oligomers was developed based on a gold electrode modified with a low background composite material of AuNPs and graphdiyne, which was further coupled with a DNA nanoprobe of tungsten selenide nanoflower as the signal enhancer (Figure 7B) [116]. With this strategy attomolar levels of α -synuclein oligomers were able to be quantified with high-to-noise signal ratio as a result of the synergistic effect between WSe_2 nanoflower and graphdiyne that can reduce the photoinduced electron-hole recombination and expedite the spatial charge separation. Nonetheless, this detection procedure was extremely extensive where α -synuclein oligomers were primarily converted to a massive output of false-target DNA through a magnetic separation. Then the false-target DNA was used for the catalytic hairpin assembly on the modified gold electrode which was previously functionalized with a triple stranded DNA. In the last step, the PEC nanoprobe was used to hybridize with the capture DNA assembled on the modified gold electrode leading to the production of photocurrent. Despite its outstanding sensitivity, this procedure resulted in an indirect detection of the analyte, lasting approximately 7 h to conclude the dual signal amplification strategy.

PEC is a relatively new branch of science developed on the basis of electrochemistry and therefore significant studies still need to be conducted to optimize these techniques for integration into POC devices. Despite the fact that the presented biosensors exhibited exceptional sensitivity with extremely low LODs, while also

displaying good performance when evaluated in serum sample, these two works involved time-consuming and labor-intensive methodologies, which can compromise its interest for integration into POC.

Simpler electrochemical methodologies, based on direct label-free assays, were developed for α -synuclein oligomers detection while also attaining a good analytical behavior capable of performing quantification within clinical relevant levels in biological fluids such as blood and serum [114, 115, 117-119, 121]. Nonetheless, further studies should be performed in order to evaluate these biosensors performance in PD samples and, therefore, their ability to differentiate between PD patients and healthy controls.

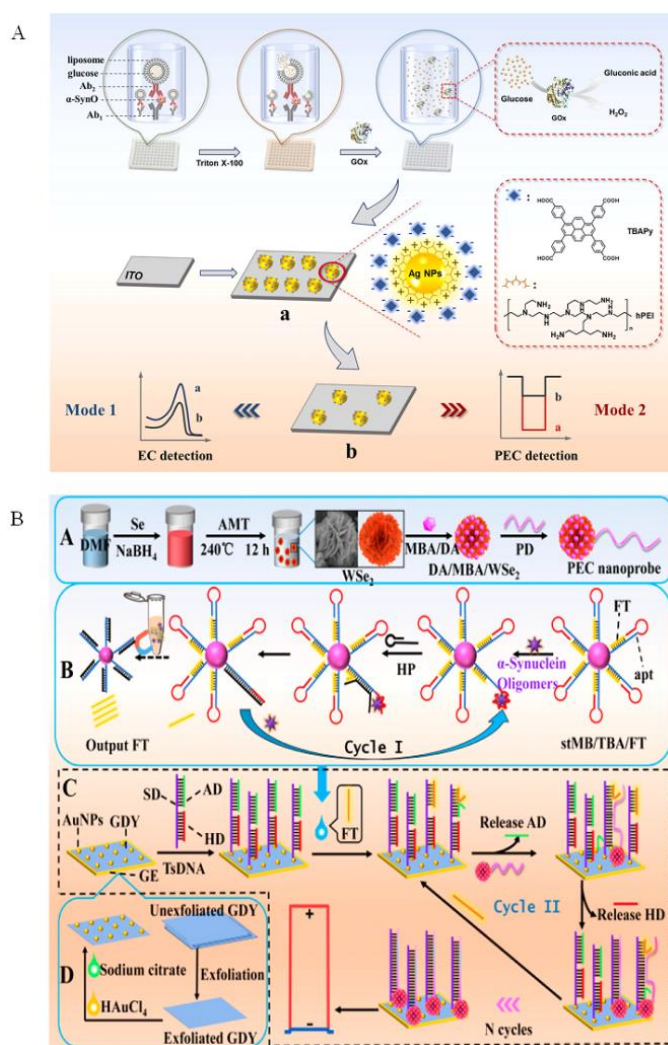


Figure 7 - Schematic representation of PEC biosensors for α -synuclein oligomers detection: A) split-type immunosensor with liposome/enzymatic reaction mediated cascaded amplification and subsequent electrode materials etching reaction [113]; B) dual-cycle amplification strategy based on a AuNPs/graphdiyne modified sensor and WSe₂ nanoflower as the signal indicator [116].

3.2. Nanostructured label-free electrochemical immunosensor for α -synuclein detection

Aggregation of α -synuclein has been recognized as one the major pathological hallmarks of PD being the focus of several cross-sectional and biosensing studies. Extensive research has been carried out to develop biosensors towards diagnosis of α -synuclein and its related species, being label-free methods the most reported. Label-free electrochemical immunosensors have emerged as important analytical tools as they are easy to operate, economic and combine the simplicity and sensitivity of electrochemical techniques with the specificity of immunoassays [43, 48, 130]. Through the nanostructuration of electrochemical transducers is possible to achieve high levels of sensitivity and specificity as nanomaterials present specific physical and chemical properties due to quantum-size effects. Ultimately, a combination of different nanomaterials can produce a synergistic effect to improve the signal transduction of electrodes as a result of their catalytic activity and conductivity, in addition to provide an effective immobilization matrix for the biological recognition events due to multivalent affinity interactions with proteins [52, 54, 130]. This way, through the nanostructuration of electrochemical transducers common limitations associated with label-free detection, as the fact that the changes occurring in the transducer surface upon target binding are less significant and need to be regarded as highly specific, can be overcome [42, 131]. Therefore, this chapter focuses on the development of a nanostructured label-free immunosensor for the detection of low levels of α -synuclein in simple and complex samples (Figure 8). The immunosensor was developed with two different electrochemical transducers namely, GCE and SPCE, being their analytical behavior studied and compared as well as their suitability for clinical application into POC devices. These materials were chosen due to their inherent characteristics such as good electrical conductivity, inertness and easy functionalization. Although the transducers were both of carbon, they presented different characteristics and formats, which directly impacted their electrochemical and analytical behavior.

From the vast range of available nanomaterials, CNTs and AuNPs were used throughout this work. From an eletroanalytical point of view, biosensors based on CNTs present high sensitivity, large surface area, fast response time due to an

ability to mediate fast electron-transfer kinetics, lower potential of redox reaction, high stability associated with less surface fouling effects and longer life time [62, 63, 132]. Another nanomaterial with positive impact in the electroanalytical behavior of biosensors are AuNPs which through combination of their excellent conductivity, high chemical stability, good biological compatibility and high surface-to-volume ratio makes them ideal materials for interfacing biological recognition events with electronic transducers [58, 133-136]. These features make CNTs and AuNPs extremely suitable for developing novel and improved electrochemical biosensing systems.

In this work, a simple methodology combining the sensitivity and fast readout of electrochemical techniques with the specificity of the antibody-antigen reaction for α -synuclein detection and quantification was successfully proposed, making this device well-suited to study and differentiate pathological from physiological levels of this biomarker. The proposed label-free electrochemical immunosensor was developed in a layer-by-layer approach and was based on a SPCE or GCE modified with SWCNTs and AuNPs in order to enhance the catalytic behavior and electron transfer capabilities of the sensing platform in addition to creating an ideal environment for the immobilization of antibodies (Figure 8). In addition, through the integration of these two nanomaterials the stability of the biosensor was further improved. Finally, the nanostructured surface was functionalized with monoclonal antibodies enabling the fast and direct detection of α -synuclein which was further studied through SWV.

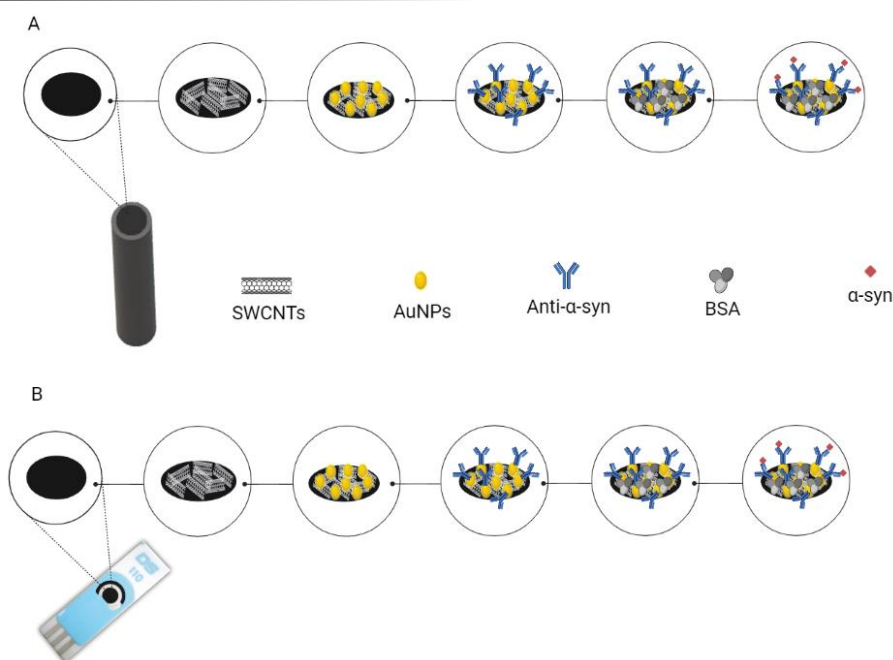


Figure 8 - Schematic illustration of the electrochemical immunosensor development in a layer-by-layer approach using a GCE (A) and a SPCE (B) [92].

3.2.1. Material and Methods

3.2.1.1. Materials

Alumina suspension 1.0 μm , 0.3 μm and 0.05 μm were purchased from Gravimetra (Spain). Sulfuric acid and absolute ethanol were purchase from Panreac (Spain). Potassium chloride and sodium chloride were purchased from Riedel-de Haën (Germany). BSA, α -synuclein, di-sodium phosphate dihydrate, dimethylformamide (DMF), fetal bovine serum, gold(III) chloride solution, graphene oxide (GO) dispersion (4 mg mL⁻¹ in H₂O), MWCNTs carboxylic acid functionalized, potassium hexacyanoferrate (III) (K₃Fe(CN)₆), potassium hexacyanoferrate (II) trihydrated (K₄[Fe(CN)₆].3H₂O), potassium phosphate monobasic, reduced graphene oxide (rGO) and SWCNTs carboxylic acid functionalized were purchased from Sigma-Aldrich (Steinheim, Germany). Mouse monoclonal antibodies to α -synuclein (ab27766) were purchased from Abcam. The electrochemical transducers used were SPCEs (DRP-110) acquired from Metrohm-Dropsens and GCE (MF-2012) acquired from Basi. Ultrapure water (18.2 M Ω cm⁻¹ resistivity) was produced by a Milli-Q Simplicity 185 system.

3.2.1.2. Electrochemical assay

Autolab electrochemical system (Eco Chemie, The Netherlands) equipped with PGSTAT-30 and NOVA software were used to perform all electrochemical studies. Two different electrochemical cells were used throughout this work: SPCEs electrochemical cell consists on a carbon working electrode (4.0 mm diameter), carbon auxiliary electrode and a silver reference electrode; GCE electrochemical cell consists on a conventional three-electrode cell which included a glassy carbon electrode (3.0 mm diameter) as a working electrode, a platinum counter electrode and a Ag|AgCl|KCl_{sat} reference electrode. Solutions of 2.5 mM and 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] and phosphate buffered saline solution (PBS, 10 mM, pH 7.4) were used as the electroactive indicator and supporting electrolyte, respectively.

Cyclic voltammetry (CV) and SWV measurements were performed by varying the potential from -0.5 to 0.7 V and -0.2 to 0.6 V, respectively, with a 0.05 V s⁻¹ scan rate. Electrochemical impedance spectroscopy (EIS) measurements were performed using a set potential of 0.2 V and a frequency range from 10⁻¹ to 10⁵ Hz with an amplitude perturbation of 10 mV.

3.2.1.3. Pre-treatment of GCE

The pre-treatment of the GCE consisted in: 1) mechanical polishing with alumina solutions 1.0, 0.3 and 0.05 µm using microcloth pads; 2) cleaning with absolute ethanol in ultrasonic bath during 10 minutes; and 3) electrochemical activation by cycling the electrode from 0.0 to 1.6 V in 0.5 mol L⁻¹ H₂SO₄ solution at 100 mV s⁻¹. Throughout all pre-treatment procedures, ultrapure water was used to rinse the electrode.

3.2.1.4. Drop cast of MWCNTs, SWCNTs, GO and rGO

Suspensions of MWCNTs, SWCNTs and rGO (1.0 mg mL⁻¹) were prepared in DMF and ultra-sonicated until homogeneous suspensions were obtained.

For the nanostructuration of the GCE surface a suitable amount of a 1 mg mL⁻¹ suspension of SWCNT was drop-casted on the surface of the GCE and set to dry for approximately 30 minutes, in a 50 °C oven to form a SWCNTs film. The

amount of SWCNT suspension to modify the electrode was optimized using four different volumes: 1.0, 3.0, 5.0 and 7.0 μL .

For the SPCE modification MWCNTs, SWCNTs, GO and rGO suspensions were diluted in ultra-pure water to the concentrations of interest. A suitable amount was drop-casted on the surface of the SPCE and set to dry at room temperature (RT). The SPCE modification with carbon nanomaterials was optimized through testing of different concentrations (0.10; 0.50 and 1.0 mg mL^{-1}) and volumes (1.0; 3.0 and 5.0 μL).

3.2.1.5. Electrodeposition of gold nanoparticles

AuNPs were prepared on the surface of the bare and modified SPCEs and GCE by chronoamperometry in a 0.1 M KNO_3 solution containing 3 mM HAuCl_4 under a -0.2 V potential for 150 s. Different HAuCl_4 concentrations (1.0; 3.0; 5.0 and 7.0 M) and electrodeposition periods (50; 100; 150; 200 and 300 s) were studied.

3.2.1.6. Antibody and BSA immobilization

To promote the antibodies immobilization on the AuNPs surface, they were modified through a thiolation process [137]. Briefly, a solution containing monoclonal antibody against α -synuclein prepared in PBS (10 mM, pH 7.4), EDTA (2.5 mM) and 2-iminethiolane (20 times molar excess over the amount of antibodies) was prepared and left to react for 1 h at RT [138].

Then, the modified GCE was immersed in 250 μL of antibody solution while 5.0 μL were drop-casted on the previously modified SPCE in a water saturated environment followed by 1 h incubation at RT and overnight incubation at 4 $^{\circ}\text{C}$. Different antibody concentrations were evaluated for the GCE (1.0, 2.5, 5.0, 7.5 and 10 $\mu\text{g mL}^{-1}$) and SPCE (50, 100, 150, 200, 250 and 300 $\mu\text{g mL}^{-1}$).

Finally, the modified GCE and SPCE were incubated in BSA for 30 min at RT in order to block the remaining adsorption-reactive sites. Different BSA concentrations were studied (0.010, 0.10, 0.50, 1.0, 1.5 and 2.0%).

3.2.1.7. Electrochemical detection of α -synuclein

The functionalized GCE and SPCE were immersed in different concentrations of α -synuclein in the range of 0.50-50 ng mL⁻¹ and 0.010-10 ng mL⁻¹, respectively. Both transducers were left to react at RT for 30 min.

All reagents and dilutions used in the electrochemical immunosensing procedure were prepared in PBS 10 mM, pH 7.4. All measurements were carried out in triplicate.

3.2.1.8. Calibration curve and Limits of detection and quantification

The calibration curve of the biosensors was built by measuring the current intensity of the redox pair at each concentration of α -synuclein and calculating the difference in current intensity between blank assays and the analyte signal. Finally, the values were fitted to a logarithmic relationship.

LOD and LOQ were defined as the concentration values of the calibration curve when LOD and LOQ were the average of current intensity reduction (X_0) of the blank plus three or tenfold the standard deviation of the blank (σ_0) [139], respectively:

$$\text{LOD} = X_0 + 3.3\sigma_0 \quad (1)$$

$$\text{LOQ} = X_0 + 10\sigma_0 \quad (2)$$

3.2.1.9. Detection in serum samples

The developed biosensor was exposed to fetal bovine serum spiked with different concentrations in order to evaluate its capability for real world application. Fetal bovine serum was diluted 10 fold in 10 mM PBS (pH 7.4) and spiked with three different α -synuclein concentrations (0.30, 1.0 and 2.0 ng mL⁻¹).

3.2.1.10. Scanning electron microscopy

Scanning electron microscopy (SEM) - FEI Quanta 400FEG ESEM / EDAX Genesis X4M - was used to study the properties and morphology of the developed biosensor. SEM studies were carried out at the Materials Centre of the University of Porto (CEMUP).

3.2.2. Results and Discussion

The goal of this work was to develop a sensitive label-free electrochemical immunosensor for α -synuclein detection to contribute to the early diagnosis and surveillance of PD. To achieve this goal, it was crucial to increase the catalytic behavior of the electrochemical transducer which was accomplished by combining different nanomaterials through a layer-by-layer approach. The great versatility of this approach enables the combination and superior control of different materials with varied characteristics that can increase the conductive properties of the electrochemical transducer in addition to creating an appropriate platform for the immobilization of thiolated biological recognition elements [140]. In this case, the GCE and SPCE nanostructuration was carried out through the drop-cast of carbon nanomaterials followed by electrodeposition of AuNPs.

3.2.2.1. Glassy carbon electrode

3.2.2.1.1. Glassy carbon electrode nanostructuration

CNTs present intrinsic electrochemical activity, high electrical conductivity and large surface area which make them ideal nanomaterials for electron transfer mediation in electrochemical biosensors [62]. In addition CNTs are easy to functionalize and biocompatible [62]. Thus, after modifying the GCE with SWCNTs a large increase in the electrode signal was observed. At this stage, four different amounts of SWCNT solution were tested. The increase of the SWV signal was calculated for each modification as shown in Figure 9. In this figure it is possible to notice that the drop-cast of SWCNTs was most successful when the added volume was 3.0 μL with an increase in signal of 252%. The percentage of increase in signal lowered when 5.0 and 7.0 μL were drop-casted while increasing the assays standard deviation.

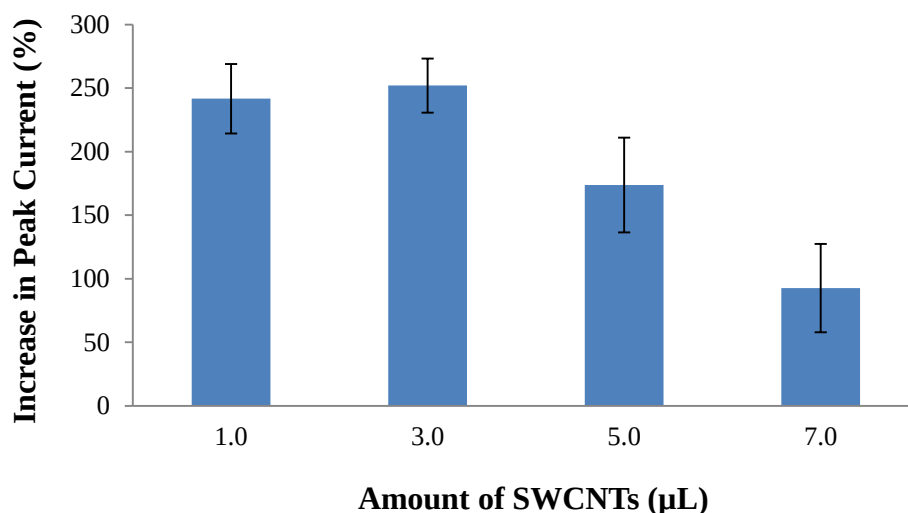


Figure 9 - Increase of the peak current (%) after modification of the bare GCE with 1.0, 3.0, 5.0 and 7.0 μL of 1.0 mg mL^{-1} SWCNTs suspension. Measurements were performed in a $5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

Figure 10 displays a SEM image of a GCE surface before and after modification with $3.0 \mu\text{L}$ of 1.0 mg mL^{-1} SWCNTs solution. The surface of the GCE is completely covered with SWCNTs, which reveals the effectiveness of the dispersing solvent to produce a very uniform distribution of SWCNTs over the electrode surface.

The next stage of the GCE nanostructuration involves the electrochemical deposition of AuNPs. Nano sized particles of noble metals have been the source of particular interest due to their distinct physical and chemical properties. The catalytic capacity of metal nanoparticles to amplify the electrochemical reactions gives them a significant priority in the design of electrochemical biosensors.

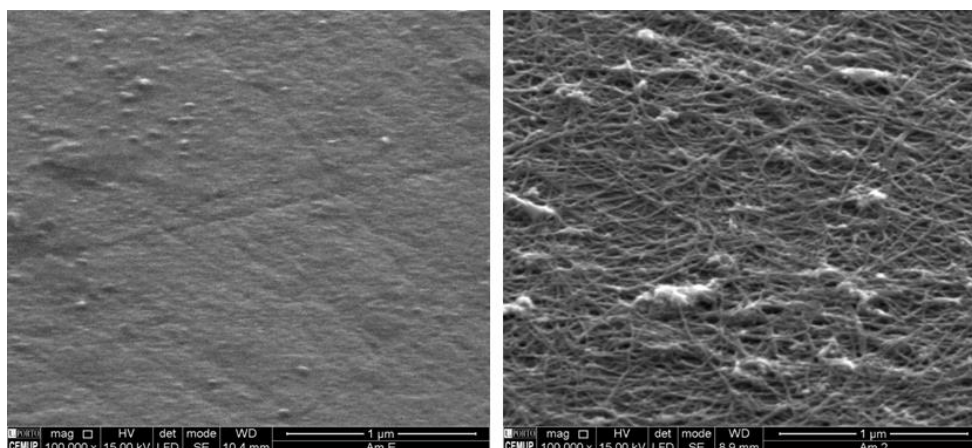


Figure 10 - SEM images of GCE after modification with SWCNTs. From left to right: Bare GCE and SWCNTs/GCE

AuNPs can promote the link between the redox centers in proteins and electrode surfaces due to their excellent conductivity and catalytic properties [141]. AuNPs are highly stable and less vulnerable to degradation caused by the solution matrix [141]. Moreover, AuNPs are non-toxic, easy to functionalize, biocompatible to both *in vivo* and *in vitro* environments and present a large surface area to volume ratio [135]. That way, AuNPs provide a great interface for biological recognition and electronic transduction which make them an essential part in bioelectrochemistry [135]. Therefore, in this work, AuNPs were employed as a substrate to immobilize antibodies providing a suitable microenvironment on the electrode and lend more freedom of orientation to enhance the direct electron transfer behavior [142].

In order to optimize the immobilization of AuNPs the electrodeposition process was conducted on the surface of the bare GCE (Figure 11) and after modification with different volumes of SWCNTs (Figure 12). In the first study, the GCE modified with AuNPs was subjected to an electrochemical stability test by treating its surface with continuous square-wave voltammetry scans in a potential range of -0.2 V to 0.6 V for various numbers of cycles in a 5 mM electrolyte solution. The immobilization of AuNPs on the surface of the bare GCE proved to be largely unstable. Despite the large increase in peak current observed during the first measurement, the signal decreased significantly after 10 measurements while after 20 measurements the signal almost returned to the original bare GCE signal.

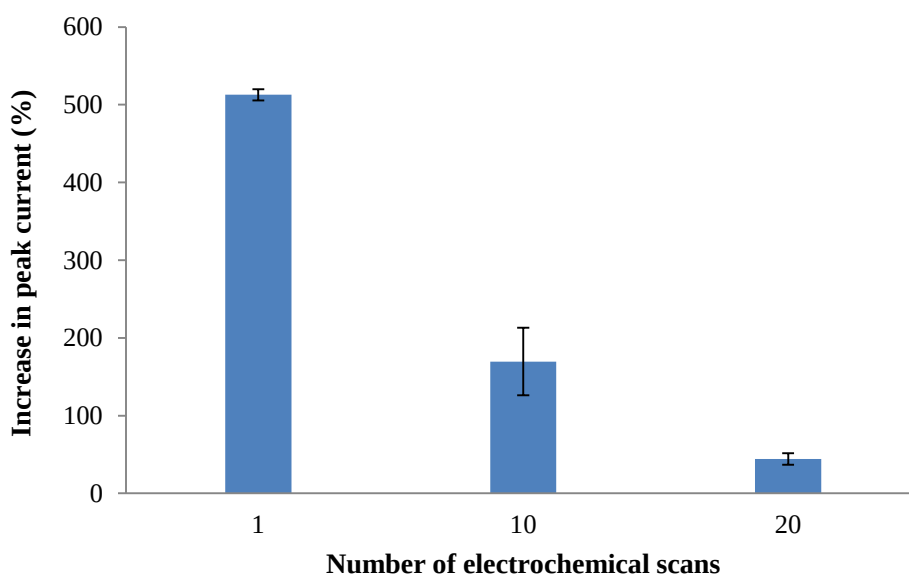


Figure 11 - Modification of the bare GCE with electrodeposited AuNPs. SWV signal after 1, 10 and 20 measurements. Measurements were performed in a 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

In the second study (Figure 12) it was possible to conclude that the method that ensured the highest improvement in the electrochemical behaviour of the GCE was the electrodeposition of AuNPs on the surface of the modified GCE with 3.0 μL of SWCNTs.

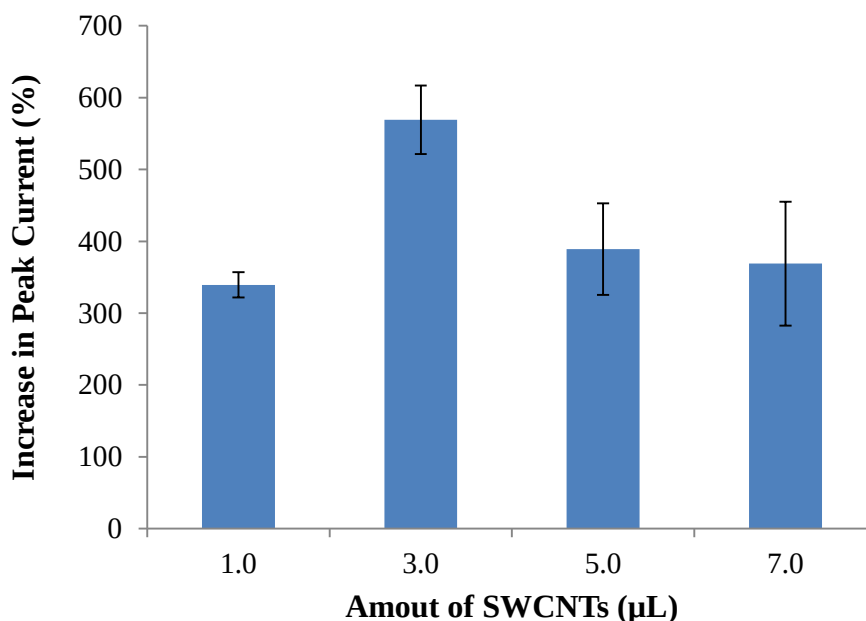


Figure 12 - Increase (%) of the peak current after electrodeposition of AuNPs. Measurements were performed in a 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

The surface of the modified AuNPs/SWCNTs/GCE was also characterized by SEM. (Figure 13). Better SEM images could not be obtained given the electrode size and equipment limitations, thus it was not possible to calculate the AuNPs distribution or size.

Finally, all of the modifications were studied using CV, SWV and EIS (Figure 14). Overall, it can be concluded that the electrochemical behavior of the biosensor was significantly improved after the modifications with nanomaterials. This fact was observed through the dramatic increase in peak current in both cyclic and square-wave voltammograms which is justified though the increase of the electrodes surface area and conductivity, favoring the electron transfer process. On the other hand, the diameter of the semicircle in the Nyquist plot, which indicated the electron transfer resistance of the layer, decreased after modification with both SWCNTs and AuNPs.

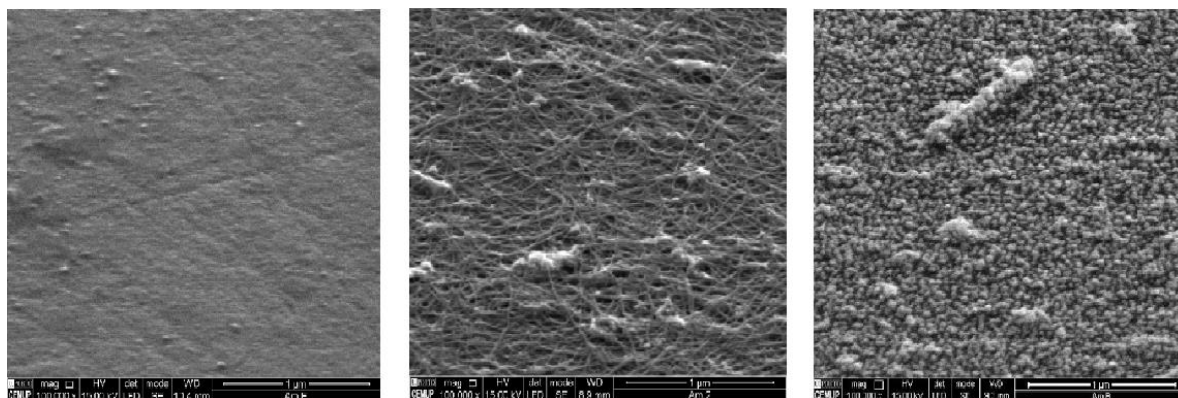


Figure 13 - SEM images of GCE after several immobilizations. From left to right: Bare GCE; SWCNTs/GCE and AuNPs/SWCNTs/GCE.

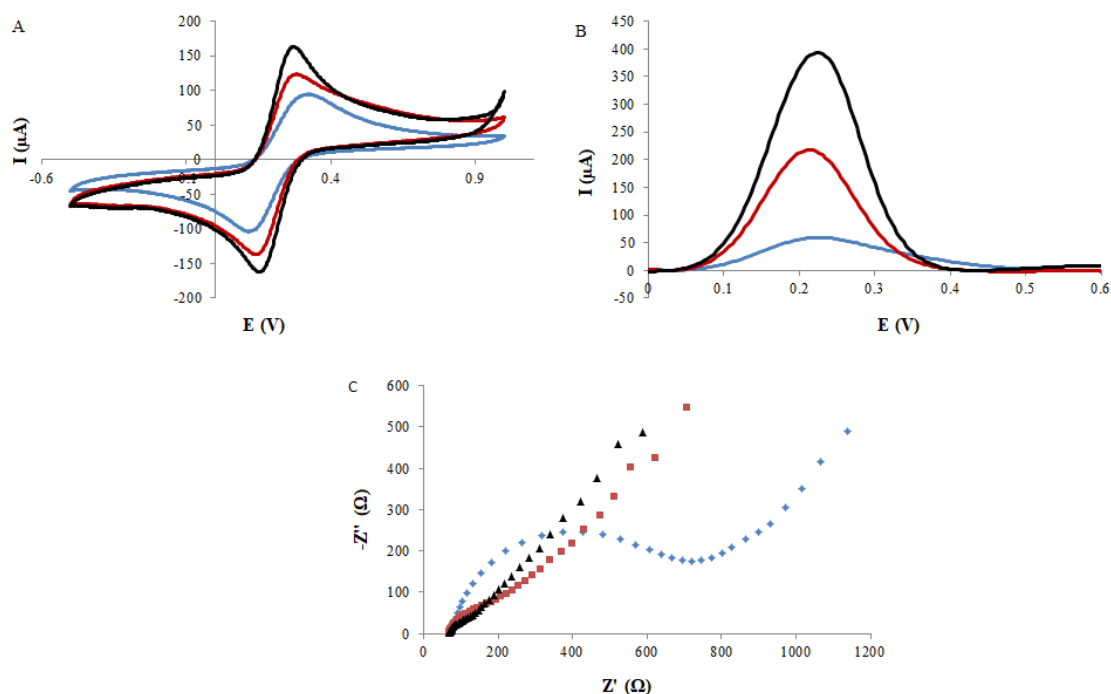


Figure 14 - CV (A), SWV (B) and EIS (C) obtained for the several steps of the biosensor development: bare GCE (blue), SWCNTs(3.0 μL)/GCE (red) and AuNPs/SWCNTs(3.0 μL)/GCE (black). Measurements were performed in a 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4)

3.2.2.1.2. Antibody and BSA immobilization

The antibodies were functionalized through chemical modification (thiolation) to promote a stable antibody immobilization on the AuNPs surface. For this purpose the antibody was treated with 2-iminothiolane. The reagent reacted with the amine groups in the antibodies to result in permanent modifications containing terminal sulfhydryl residues. Five concentrations (1.0, 2.5, 5.0, 7.5 and 10.0 μg mL⁻¹) of antibody were

tested and a large signal reduction for the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox couple was observed (Figure 15). This peak reduction is caused by the partial blockage of the AuNPs/SWCNTs/GCE surface by the antibodies making the electron transfer process between the electrode and the solution slower and more difficult.

From Figure 15 it is possible to conclude that electrochemical signal saturated at a concentration of $5.0 \mu\text{g mL}^{-1}$ causing an average reduction of 61 % while displaying low variability between assays with a 4.0 % RSD.

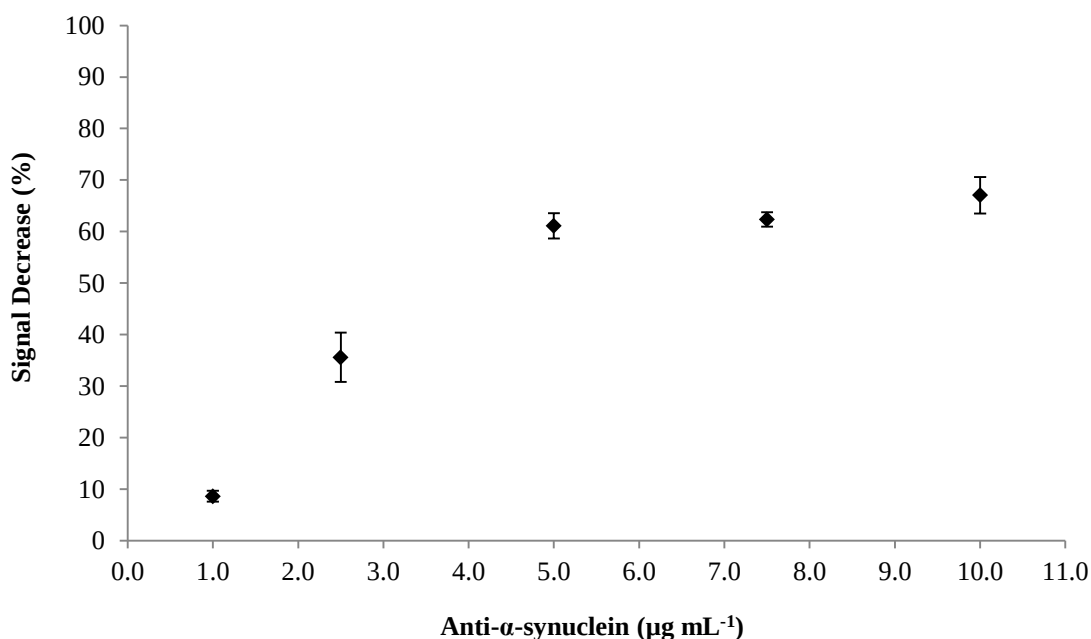


Figure 15 - Optimization of antibody immobilization with different concentrations through overnight incubation at 4°C ($n=3$). Measurements were performed through SWV in a $5 \text{ mM Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

In the last stage of the transducer optimization, BSA was immobilized on the surface of the modified GCE in order to minimize non-specific adsorptions. This is a crucial step, particularly when applied to real samples such as blood, CSF or saliva, in order to ensure an analytical measurement of the biomarkers of interest with high sensitivity and specificity. Four BSA concentrations were evaluated and it was possible to conclude that the SWV signal saturated at a 0.5 % BSA concentration causing a signal reduction of approximately 57 % with a 0.36 % RSD in relation to the antibody modified GCE signal (Figure 16).

From Figure 17 it is possible to conclude that immobilization of both antibody and BSA caused a signal reduction associated with the formation of an insulating protein

layer that hindered the electron transfer process between the transducer surface and electrolyte.

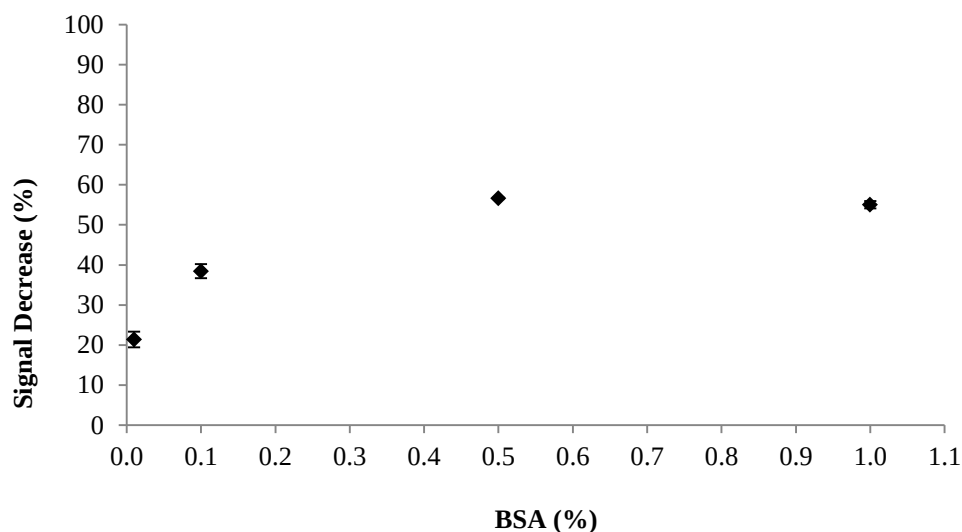


Figure 16 - Optimization of BSA immobilization with different concentrations through 30 min incubation at RT (n=3). Measurements were performed through SWV in a 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

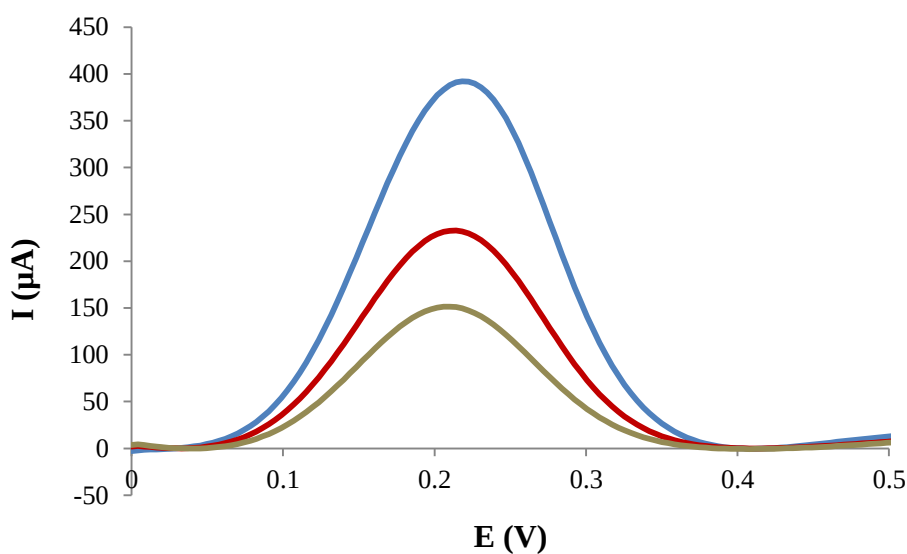


Figure 17 - SWV obtained for AuNPs electrodeposition (blue), anti- α -synuclein ($5.0 \mu\text{g mL}^{-1}$) (red) and BSA (0.5%) (grey) immobilization. Measurements were performed in a 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

3.2.2.1.3 Calibration curve

Under optimized conditions, the label-free detection of α -synuclein was carried out by incubating the immunosensor with different α -synuclein concentrations for 30 min at

RT in order to ensure the successful antibody-antigen reaction. Figure 18 depicts the analytical calibration curve in which the SWV signal decreases as a result of the alterations to the interface properties of the biosensor due to the capture of increasing α -synuclein concentrations by the monoclonal antibody. The calibration plot exhibited an exponential relationship between the α -synuclein concentrations ($0.50\text{--}50\text{ ng mL}^{-1}$) and the SWV response with the analytical curve presenting a wide concentration range with a correlation coefficient (r) of 0.996 ($n = 4$). Based on the analytical data, LOD and LOQ were calculated to be 0.43 ng mL^{-1} (30.7 pM) and 0.94 ng mL^{-1} (67.1 pM). Despite being able to detect values as low as 0.5 ng mL^{-1} with clinical relevance it is possible to see from Table 1 that the attained LOD is higher than most of the reported biosensors. In addition, a recovery assay was conducted at a controlled α -synuclein concentration of 5.0 ng mL^{-1} in 10 mM PBS (pH 7.4) obtaining an acceptable recovery of 89.1 % although with some variation between the three independent assays with a reported RSD of 10.9 %, highlighting the reproducibility problems of working with GCE. In fact, the demands of a GCE that require repeated polishing procedures may influence its electrochemical performance over time being difficult to ensure the same surface conditions for a group of electrodes.

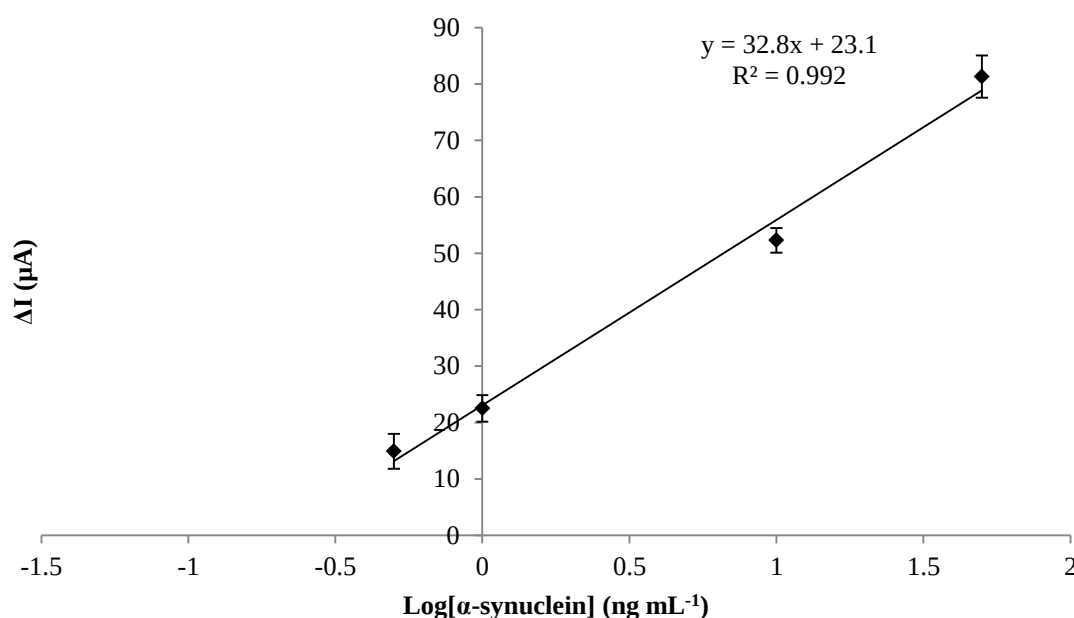


Figure 18 - Calibration curve based in the SWV signal reduction after detection of α -synuclein concentrations ($0.50, 1.0, 10,$ and 50 ng mL^{-1}) ($n=4$). Measurements were performed in a 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

3.2.2.2. Screen-printed carbon electrode

3.2.2.2.1. Screen-printed carbon electrode nanostructuration

In order to improve the biosensor analytical behavior and reduce the variability found with GCE the nanostructured immunosensor was adapted to a SPCE platform. In fact, SPCE can be safely used for successive analyses as its disposable features prevent electrode poisoning from recurrent use being reproducibility of results after consecutive use a salient feature of SPCE.

With the goal of translating the developed immunosensor into a SPCE platform all conditions for the immobilization of each layer were optimized. From Figure 19A to 19D it is possible to observe the impact of the different nanomaterials on the SPCE signal leading to the conclusion that the combination of CNTs (deposition of 3.0 μL volume) with AuNPs (electrodeposition at -0.2 V during 150 s using 3.0 mM HAuCl_4) produced the best results and that lower concentrations of carbon nanomaterials were far more effective in promoting a better electrochemical behavior of the transducer. In fact, the signal increase from the immobilization of SWCNTs, MWCNTs and rGO was overall not significant at the high concentration of 1.0 mg mL^{-1} , which can be associated with the presence of residues of the organic solvent DMF that at higher concentrations may not completely evaporate hindering the electron transfer. On the other hand, the SPCE modification with 0.10 mg mL^{-1} concentrations had an effective signal increase associated with the excellent conductivity and electrochemical activity of the carbon nanomaterials, which facilitated the redox process at the transducer interface resulting in a signal increase of 267.2, 365.8 and 152.8% for SWCNTs, MWCNTs and rGO immobilization with relative standard deviations (RSD) of 1.2, 1.5 and 26.7%, respectively. The highest value of RSD for the immobilization of rGO might be associated with the difficulty in achieving a homogenous suspension of rGO even after sonicating for long periods of time. In contrast, the SPCE modification with GO led to a signal reduction as GO exhibits lower conductive properties when compared with the other tested nanomaterials. Nevertheless, GO can have different and important applications as for example in the immobilization of biomolecules as a result of the presence of various oxygen functional groups in its structure [143-145].

Following this first stage of SPCE functionalization, and despite clear advantages in the immobilization of MWCNTs and SWCNTs, AuNPs were electrodeposited in all modified SPCEs in order to evaluate the synergistic effect of coupling different

nanomaterials. It is possible to observe from Figure 19A to 19D that the formation of the second layer caused an even larger increase in signal across all modified SPCEs due to the excellent conductivity and high surface area of AuNPs [146]. Similar results were achieved for the combination of AuNPs with SWCNTs ($472.9 \pm 2.3\%$) and MWCNTs ($519.4 \pm 3.7\%$), while the worst performance was reached with AuNPs/GO in compliance with the previous GO modification data. The nanomaterials that were chosen to develop the immunosensor were SWCNTs and AuNPs as they combined the ability to greatly enhance the electrochemical behavior of the SPCEs with the high stability of the SWCNTs suspension. In fact, the diluted suspensions of MWCNTs and rGO appeared to start aggregating after a few minutes, which could negatively impact the reproducibility when preparing simultaneously several SPCEs for real application.

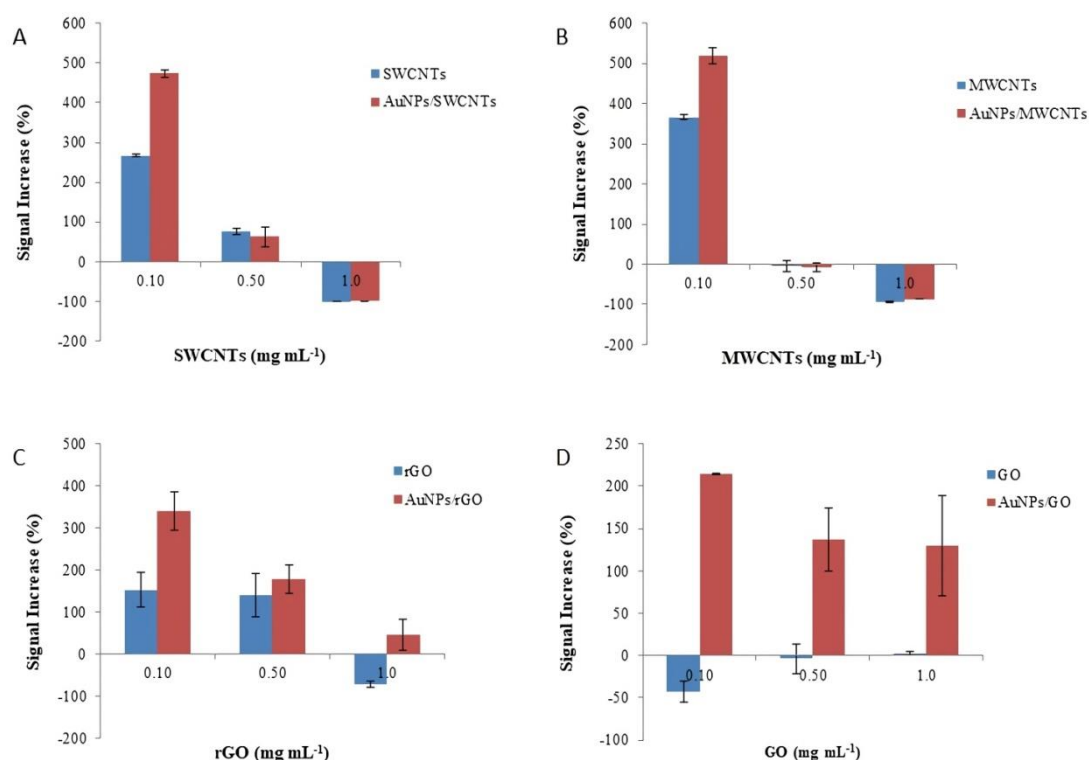


Figure 19 - Results for SPCE modification with 3.0 μ L of SWCNTs (A), MWCNTs (B), rGO (C) and GO (D) with different concentrations (0.10; 0.50 and 1.0 mg mL⁻¹) and further AuNPs electrodeposition (-0.2 V; 150 s; 3.0 mM H₂AuCl₄) (n=3). Measurements were performed through SWV in a 2.5 mM Fe(CN)₆^{3-/4-} solution in 10 mM PBS (pH 7.4).

To complete the SPCE nanostructuration, studies concerning the volume of SWCNTs drop-casted, H₂AuCl₄ concentration, electrodeposition time and supporting electrolyte were carried out. In addition, AuNPs were also

electrodeposited on the SPCEs bare surface to prove the advantages of coupling AuNPs with SWCNTs. The conjugation of these nanomaterials was more efficient when AuNPs were electrodeposited on the SPCE modified with 3.0 μL of SWCNTs which resulted in a significant signal increase (Figure 20).

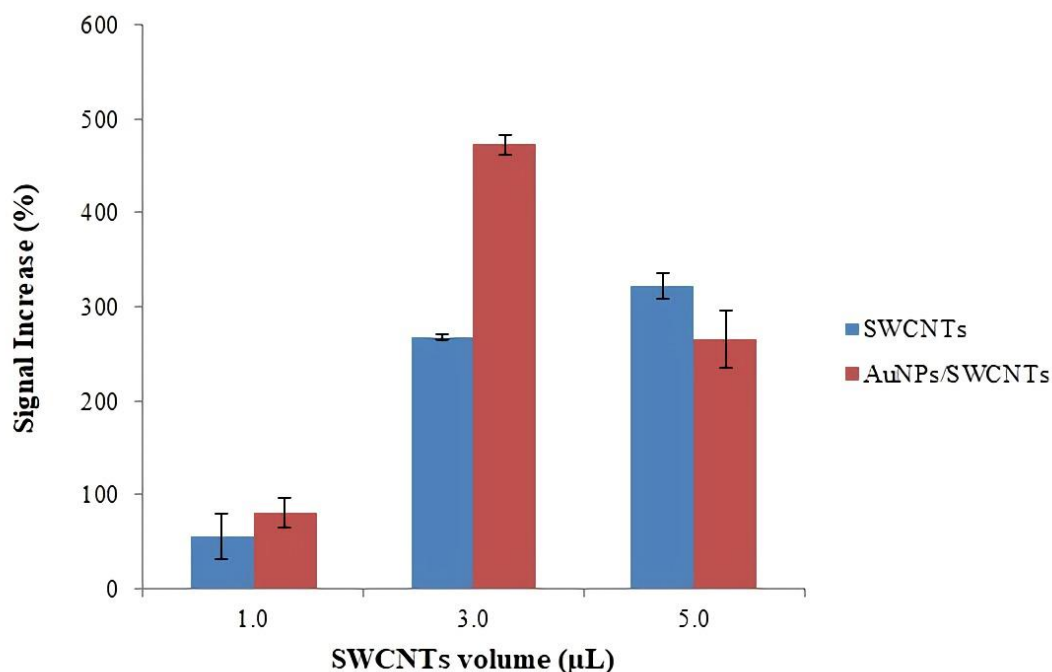


Figure 20 - Results for SPCE modification with different SWCNTs (0.10 mg mL^{-1}) volumes and electrodeposited AuNPs (-0.2 V ; 150 s ; 3.0 mM H AuCl_4) ($n=3$). Measurements were performed through SWV in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in $10 \text{ mM PBS (pH 7.4)}$.

Regarding the electrodeposition process, saturation was attained by applying a -0.2 V potential in a 3.0 mM H AuCl_4 solution (Figure 21) for 150 s (Figure 22) which was further confirmed through SEM where AuNPs electrodeposited for 150 s were more densely distributed across the SPCE surface (Figure 23). Finally, the impact of a supporting electrolyte for the electrodeposition process was studied and despite presenting similar electrochemical behavior (Figure 24A), there were clear morphological differences between AuNPs electrodeposited in ultra-pure water and KNO_3 (Figure 24B). AuNPs electrodeposited in the presence of KNO_3 presented a sphere-like shape while exhibiting less variation in size and being more densely distributed across the modified SPCE surface (Figure 24B).

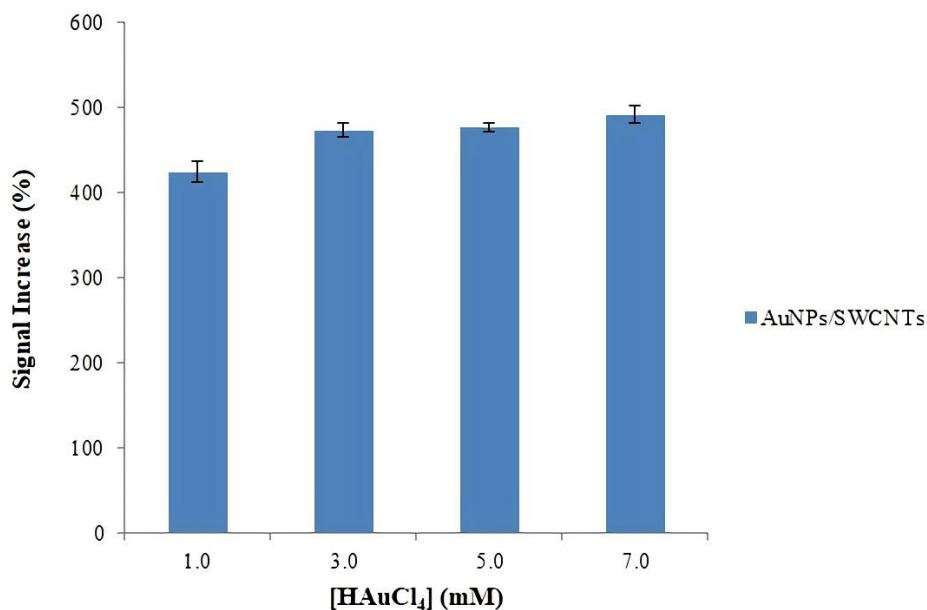


Figure 21 - Results for SPCE modification with SWCNTs (0.10 mg mL^{-1} ; $3.0 \text{ }\mu\text{L}$) and electrodeposited AuNPs (-0.2 V ; 150 s) with different HAuCl_4 concentrations ($n=3$). Measurements were performed through SWV in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

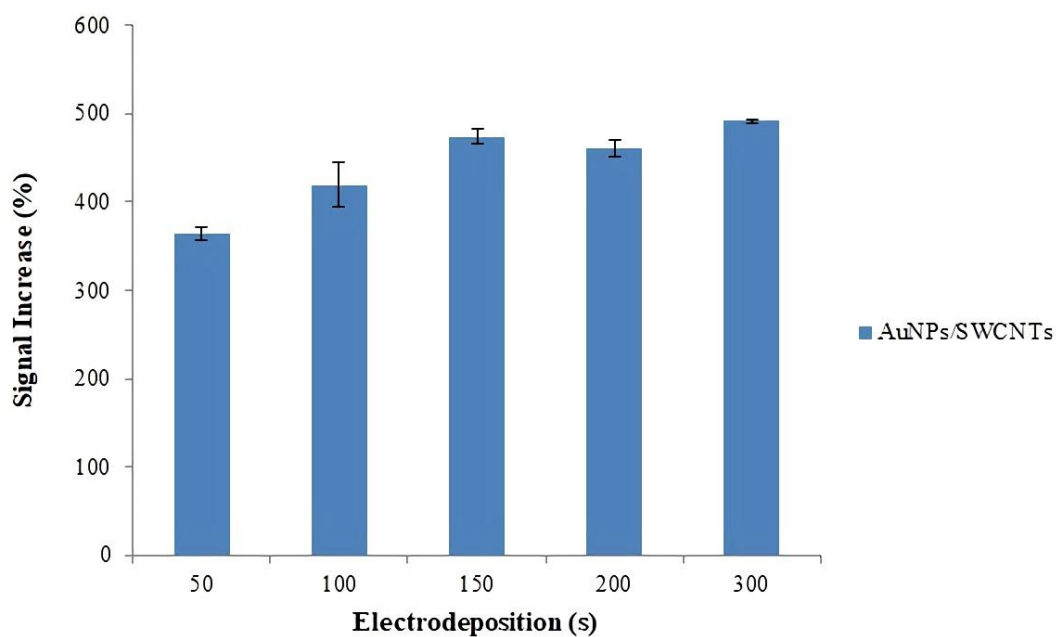


Figure 22 - Results for SPCE modification with SWCNTs (0.10 mg mL^{-1} ; $3.0 \text{ }\mu\text{L}$) and electrodeposited AuNPs (-0.2 V ; 3.0 mM HAuCl_4) through different electrodeposition periods ($n=3$). Measurements were performed through SWV in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

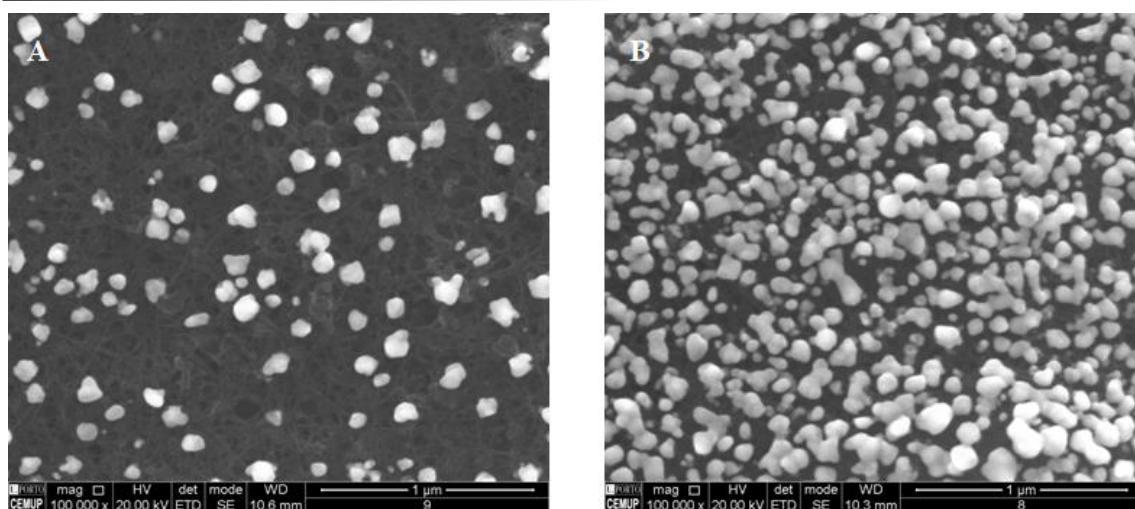


Figure 23 - SEM images of the SPCE modified with SWCNTs ($3.0 \mu\text{L}$; 0.10 mg mL^{-1}) and electrodeposited AuNPs (HAuCl_4 , 3 mM ; -0.2 V) for: A) 50 s ; B) 150 s .

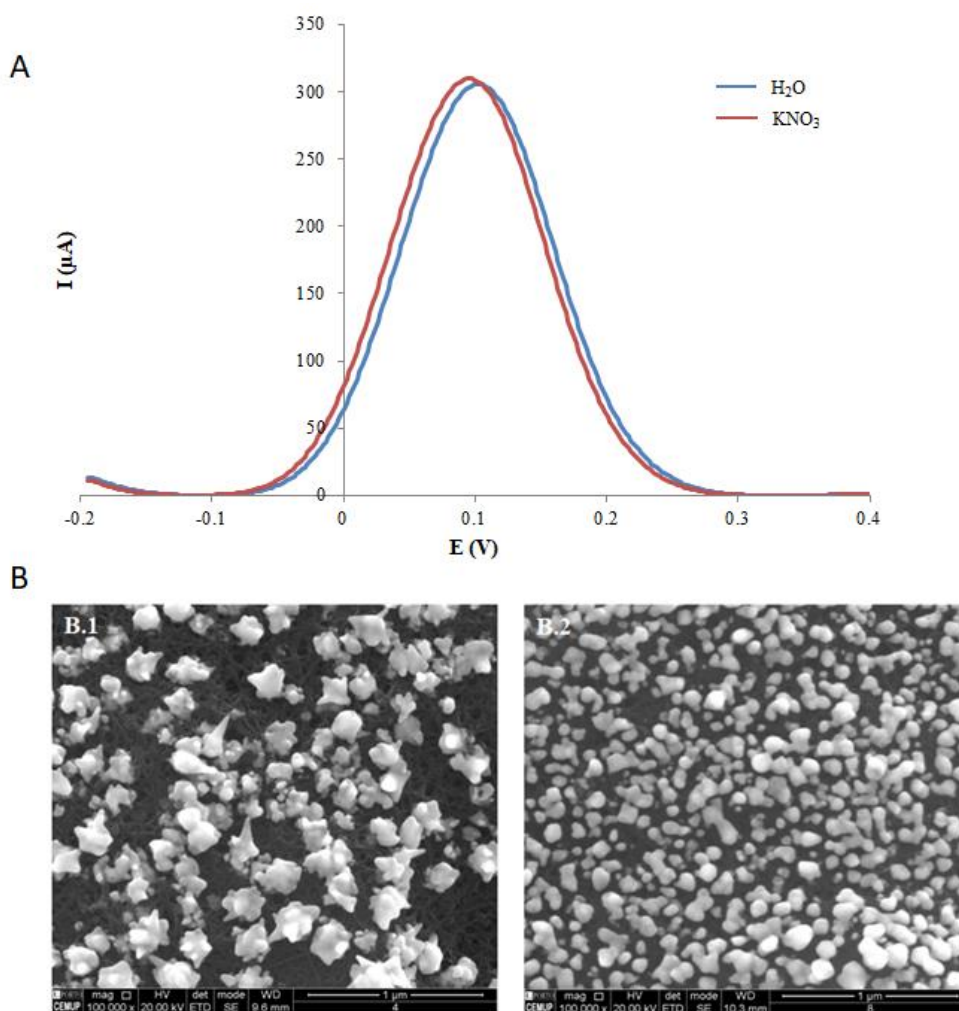


Figure 24 - SWV (A) and SEM (B) of SPCE modified with SWCNTs ($3.0 \mu\text{L}$; 0.10 mg mL^{-1}) and electrodeposited AuNPs (3 mM HAuCl_4 ; -0.2 V ; 150 s) in ultra-pure H_2O (B.1) and KNO_3 (B.2). SWV measurements were performed in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

This way, for the successful SPCE nanostructuration, a 3.0 μL volume of a 0.10 mg mL^{-1} SWCNTs suspension was used while the electrodeposition process was promoted by applying a -0.2 V potential during 150 s in a 3.0 mM HAuCl_4 solution with 0.1 M KNO_3 as the supporting electrolyte. Finally, bare SPCEs were modified with electrodeposited AuNPs which caused a signal enhancement of 444% with a 2% RSD, presenting an excellent electrochemical behavior, although not as good as the electrochemical behavior displayed by the SPCE modified with SWCNTs and AuNPs (Figure 25). In addition, the great advantage of combining these two nanomaterials was again revealed in the stability of the nanostructured sensor as following an overnight incubation at 4 °C, SPCEs modified with SWCNTs and AuNPs presented a signal decrease of only 2% in contrast to the 19% exhibited by the AuNPs modified SPCEs (Figure 25).

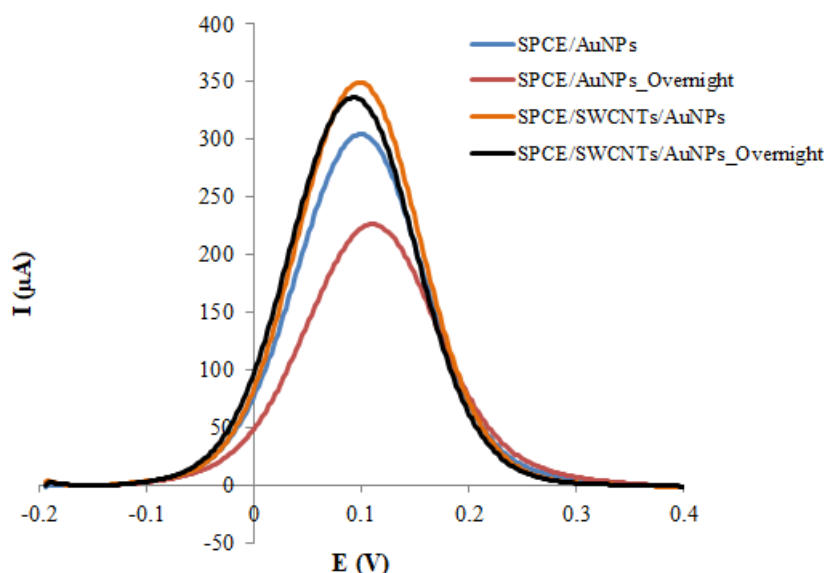


Figure 25 - SWV signal of the SPCE modified with AuNPs and with SWCNTs and AuNPs immediately after the nanostructuration and after an overnight incubation at 4 °C. Measurements were performed in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

Using the optimized parameters, it is possible to observe in Figure 26 the formation of a densely packed film of nanomaterials with clear differences between each layer. The generated AuNPs were well distributed on the SWCNTs surface, which further enhanced the current intensity, while their morphology extended the reach of the sensing interface further into the sample solution, which has been proved to reduce the response time and lower detection limits by enhancing the accessibility during biorecognition events [147, 148].

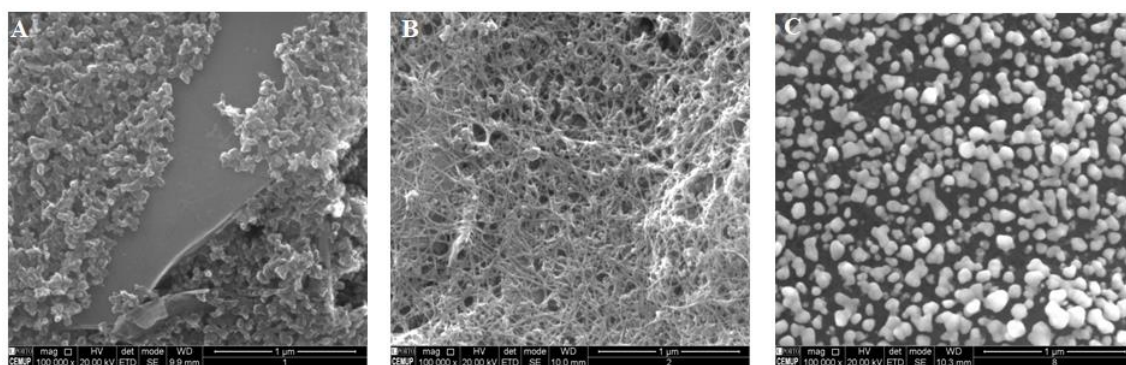


Figure 26 - SEM images of the SPCE nanostructuring: A) SPCE; B) SWCNTs/SPCE and C) AuNPs/SWCNTs/SPCE.

To conclude, the performance of the electrochemical transducer was improved by combining the excellent physical and catalytic properties of CNTs with the electronic properties of AuNPs. All of these modifications were integrated with experimental simplicity as a result of the ease of functionalization of each layer. In fact, SWCNTs were drop-casted on the surface of screen-printed carbon electrodes and set to dry at RT which took approximately 45 min for the formation of a stable film. Due to the versatility and ease of functionalization of CNTs, AuNPs were electrodeposited on their surface through a very simple and fast procedure that promotes the synthesis and simultaneous deposition of AuNPs. So, the full nanostructuring of the SPCE can be accomplished in less than 1 h. Moreover, the electrochemical behavior of each layer was studied, which led to the conclusion that the electrochemical performance and stability of these two nanomaterials combined were superior to their isolated activity. This was a critical stage of the immunosensor development in order to ensure an exceptional electrochemical behavior and intensify the signal processing, in addition to promoting an appropriate surface for the successful immobilization of the monoclonal antibodies through chemisorption. All these parameters were crucial to achieve a better evaluation of the changes occurring at the transducers surface, in particular the antibody-antigen interactions.

3.2.2.2.2. Antibody and BSA immobilization

For the immobilization of the biological recognition element, it was crucial to ensure the formation of a stable layer of antibodies on the AuNPs/SWCNTs/SPCE in order to achieve a sensitive and specific recognition of

α -synuclein. For this purpose, the antibodies were chemically modified through a thiolation process, following a protocol previously adopted [149], to create sulfhydryl groups which present strong affinity with metals being ideal for immobilization on the previously modified SPCE. In this procedure, 2-imminothiolane reacts with amine groups in proteins in a ring-opening reaction to result in covalent modification containing terminal sulfhydryl residues while retaining the divalent nature of the antibody molecule [138]. Following this chemical modification, proteins can be subjected to disulfide formation upon sulfhydryl oxidation which can cause unwanted conjugation and precipitation of the protein [138]. This way the addition of EDTA will prevent metal-catalyzed oxidation and maintain sulfhydryl stability [138]. For the formation of a stable layer of antibodies on the nanostructured SPCE, six concentrations were studied ($50\text{--}300\text{ }\mu\text{g mL}^{-1}$). From Figure 27 it is possible to conclude that saturation occurred at a concentration of $200\text{ }\mu\text{g mL}^{-1}$ with a signal decrease of 46.8% comparing to the signal of the nanostructured SPCE, from which no significant changes were observed, being this concentration selected as the optimum value for the development of the immunosensor. In addition, the unmodified antibody was also immobilized on the surface of the nanostructured SPCE, overnight at a concentration of $200\text{ }\mu\text{g mL}^{-1}$, causing 13% less signal reduction when compared to the thiolated antibody proving the efficiency of the chemical modification (Figure 28).

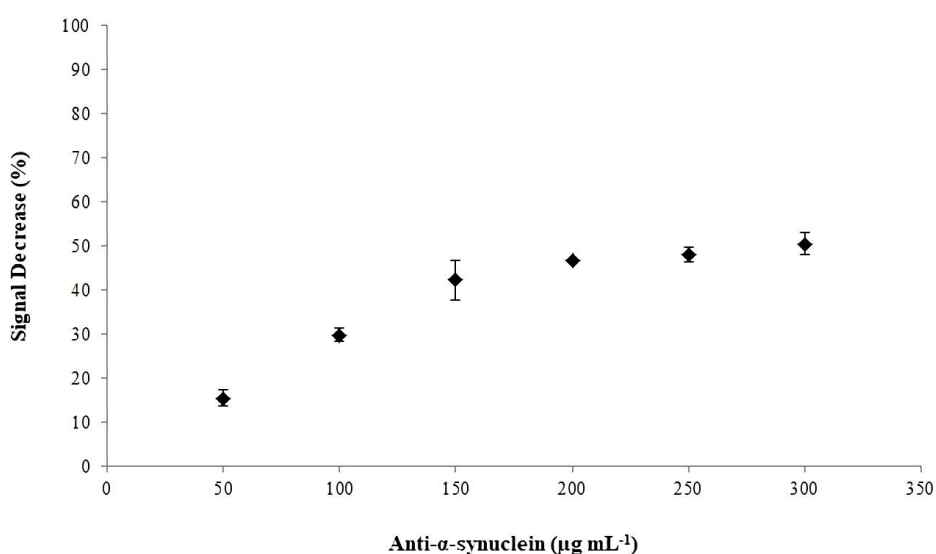


Figure 27 - Optimization of the thiolated antibody immobilization with different concentrations through overnight incubation at $4\text{ }^{\circ}\text{C}$ ($n=3$). Measurements were performed through SWV in a $2.5\text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

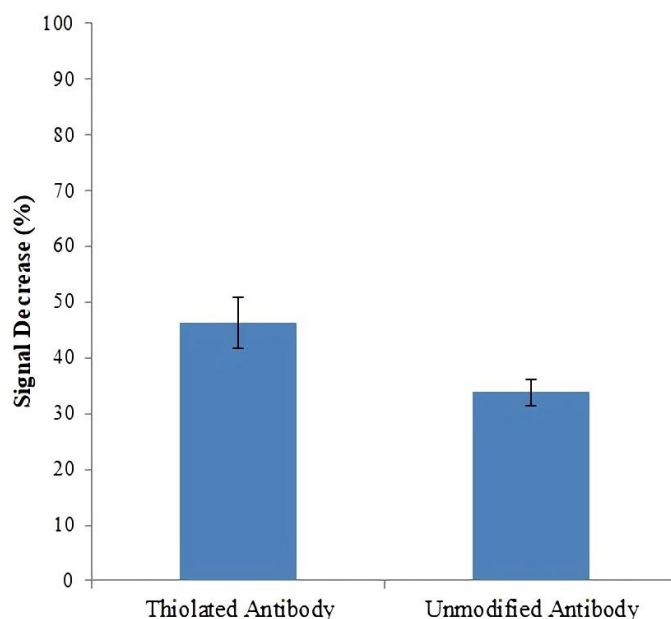


Figure 28 - Results for the overnight incubation of $200 \mu\text{g mL}^{-1}$ of thiolated and unmodified antibody ($n=3$). Measurements were performed through SWV in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

Finally, another critical issue relates to the minimization of nonspecific binding of interfering species in samples such as serum, blood or CSF. For this purpose, five BSA concentrations were evaluated (0.10 , 0.50 , 1.0 , 1.5 and 2.0%) with the optimum results reached at a concentration of 1% with a signal decrease of 51.7% in relation to the signal of the antibody modified SPCE (Fig. S10).

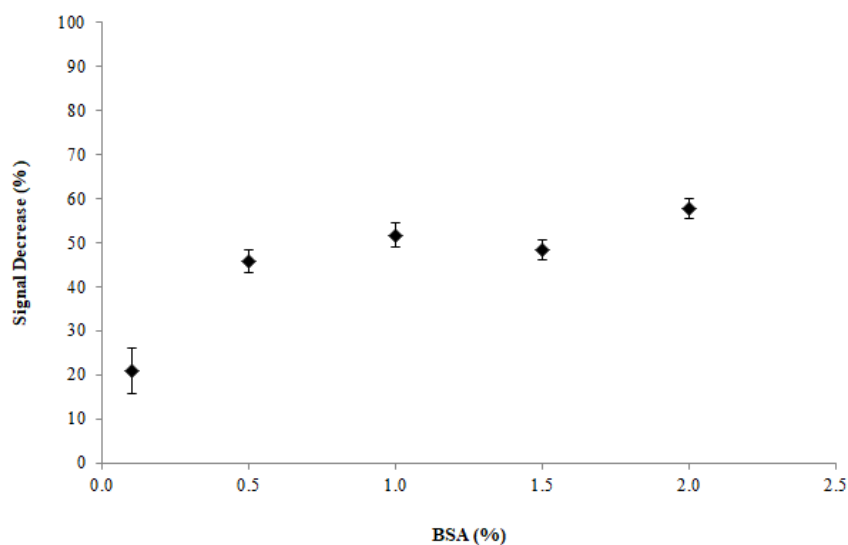


Figure 29 - Optimization of BSA immobilization with different concentrations through 30 min incubation at RT ($n=3$). Measurements were performed through SWV in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

3.2.2.2.3. Electrochemical characterization of the immunosensor

The proposed immunosensor was developed in a layer-by-layer approach, being extensively characterized through SWV (Figure 30), CV (Figure 31) and EIS (Figure 32), which enabled the precise manipulation of the characteristics of each layer through control of the materials and chemical groups exposed on the surface [140, 150]. In the case of SWV and CV which evaluate the charge transfer at the electrode/electrolyte interface, clear signal enhancements were observed (Figure 30 and 31) after the nanostructuration of the SPCE as nanomaterials favored direct and fast electron transfer, functioning as electron conduction pathways that decrease the overpotential of electroanalytical reactions [58, 151, 152]. SWCNTs were integrated on the transducer surface due to their electronic properties [62, 66, 153, 154] such as high conductivity along their length [66, 153] providing an ideal medium for electron transfer in electrochemical biosensors [62, 66], high surface area which facilitates further integration with more components [62, 153] and excellent chemical and thermal stability [62, 66, 153]. The electrodeposition of AuNPs promoted an even better electron transfer process as a result of their excellent electrical conductivity, amplifying the electrochemical response of the transducer while also providing an excellent matrix for the immobilization of the biological recognition elements due to their biological compatibility, chemical stability and high surface-to-volume ratio [66, 155]. In addition, the coupling of AuNPs with SWCNTs improved their long-term stability. This first stage of the immunosensor development was crucial to significantly enhance its electrochemical behavior since all further modifications resulted in a signal decrease. In fact, after the immobilization of antibodies and BSA on the surface of AuNPs, a large decrease in signal was observed as a result of the successful formation of a protein layer that hindered the electron transfer (Figure 30 and 31). The antibodies were immobilized through thiol bonds on the AuNPs surface promoting the formation of a dense and stable layer of biological recognition elements which is going to increase the sensitivity of the immunosensor while the immobilization of BSA minimized the non-specific interaction ensuring that the final changes in the electrochemical signal result from the specific antibody-antigen interactions. Finally, after applying the developed immunosensor to α -synuclein detection, the

signal decreased even more as a result of the immunocomplex formation. Further voltammetric measurements support this evidence (Figure 31).

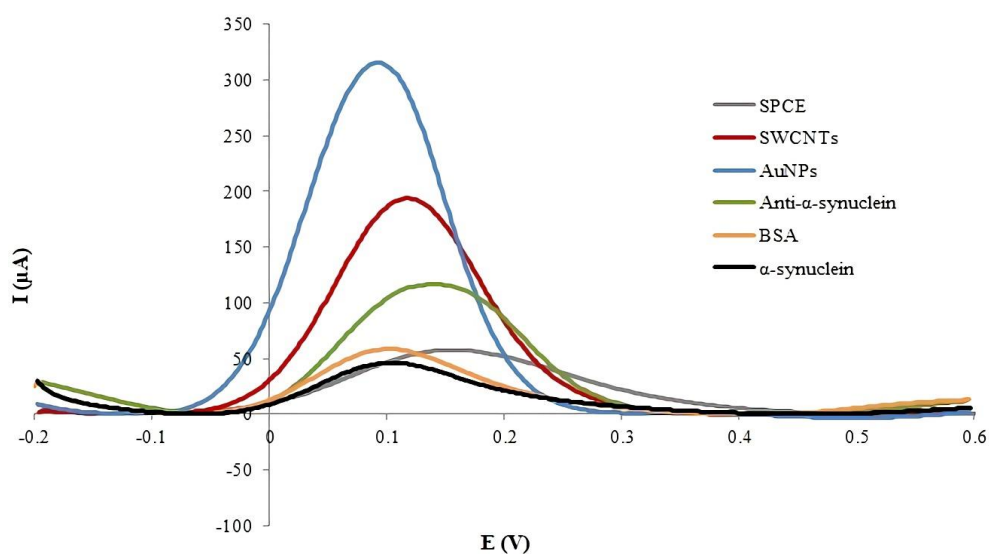


Figure 30 - SWV results for the optimized stages of the immunosensor development. Measurements were performed in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

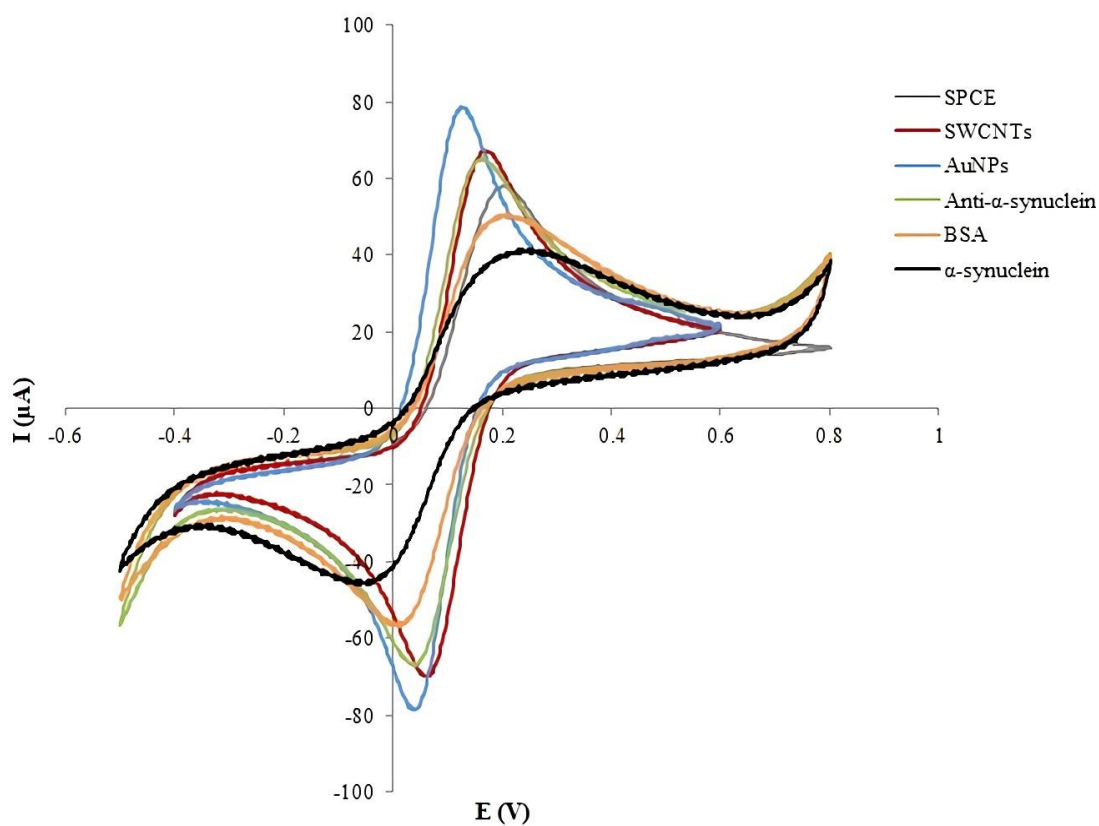


Figure 31 - CV results for the optimized stages of the immunosensor development. Measurements were performed in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

To further complete the electrochemical characterization of the immunosensor development, each stage of the SPCEs modification was studied through EIS (Figure 32). In contrast with SWV and CV, EIS measures the ability of a circuit to resist the flow of electrical current. EIS measurements allow the characterization of heterogeneous and complex systems with different electric and structural properties by using equivalent circuits as models [156, 157]. The resulting impedance spectra are composed of two portions, a semi-circle at higher frequencies corresponding to the electron-transfer resistance and a linear part at lower frequencies related to diffusion control of species called Warburg impedance [157]. From the EIS spectra (Figure 32) it is possible to observe clear differences in signal after integration of each layer on the SPCE surface starting with the bare SPCE, which exhibited a semi-circle with a small diameter at high frequencies representative of some resistance of the SPCE towards electron transfer. Once modified with nanomaterials there was a gradual decrease in the electron transfer resistance confirmed by the disappearance of the semi-circle which, after electrodeposition of AuNPs, was replaced by a straight line representative of a diffusion process. Following SPCE functionalization with antibodies, further blockage with BSA and formation of the immunocomplex, there was a clear enlargement of the semi-circles diameters as a result of the formation of an insulating protein layer which covered the surface of the nanomaterials resulting in an increase of electron transfer resistance.

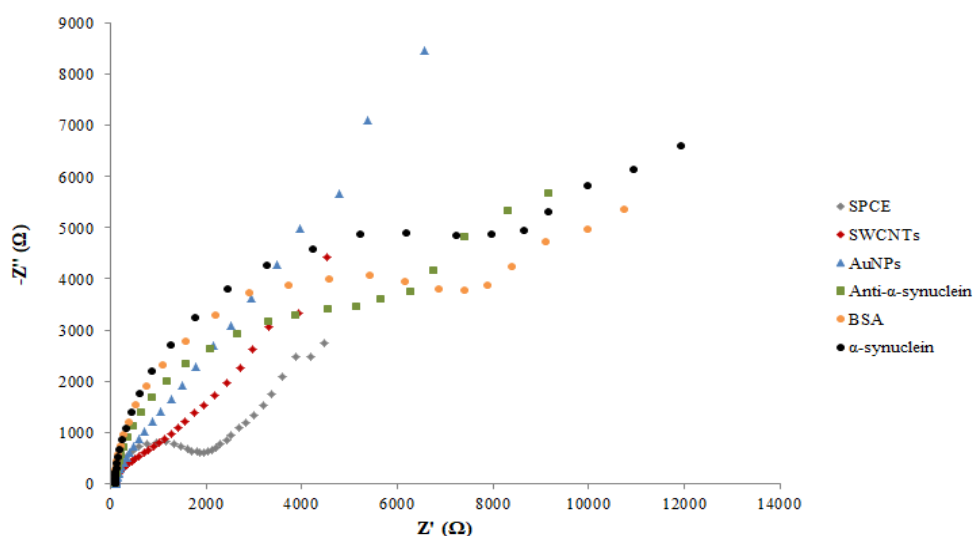


Figure 32 - EIS results for the optimized stages of the immunosensor development. Measurements were performed in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

3.2.2.2.4. Electroanalytical performance of the immunosensor

Under optimized conditions, the label-free detection of α -synuclein was carried out by incubating the immunosensor with different α -synuclein concentrations for 30 min at RT in order to ensure the successful antibody-antigen reaction. Figure 33A and B depicts the analytical calibration curve in which the SWV signal decreases as a result of the alterations to the interface properties of the biosensor due to the capture of increasing α -synuclein concentrations by the monoclonal antibody. The calibration plot exhibited an exponential relationship between the α -synuclein concentrations (0.010-10 ng mL⁻¹) and the SWV response with the analytical curve presenting wide linearity and good distribution of the data, even at low levels, with a correlation coefficient (r) of 0.996 ($n=5$). Based on the analytical data, limits of detection (LOD) and quantification (LOQ) were calculated to be 4.1 pg mL⁻¹ (0.29 pM) and 12.6 pg mL⁻¹ (0.9 pM). In addition, the immunosensor presented good sensitivity by being able to discriminate between small changes in concentration [147] which is very important for PD diagnosis as α -synuclein levels that distinguish healthy from PD patients have been reported between 1.0 and 2.0 ng mL⁻¹ [158, 159].

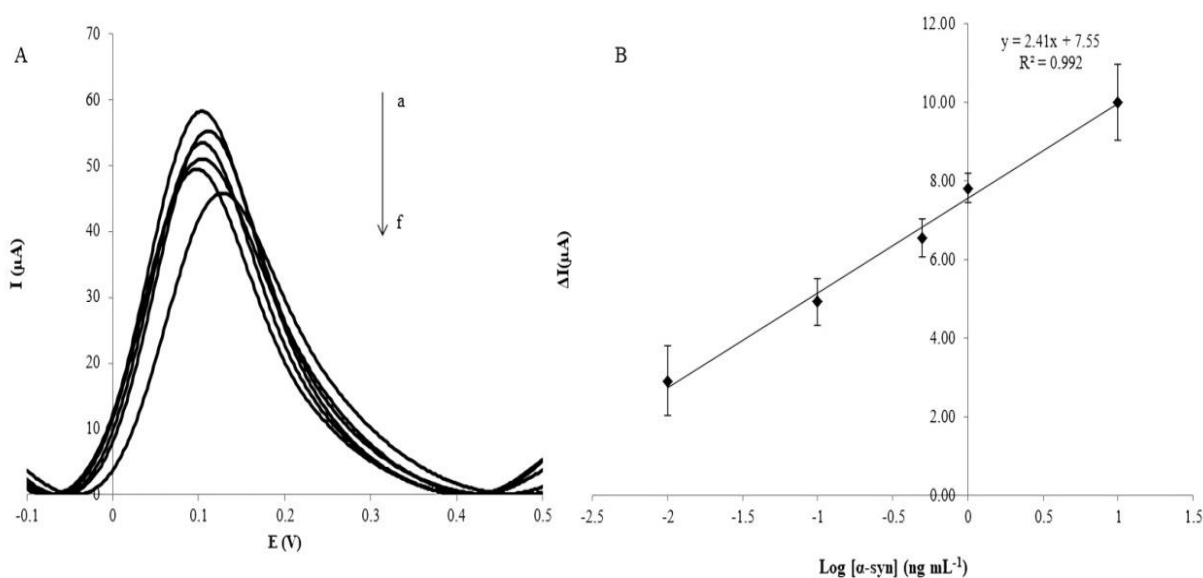


Figure 33 - A) SWV response of the immunosensor to different α -synuclein concentrations (from a to f: 0, 0.010, 0.10, 0.50, 1.0 and 10 ng mL⁻¹); B) Calibration curve based in the SWV signal reduction after detection of α -synuclein concentrations (0.010, 0.10, 0.50, 1.0 and 10 ng mL⁻¹) ($n=3$). Measurements were performed in a 2.5 mM Fe(CN)₆^{3-/4-} solution in 10 mM PBS (pH 7.4).

From Table 1, it is possible to conclude that several works were able to attain low LODs for α -synuclein detection to which the proposed immunosensor compares favorably by coupling its low LOD and LOQ with good sensitivity, short measurement time, small sample volume and simplicity of process, important characteristics for integration into point-of-care devices. All of these characteristics were achieved by functionalizing SPCEs with SWCNTs, AuNPs and monoclonal antibodies through simple and scalable experimental procedures. In fact, SPCEs have attracted increased interest for application in electrochemical immunosensing as a result of their low cost of fabrication and possibility of large scale production [48]. In addition, their miniaturized features enable the reduction of reagent consumption as every modification can be performed through the use of a small drop. All of the modifications in this study were performed using 3 or 5 μ L. Other advantages of these type of transducers are related to their easy functionalization, reliability, reproducibility, and possibility of integrating different materials, both electroactive and biological, which enables the formulation of various detection schemes [48]. The main factors that can influence a successful surface biofunctionalization are the efficiency and stability of the physico-chemical modifications and immobilization of bioreceptors [160]. This way, functionalizing the SPCEs through a layer-by-layer approach enabled to control film characteristics at the molecular level highlighting the properties of each material as opposed to the formation of a nanocomposite, in which the materials are randomly distributed and there is no possibility of controlling the properties that are exposed on the transducers surface.

The combination of SWCNTs and AuNPs created a synergistic effect that significantly improved the electrochemical behavior of the transducer as a result of the exceptional catalytic and conductive properties of both nanomaterials. In addition, the integration of both nanomaterials clearly improved the stability of the nanostructured sensor, which was essential to immobilize a high density of monoclonal antibodies on the surface of the AuNPs through chemisorption. Using this chemical process, a more stable immobilization of antibodies was promoted with improved epitope presentation and thereby better reproducibility of the antibody-antigen binding events. Moreover, these modifications shortened the diffusion distances causing the binding events to occur in close contact with the surface of the electrochemical transducer, which promoted a more rapid and

sensitive assay. Finally, by being based on a label-free detection process, it obviates the need for expensive and time-consuming amplification procedures such as biotin or enzyme-linked antibody or the conjugation of secondary antibodies with different nanomaterials.

3.2.2.2.5. Precision, stability and specificity of the immunosensor

The precision and stability studies of the immunosensor were estimated at a controlled α -synuclein concentration of 0.30 ng mL^{-1} in PBS. In this study, the precision was studied through an intermediate process by evaluating the dispersion of results of independent assays using identical samples with the same method, in the same laboratory with a group of modified SPCEs throughout different periods of time. The developed immunosensor presented excellent results for the precision assays attaining a RSD of 3.5% for the intra-day ($n=3$) studies, which were assays performed in the same day within short intervals of time, while for the inter-day ($n=3$) studies, which were performed in three consecutive days, an average 4.7% RSD was obtained. The stability ($n=3$) (Figure 34) of the immunosensor was tested by recording SWV responses every week for one month for electrodes stored at 4°C . A recovery of 103.8% with a RSD of 0.5% was attained after one month storage, proving the stability of the electrochemical immunosensor. This analytical parameter of the immunosensor development was a significant improvement from previous work, where a gold electrode was modified with a mercaptopropionic acid SAM, followed by AuNPs electrodeposition [149]. In this work, the AuNPs did not remain immobilized on the surface of the electrode over an extended period of time which reduced the analytical stability of the immunosensor to one week.

Finally, specificity studies were performed by exposing the developed immunosensor to a mixed solution of $\text{A}\beta$ 42 (1.0 ng mL^{-1}) and $\text{A}\beta$ 40 (10 ng mL^{-1}) in fetal bovine serum diluted 10 times in 10 mM PBS (pH 7.4) registering no significant changes in the SWV signal, corroborating the specificity of the immunosensor for α -synuclein (Figure 35). $\text{A}\beta$ 42 and $\text{A}\beta$ 40 variants are important Alzheimer's disease (AD) biomarkers [161, 162] being that $\text{A}\beta$ 42, which constitutes less than 10% of total $\text{A}\beta$, seems more prone to aggregate contributing to the modification of the ratio between the two variants, making

them crucial biomarkers for the detection of A β pathology and therefore attest the presence of AD [162], the most prevalent neurodegenerative disease [163].

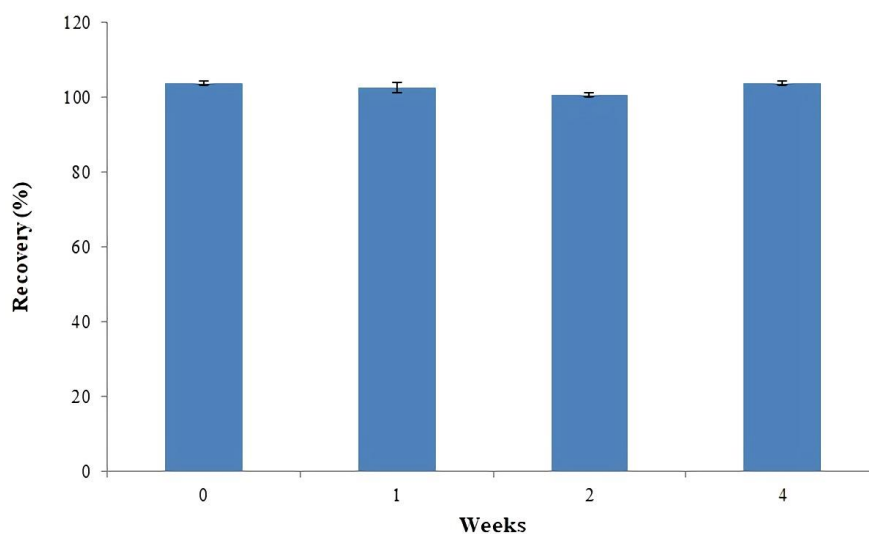


Figure 34 - Stability assay performed for a controlled α -synuclein concentration (0.30 ng mL^{-1}) through a 4 week period with storage at 4°C ($n=3$). Measurements were performed through SWV in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

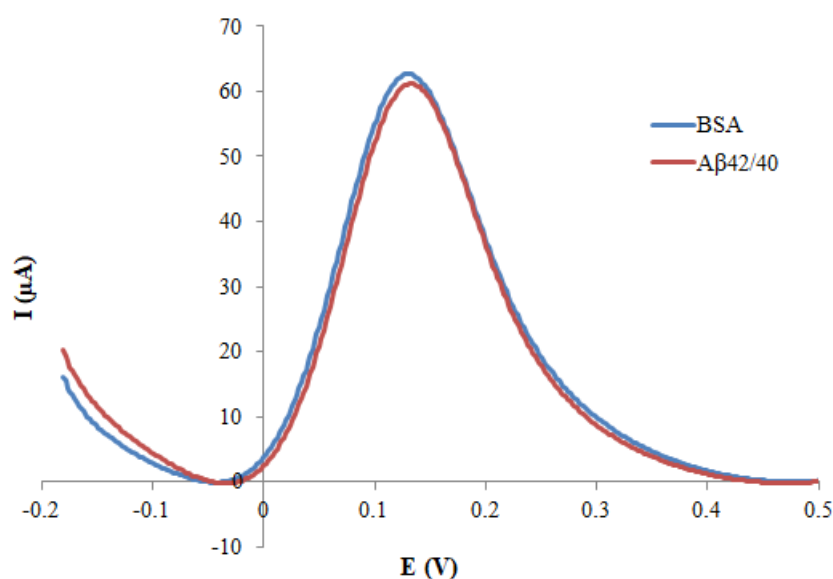


Figure 35 - Specificity assay performed by exposing the developed biosensor to fetal bovine serum spiked with A β 42 and A β 40 at a 1:10 ratio. Measurements were performed through SWV in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in 10 mM PBS ($\text{pH } 7.4$).

3.2.2.2.6. α -synuclein detection in fetal bovine serum

To study the performance of the developed immunosensor in complex samples, three concentrations were spiked into fetal bovine serum which was

diluted 10 times in 10 mM PBS (pH 7.4). Recoveries of 96.7, 106.2 and 102.9% (Table 2) were attained for the concentrations of 0.30, 2.0 and 3.0 ng mL⁻¹, respectively. This way, the developed immunosensor exhibited excellent sensitivity in detecting and distinguishing small changes in concentration despite registering a higher RSD for the lowest spiked concentration. While α -synuclein is found in the pM range, proteins present in serum can be found in the μ M to mM range which can lead to some cross reactivity [164]. Therefore, the serum had to be diluted in order to minimize non-specific interactions which were decreasing the biosensor’s sensitivity.

Table 2 - Recovery assays in fetal bovine serum spiked with α -synuclein (n=3).

Sample	Standard value (ng mL ⁻¹)	Found (ng mL ⁻¹)	Recovery (%)	RSD (%)
1	0.300	0.290	96.7	7.4
2	2.00	2.12	106.2	4.7
3	3.00	3.09	102.9	2.8

Although further studies should focus on testing in biological samples, such as CSF and blood, the results obtained in fetal bovine serum seem promising being that high selectivity was attained due to the high binding affinity between the monoclonal antibodies and α -synuclein. Several studies suggest the potential diagnostic and prognostic value of CSF and blood biomarkers that can reflect the pathophysiology of Parkinson’s disease. In addition, the levels of α -synuclein in CSF are the most reported in several longitudinal studies using its levels to establish cut-off points that can distinguish controls from Parkinson’s disease patients [165-167]. Nevertheless, the use of CSF requires a lumbar puncture, which is an invasive procedure. For that reason, several studies are also focusing its attention on the study of α -synuclein levels in blood which would provide a non-invasive tool for the differential diagnosis of Parkinson’s disease.

As a final point, the proposed electrochemical immunosensor exhibited excellent sensitivity with low LOD and LOQ, which coupled to its fast and simple label-free detection process showed excellent potential as a tool to study the aggregation mechanisms of this important PD biomarker and further contribute to the effective diagnosis of this neurodegenerative disease.

3.2.2.3. GCE vs SPCE

GCE and SPCE present different properties which had a direct impact on the immunosensors analytical behavior. Following the same design of biosensor both electrochemical transducers exhibited significant differences concerning the optimization process of each layer (Table 3). In fact, while GCE was modified with a 1 mg mL^{-1} suspension of SWCNTs and set to dry at 50°C , the SPCE modification required 1/10 of the concentration of the same suspension dried at RT. Actually, in this case the GCE exhibited more robustness since the SWCNTs modified SPCE could not be exposed to higher concentrations of DMF even when dried at 50°C . AuNPs electrodeposition was the only step that was carried out under the same conditions through the application of -0.2 V for 150 s while the transducers surface were exposed to a 3 mM H AuCl_4 solution in 0.1 M KNO_3 .

When both transducers were studied after the optimized nanostructuration, it is possible to observe that GCE had to be modified with a more concentrated suspension of SWCNTs while been evaluated in a $5 \text{ mM K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ solution to attain a similar electrochemical behavior to the SPCE studied in a 2.5 mM electrolyte solution. Nonetheless, after minimizing the surface area effect, it is possible to observe that both transducers exhibited similar electrochemical behavior when studied under the exact same conditions (Figure 36): modification with a 0.10 mg mL^{-1} SWCNTs suspension and analyzed in a $2.5 \text{ mM K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ solution. Following deposition of AuNPs the biggest differences between the functionalization of both electrodes was registered in the immobilization of the monoclonal antibodies. Since these transducers present different surface areas (Table 3), with the SPCE exhibiting almost twice the area of a GCE, the concentrations required to promote a successful immobilization of the antibodies were significantly different being that the SPCE required a concentration 40-fold higher than the GCE. This fact may have had a direct impact on the immunosensors analytical behavior since higher concentrations of the biological recognition elements can favor the direct determination of the analytes. In fact, the immunosensor developed with SPCE presented a better and more reproducible analytical behavior, being able to detect α -synuclein levels one order of magnitude lower. In addition, another important fact concerning the functionalization of both transducers with antibodies is the necessary volume required to perform their immobilization with success. A major limitation associated with GCE is associated with

their size being necessary to immerse the electrodes in a 250 μL volume to carry the successful antibody immobilization overnight. On the other hand, SPCE only required a 5 μL volume of antibody solution to cover the electrodes surface while maintaining the electrodes in a water saturated environment to prevent evaporation of the solution. Finally, it is possible to conclude that the nanostructured immunosensor based on a SPCE platform exhibited a better analytical and electrochemical behavior being able to detect lower concentrations of α -synuclein while requiring less reagent consumption to perform the immunosensor development. GCE is a more robust electrochemical transducer that enables repeated use over time being its surface very reactive once activated via polishing. Nonetheless, the repeated polishing processes may negatively impact the transducers electrochemical behavior causing significant changes in the electrochemical signal. In addition, when working with a set of GCE it is very difficult to ensure the same surface state between them after the cleaning procedures.

Table 3 - Comparison between GCE and SPCE concerning the optimized parameters for the immunosensor development.

	GCE	SPCE
Area (mm^2)	7.1	12.6
SWCNTs concentration (mg mL^{-1})	1.0	0.10
SWCNTs volume (μL)	3.0	3.0
Antibody concentration ($\mu\text{g mL}^{-1}$)	5.0	200
BSA (%)	0.50	1.0
Calibration curve (ng mL^{-1})	0.50–50	0.010 - 10
LOD (pg mL^{-1})	43	4.1
LOQ (pg mL^{-1})	940	12.6
Antibody and Sample volume (μL)	~250	5.0
Electrolyte concentration	5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$	2.5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$

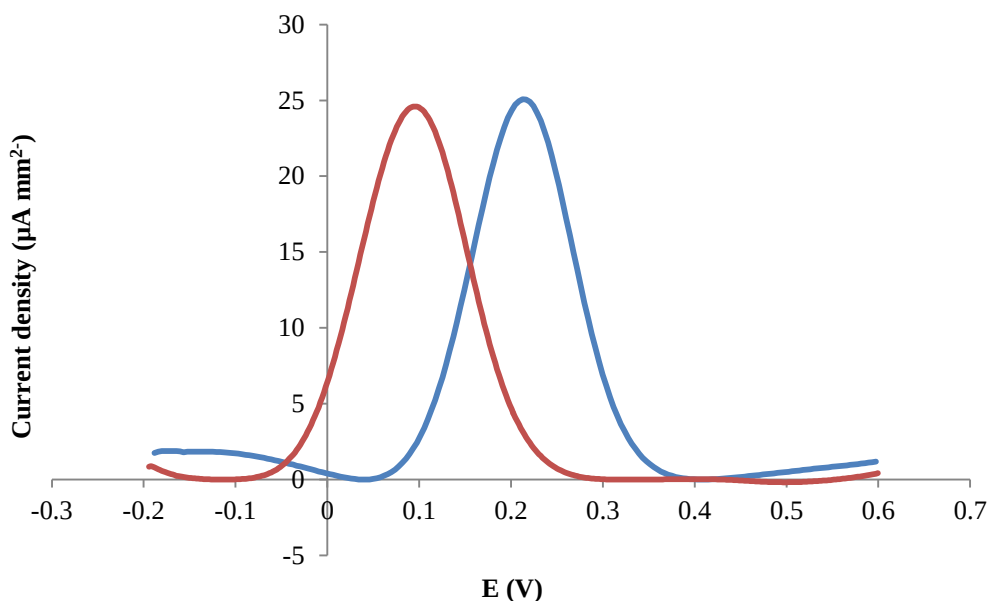


Figure 36 - SWV results for the GCE (blue) and SPCE (red) modified with 0.1 mg mL^{-1} SWCNTs and AuNPs under optimized conditions. Measurements were performed in a $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ solution in $10 \text{ mM PBS (pH 7.4)}$.

3.2.3. Conclusion

An electrochemical immunosensor developed in a layer-by-layer approach using a GCE and SPCE as the electrochemical transducers was proposed for the detection of α -synuclein, an important pathological hallmark of PD. The adopted approach enabled to control the characteristics of each layer at the molecular level with experimental simplicity, thus contributing to the assembly of different nanomaterials. Moreover, the extensive optimization of each layer maximized the synergistic effect of the modifications, which led to a significant improvement in the sensitivity of the label-free immunosensor. This way, the modifications with SWCNTs and AuNPs promoted the electron transfer process at the electrode/electrolyte interface improving the electrochemical response of the transducers while also increasing the long-term stability of the nanostructuration. In addition, due to the chemical stability and biological compatibility of AuNPs, an appropriate matrix for the immobilization of monoclonal antibodies was promoted, which retained their bioactivity and immunoreagent stability upon immobilization. The combination of all these layers promoted a sensitive and specific quantification of α -synuclein, with low LOD and LOQ, using simple, fast and relatively inexpensive instrumentation. In addition, SPCE exhibited a

significant better electrochemical behavior enabling the development of a more sensitive and reproducible immunosensor while only requiring a small sample volume. Therefore, this can constitute a novel approach in the detection and quantification of PD biomarkers and have a positive impact in its diagnosis. Nonetheless, due to the multifactorial nature of PD future studies will focus on adapting the developed immunosensor into a platform capable of simultaneously detect and quantify multiple biomarkers, thus contributing to a more precise and specific diagnostic process.

Chapter 4. Alzheimer's disease biosensors

*Adapted from original publications: (1) Carneiro, P, Morais S and Pereira MC, Biosensors on the road to early diagnostic and surveillance of Alzheimer's disease. *Talanta* 211 (2020) 120700. (2) Carneiro, P, Morais S and Pereira MC, Nanomaterials towards biosensing of Alzheimer's disease biomarkers. *Nanomaterials* 9 (2019) 1663.

Abstract

Alzheimer's disease (AD) is an incurable and highly debilitating condition characterized by the progressive degeneration of nerve cells, which leads to manifestation of disabilities in cognitive functioning. Recently, substantial progress has been made in identifying PET and CSF biomarkers for AD, but their use is limited by invasiveness of procedures and high costs, restricting their use as front-line diagnostic tools. Therefore, it is of utmost importance to develop analytical tools that can contribute to the identification and determination of biomarkers for AD in order to facilitate the diagnostic process and identification of patients at earlier stages, as well as monitor biochemical effects of treatments. In this regard, this chapter addresses the topic of biosensors towards detection of AD biomarkers, from state of the art to experimental work. This way, this chapter is organized in two sections. In the first part, a comprehensive review of the biosensors developed towards detection of AD main biomarkers is presented, focusing on the advances, challenges and recent trends of AD biosensing with a strong focus towards the integration of nanomaterials in the biosensors design. In the second part of this chapter, one of the most common problems associated with biosensing was addressed. This problem is associated with the negative impact of biofouling upon exposure of the biosensing surfaces to biological fluids, which frequently affects the analytical behavior of the developed sensing platforms. This problem is one of the biggest obstacles limiting the commercialization and clinical application of the vast number of developed biosensors. This way, in this experimental section two antifouling strategies were developed and compared with the goal of developing surfaces resistant to non-specific binding events while also keeping intact the transducers functioning. Finally, taking advantage of the optimized antifouling coating an electrochemical immunosensor was developed toward detection of A β 42. This immunosensor was based on a sandwich detection system with novel immunosensing probes being presented for the production of the analytical signal specifically related with the concentration of the analyte of interest. These probes were based on colloidal nanoparticles functionalized with monoclonal antibodies specific to A β 42 and labeled with metal ions (Pb²⁺) for the electrochemical signal amplification. Despite being able specifically detect low levels of A β 42, the proposed immunosensor demonstrated reproducibility problems related with the instability of the developed immunosensing probes.

4.1. State of the art

Alzheimer's disease (AD) is the most common human NDD that commonly manifests itself in the form of dementia [1-3]. Its incidence increases with age and, like most NDDs, AD is considered a multifactorial condition mainly characterized by the aggregation of a small number of proteins [1-3]. Approximately 40 million people, mostly older than 60 years, have dementia and this number is expected to triple by 2050, being recognized by the World Health Organization as a global public health priority [2, 5, 163, 168-170].

AD is the most common cause of dementia with severe impacts on individuals and society [2, 171-174]. AD is thought to begin 20 years or more before symptoms arise, with small changes in the brain that are unnoticeable to the person affected. Only after years of brain changes do individuals experience noticeable symptoms, such as memory loss and language problems. Symptoms occur because nerve cells in parts of the brain involved in cognitive function have been damaged or destroyed. Individuals typically live with AD symptoms for years. Over time, symptoms tend to increase and start interfering with individuals' ability to perform everyday activities.

Currently, the field of drug development concerning AD is directing its efforts towards the development of disease-modifying drugs being these treatments more likely to succeed when administrated in the early stages of disease. However, several clinical and neuropathological studies have classified the criteria for AD diagnosis to be suboptimal as this process is mostly based on clinical assessment of symptoms, which can lead to misdiagnosis or diagnosis made in advanced stages of the pathology, which in turn can influence the course of several drug candidates when tested in clinical trials [5, 175]. Therefore, the development of diagnostic tools capable of performing an early, accurate and differential diagnosis is of vital importance [175].

4.1.1. Alzheimer's disease biomarkers

Research, in recent decades, has culminated in the evidence that the formation of extracellular amyloid β ($A\beta$) plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau protein are the main causes for the development of AD [2, 161, 169, 171, 176, 177]. Moreover, these two processes are usually accompanied by neuroinflammation and oxidative stress, all of which contribute to the loss of synaptic integrity and neural network connectivity [2, 161, 169, 171, 178, 179]. Amyloid plaques

are extracellular accumulations of abnormally folded A β peptides, A β 40 (40 amino acids) and A β 42 (42 amino acids), derived from proteolytic cleavage of the glycoprotein amyloid precursor protein (APP) by β - and γ -secretase activities, being the variant with 42 amino acids more prone to aggregate [2, 161, 169, 174, 177, 178]. Research gives support to the fact that the primary event in AD may result from an imbalance between the production and clearance of A β in the brain, leading to the overexpression of A β 42 and consequent aggregation, from which all other stages of the disease unfold [174, 180]. Although the formation of amyloid plaques composed of A β aggregates has been thought for decades to be the critical pathological hallmark of AD, a lot of evidence has brought to light that A β oligomers may be its most pathological form [174, 181]. The fact that one-third of the elderly die with evidence of A β accumulation without noticeable symptoms proves that while remaining essential for AD diagnosis A β deposition on its own is not enough to cause dementia in AD [163, 174, 182].

Neurofibrillary tangles made of intracellular aggregations of paired helical filaments of abnormal phosphorylated tau protein, have also been investigated as a biomarker [2, 174, 183-186]. Tau in AD undergoes abnormal phosphorylation becoming unable to bind and stabilize microtubules, leading to protein misfolding and subsequent aggregation as neuropil threads in dystrophic neurites and as neurofibrillary tangles which, eventually, results in loss or decline of axonal or dendritic transport [184-186]. Tau protein undergoes several modifications being phosphorylation on multiples sites considered to be a major early event in the formation of tau inclusions [187]. To get a comprehensive overview of these post-translational modifications, several modified sites have to be assessed at a qualitative and quantitative level which can be extremely challenging due to the large number of modified sites and existence of multiple types of modifications [187]. Current strategies struggle to distinguish these post-translational modifications of tau protein mainly associated with epitopes variability which affects the affinity of the biological recognition elements that in turn disturbs the rigorous quantification of tau protein [187].

The identification of mutations in gene coding for proteins involved in A β deposition/clearance pathways, as the case of apolipoprotein E (ApoE), constitutes another pathological hallmark of the disease. ApoE is a 299 amino acid protein produced primarily by astrocytes and microglia with three common isoforms in humans (ApoE2, ApoE3 and ApoE4) that only differ by 1 or 2 amino acids impacting the charge and structural properties of the protein and, ultimately, their functional properties [171,

188-191]. Studies have revealed that deposition of A β into plaques can be influenced by isoforms and amount of ApoE present in the central nervous system, inevitably impacting the onset and progression of AD [189]. This way, ApoE4 increases the risk of developing AD and it is also associated with earlier onset of the disease [171, 188, 189, 191]. On the other hand, ApoE2 reduces the risk of developing AD by decreasing the deposition of A β and delaying the onset time [171, 189, 191].

Increasingly, AD is being classified as a multifactorial disorder that arises from the interaction of multiple pathogenic factors, such as amyloid precursor protein, A β , Tau protein, ApoE and other co-morbidities.

Biomarkers such as A β and tau protein can have a pivotal role in developing more accurate diagnosis tools for AD and their use was recommended in recent research diagnostic guidelines for AD by the National Institute on Aging–Alzheimer's Association [175, 192] and International Work Group 2 criteria [175, 193]. To date, the only biomarker that has been approved by the Food and Drug Administration was the evaluation of A β through positron emission tomography scans providing a visual aid to assess the presence of A β plaques [175]. On the other hand, this is an expensive and specialized procedure with radioactive burden on the patient. Therefore, cerebrospinal fluid (CSF) biomarkers appear as a viable alternative as several studies have shown unequivocally that AD is associated with lower CSF levels of A β 42 and higher CSF levels of total tau (t-tau) and phosphorylated (p-tau) being able to distinguish patients with AD *versus* controls, also in the early stages [163, 194, 195]. In addition, the evaluation of the ratio A β 42/A β 40 has proven to increase diagnostic accuracy [162].

Therefore, it is of the utmost importance to develop analytical tools that can contribute to the identification and determination of biomarkers for AD in order to facilitate the diagnostic process and identification of patients at earlier stages, as well as monitor biochemical effects of treatments. Moreover, with the implementation of new and more efficient diagnostic techniques, it will be possible to provide patients a healthier and more reliable life by slowing or preventing the disease progression in its early stages.

4.1.2. Alzheimer's disease biosensors

Over the last decade, AD has been the most intensively studied neurodegenerative disease with regard to biomarkers detection, in blood and CSF, of mainly A β [149, 196-

218], tau protein [219-229], ApoE [230-236] and multiple detection of these same biomarkers [237-246]. While the roles of A β and Tau protein in AD are well described in literature [2, 163, 171], ApoE, and particularly the isoform ApoE4, has been receiving a lot of attention as a major genetic risk for AD as it can predict accumulation of A β in the brain [2, 171, 189, 190]. Configuration, analytical performance and application of the biosensors developed in the last decade for AD biomarkers are displayed in Tables 4-7. Table 4 characterizes the biosensors for A β biomarker and related variants [149, 196-218]; Table 5 focuses on the biosensors for tau protein determination [219-229]; Table 6 exhibits the biosensors for ApoE detection [230-236] and Table 7 shows the biosensors capable of performing multiple determination of the aforementioned biomarkers [237-246].

As already referred electrochemical immunosensors are the most reported for determination of the selected biomarkers. The most common transducers used throughout the reviewed studies (Tables 5-7) were GCE [199, 204, 231, 239] (Tables 4, 6 and 7), gold electrode (AuE) [149, 215-218, 220-223, 237, 238] (Tables 4, 5 and 7), indium tin oxide (ITO) [212, 233-236] (Tables 4 and 5) and SPCEs [200-202, 224, 230, 245] (Tables 4-7). Nevertheless, other materials such as anodic aluminum oxide membrane (AAO) [203] (Table 4), silicon wafer [205, 206, 208] (Table 4) and screen-printed gold electrodes (SPGE) [213] (Table 4) were also used.

In recent decades nanomaterials have become an integral part of almost every biosensor development. This way, the transducers used in the reviewed studies were commonly modified with various nanomaterials such as AuNPs [149, 200-204, 207-209, 212, 223, 235-237, 239, 244, 245] (Tables 4-7), AgNPs [243] (Table 7) and silica nanoparticles [225] (Table 5), magnetic beads/spheres [230, 232, 245] (Tables 6 and 7), polymers [197, 198, 206, 212, 213, 216, 242, 244] (Tables 4 and 7) and carbon nanomaterials such as GO [224, 231, 234] (Tables 5 and 6) and CNTs [196, 199, 228, 239] (Tables 4, 5 and 7) but other nanomaterials, such as dendrimers [224] (Table 5) and quantum dots (QDs) [232, 234] (Table 6) have also been used but to a lesser extent.

Gold nanoparticles were the most applied nanomaterials as they exhibit excellent electronic and optical properties in combination with good biological compatibility, ease of functionalization, controllable morphology and size, chemical stability, large surface area and high surface-to-volume ratio [16, 59, 247]. Carbon nanomaterials such as GO and CNTs have also been commonly applied in the development of electrochemical and optical biosensors. GO is a planar sheet of carbon atoms displaying

large surface area, high mechanical strength, high electrical and thermal conductivity and high elasticity [144, 248, 249]. In addition, GO is also characterized by the presence of various oxygen functional groups [250]. On the other hand, CNTs are cylindrical molecules that consist of rolled-up sheets of single-layer carbon atoms that can be classified as SWCNTs or MWCNT being characterized by their high surface-to-volume ratio, exceptional electronic properties, presence of edge-plane-like defects and ease of functionalization, all which contribute for the development of high performance biosensors [59, 154, 251]. At last, other type of nanomaterials that has been extensively applied for the development of biosensors were polymers mainly due to their ease of functionalization and high stability [16]. In addition, some polymers such as polypyrrole which are classified as conducting polymers also exhibit inherent electrical conductivity which can be highly relevant for electrochemical biosensors [16, 252, 253].

Another crucial parameter that requires extensive focus and optimization is related to the immobilization of the biological recognition elements. This way, different methods were applied to successfully immobilize the required biorecognition elements. Random physical adsorption has been one of the most commonly applied methods for antibody immobilization [196, 207, 211, 215, 228, 230, 232, 233, 235, 254] (Tables 4, 5 and 6). Nonetheless, the most common method to promote immobilization of biological recognition elements on AD biosensors was based on covalent bonding through EDC/NHS coupling [200, 203-205, 210, 216, 220, 222-224, 226, 227, 229, 231, 234, 236-240, 244, 245] (Tables 4-7). Other methods such as conjugations through thiol groups [149, 208] (Table 4), glutaraldehyde [197, 198, 224] (Tables 4 and 5) or polymers [206] (Table 4) have also been used. Despite being widely applied for immobilization of the biological recognition elements, the aforementioned methods face a big problem with uncontrolled anchoring of the biorecognition elements which can affect the function of their active sites. This way, to overcome this problem some works applied an affinity based method through the biotin-strept(avidin) interaction where biotinylated biomolecules can be attached to substrates via avidin (or streptavidin) bridges [55, 68, 201, 213, 242] (Tables 4 and 7). Another commonly used affinity based immobilization tag was through binding of antibodies to protein G [202, 221, 225] (Tables 4 and 5).

Different detection schemes and techniques were applied for detection of the main AD biomarkers mostly based on electrochemical and optical techniques. The main electrochemical techniques used throughout the reviewed works were SWV [149, 212,

217, 218, 240] (Tables 4 and 7), DPV [199, 200, 223, 224, 231, 234, 239] (Tables 4-7) and EIS [202, 203, 211, 213, 214, 216, 219-222, 235]. The remaining studies were mainly distributed between optical techniques, which relied on SPR (Tables 4-7) [210, 226-228, 242] and LSPR (Tables 4-7) [209, 225, 235, 244], fluorescent detection (Tables 4, 6 and 7) [205, 206, 234, 246], colorimetric methods (Tables 4, 6 and 7) [207, 236, 243] and microscopic techniques such as scanning tunneling microscopy (STM) (Table 4) [208].

Throughout this review we have highlighted the studies that presented the best analytical behavior and lowest LODs focusing particularly in the studies that were able to detect levels lower than the cut-off values in CSF of the main AD biomarkers.

Table 4 - Configuration, analytical performance and application of the developed biosensors for A β biomarker and related variants determination.

Biomarker	Transducer	Detection Method	Sample	Detection range (nM*)	LOD (nM*)	References
A β 42	CNTs-Metal semiconductor field effect transistor structure modified with E. coli outer membrane with autodisplayed Z-domains and immobilized antibodies	Electrical conductance	Human Serum	$2.2 \times 10^{-4} - 0.22$ *	2.2×10^{-4} *	[196]
A β 42	Interdigitated microelectrode with SiO ₂ surface functionalized with APMES, polyvinyl pyrrolidone-aldehyde solution, sodium borohydride, glutaraldehyde and antibodies	Ion concentration polarization-based preconcentration - Measurement of impedance	Buffer	$2.2 \times 10^{-6} - 0.22$ *	8.15×10^{-6}	[197]
A β 42	Interdigitated microelectrode with a SiO ₂ layer which was later treated with APMES, polyvinyl pyrrolidone-aldehyde solution, sodium borohydride, glutaraldehyde and antibodies	Measurement of impedance	Buffer and mouse plasma	$2.2 \times 10^{-5} - 0.022$ *	NR	[198]

Cu ²⁺ ; A β 42	GCE functionalized with PDDA/SWCNTs combined with neurokinin B	ABTS- DPV	Buffer, Blood and Hippocampus of rats	Cu ²⁺) 100 – 10000 A β 42) 0.220 – 8.42 * 2.20 – 84.2 * 22.0 – 842 *	Cu ²⁺) 40 A β 42) 0.11 *	[199]
A β 42	SPCE functionalized with AuNPs/mixed monolayers of PEG-SH and MPA and antibodies	Sandwich assay with ALP tagged antibody for DPV signal amplification	Buffer, Diluted human serum and Plasma	1 $\times$ 10 ⁻⁴ – 0.025	1 $\times$ 10 ⁻⁴	[200]
A β 42	SPCE modified with AuNPs and conjugate streptavidin-biotin to promote A β 42 immobilization on the surface. Recognition antibodies and ALP labeled detection antibodies were used to perform the immunoassay. Biotin-A β 42 and A β 42 compete for the recognition antibody	Competitive immunoassay using anodic stripping of enzymatically generated silver by CV	Buffer	0.110 – 110 *	0.022 *	[201]
A β 42	Carbon disposable electrochemical printed chip modified with	EIS	Buffer	0.010 – 10	0.57	[202]

	AuNPs/MHDA SAM, Protein G and antibodies					
A β 42	AAO substrate sputtered with a thin gold film functionalized with AuNPs, MUA and antibody immobilization through EDC/NHS interaction	EIS	Buffer	$2.2 \times 10^{-4} - 2.2 *$	$2.2 \times 10^{-6} *$	[203]
A β 42	AuE modified with a MPA SAM, AuNPs and chemically modified antibodies	SWV	Buffer	$2.2 \times 10^{-3} - 0.022 *$	$1.2 \times 10^{-3} *$	[149]
A β 42	GCE modified with Ru(bpy) $_3^{2+}$ caged in a 3D zinc oxalate MOFs and antibodies	Sandwich assay using antibodies conjugated with AuNPs and NiFe based nanocube MOFs - ECL	Buffer and aCSF	$2.20 \times 10^{-5} - 11.1 *$	$3.0 \times 10^{-6} *$	[204]
A β 42	Silicon wafer treated through silanization with carboxylated alkyltrichlorosilane modified with antibodies through EDC/NHS interaction	Immunoassay using a recognition and Cy5 labeled antibody -	Buffer-Tween	$550 - 1660 *$	$66 *$	[205]

		Fluorescent detection				
A β 42	Silicon microarray platform coated with poly(DMA-co-NAS-co-MAPS) modified with antibodies	Biotin-labeled secondary antibody and Cyanine 3 labelled with streptavidin - Fluorescent detection	aCSF	0.022 – 1.1 *	0.016 *	[206]
A β 42	AuNPs functionalized with antibodies against A β 42	Colorimetric sandwich immunosensor	Buffer and Serum samples	7.5 – 350	2.3	[207]
A β 42	Sputtered gold on a silicon wafer that was later modified with ODT SAM and antibody fragments	Sandwich Immunoassay using AuNPs functionalized with antibodies - STM detection	Buffer	$2.20 \times 10^{-6} - 220 *$	$2.2 \times 10^{-6} *$	[208]
A β 42	Glass slides treated with APTES and	LSPR	CSF	$1.0 \times 10^{-3} - 10^6$	1.5×10^{-3}	[209]

	absolute ethanol and modified with AuNPs modified with A β 42 and ApoE4 as chaperone		buffer			
A β 42	Waveguide-coupled bimetallic chip modified with a chloroform SAM and antibodies immobilized via a covalent bond through EDC/NHS	SPR	Buffer	0.022 – 0.44 *	0.048 *	[210]
A β 40	Interdigitated AuE modified with DTSP SAM and antibodies	EIS	Buffer	0.010 – 100	0.010	[211]
A β 40	ITO coated glass substrates modified with AuNPs and PEG	SWV	Buffer and Brain samples	100 – 500	4.78	[212]
A β oligomers	SPGE modified with biotinylated PrP ^C (95–110) peptide tethered via biotin-NeutrAvidin in POPA co-polymer	EIS	DMSO/F12 medium and Chinese hamster ovary cell line	10 ⁻³ – 1000	5.0 $\times$ 10 ⁻⁴	[213]

A β oligomers	Curcumin-Ni was electropolymerized on Ni foam substrate	EIS	Buffer and aCSF	0.0010 – 5.0	1.0×10^{-3}	[214]
A β oligomers	AuE modified with cysteine- PrP (95–110). MU was used to block the unreacted gold surface	Sandwich assay using ALP-PrP(95–110) with CV detection	Buffer and Serum samples	$5.0 \times 10^{-3} - 2$	3.0×10^{-3}	[215]
A β oligomers	AuE modified with Poly (pyrrole-2-carboxylic acid) linking agent and PrP ^C receptor	EIS	Buffer and Brain samples	$10^{-7} - 10$	10^{-7}	[216]
A β 42 oligomer	AuE modified with peptide probe (MUA – Ferrocene, I10 peptide), TCEP and MNH	SWV	Buffer and Serum samples	0.48 – 12	0.24	[217]
A β 42 oligomer	AuE modified with assembly solution (protein binding peptide and TCEP) and MNH	SWV	Buffer	0.50 – 2.5	0.048	[218]

*Value was expressed in $\mu\text{g mL}^{-1}/\text{ng mL}^{-1}/\text{pg mL}^{-1}$ and converted to nM.

AAO, Anodic aluminum oxide; ABTS-PDDA/SWCNTs, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)-poly(diallyldimethylammonium chloride)-bi functionalized single-walled carbon nanotubes composite; aCSF, Artificial cerebrospinal fluid; ALP, Alkaline phosphatase; APMES, 3-(ethoxydimethylsilyl) propylamine; APTES, 3-aminopropyltriethoxysilane; AuE, Gold electrode;

AuNPs, Gold nanoparticles; CNTs, Carbon nanotubes; CSF, Cerebrospinal fluid; CV, Cyclic voltammetry; DMSO, Dimethyl sulfoxide; DPV, Differential pulse voltammetry; DTSP, Dithiobis (succinimidyl propionate); ECL, Electrochemiluminescence; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EIS, Electrochemical impedance spectroscopy; GCE, Glassy carbon electrode; ITO, Indium tin oxide; LSPR, Localized surface plasmon resonance; MHDA, 16-mercaptohexadecanoic acid; MNH, 9-mercapto-1-nonanol; MOFs, Metal-organic frameworks; MPA, Mercaptopropionic acid; MU, 11-mercapto-1-undecanol; MUA, 11-mercaptopundecanoic acid; NHS, N-hydroxysuccinimide; NR, Non-reported; ODT, 1,8-octanedithiol; PEG, Polyethylene glycol; poly(DMA-co-NAS-co-MAPS), Ter-copolymer made with an optimised composition of dimethylacrylamide (DMA), 3-(trimethoxysilyl)propyl methacrylate (MAPS) and N-Acryloyloxy succinimide ester (NAS); POPA, Co-polymer of polytyr- amine/3-(4-hydroxyphenyl) propionic acid; SAM, Self-assembled monolayer; SPCE, Screen-printed carbon electrode; SPGE, Screen-printed gold electrode; SPR, Surface plasmon resonance; STM, Scanning tunneling microscopy; SWV, Square-wave voltammetry; TCEP, Tris(2-carboxyethyl)phosphine.

Table 5 - Configuration, analytical performance and application of the developed biosensors for Tau protein and related variants determination.

Biomarker	Transducer		Detection Method	Sample	Detection Range (nM)	LOD (nM)	References
Tau protein	384-multiwell	microelectrode array	EIS	Cell culture medium	1.00 – 100	NR	[219]
Tau protein	AuE functionalized with lipoic acid and NHS for tau protein immobilization with		EIS through analysis of tau-	MES+NaCl +EDTA	200 – 1000	200	[220]

	subsequent blocking with ethanolamine and backfilling with hexanethiol	tau binding					
Tau protein	Microband AuE covered with a layer of DTSSP SAM, protein G and antibodies	EIS	Buffer and Human serum	$1.0 \times 10^{-5} - 0.010$	3.0×10^{-5}	[221]	
Tau protein	AuE functionalized with antibodies through NHS coupling	EIS	Buffer	NR	1100 *	[222]	
Tau protein-381	AuE modified with MPA SAM and antibodies	Sandwich immunoassay with aptamer-CS-AuNPs conjugate - DPV	Buffer and Human serum	$5.0 \times 10^{-4} - 0.10$	4.2×10^{-4}	[223]	
Tau protein	SPCE with graphene oxide and amine functionalized 1 st generation trimethylolpropane tris[poly(propyleneglycol)] (pPG) dendrimers and antibodies for simultaneous detection of Myelin Basic and Tau proteins	Sandwich immunoassay with antibody conjugated carboxyl functionalized 3.5th generation	CSF and Serum from Multiple Sclerosis patients	0.250 – 250	0.150	[224]	

		pPG/CdS and pPG/PbS probes - DPV				
Tau protein	Chromium and gold deposited on glass slides that were functionalized with a DDA SAM, amino-surface-modified silica nanoparticles, gold layer, protein G and antibodies	LSPR	Buffer	$1.7 \times 10^{-4} - 1.7 *$	$1.7 \times 10^{-4} *$	[225]
t-Tau p-Tau	AuE modified with MUA SAM and antibodies	SPR	Buffer and Human serum	t-Tau) $2.2 \times 10^{-4} - 0.051 *$ p-Tau) $8.18 \times 10^{-3} - 3.57 *$	t-Tau) $5.3 \times 10^{-7} *$ p-Tau) $1.3 \times 10^{-3} *$	[226]
Tau Protein-381	Gold chip functionalized with mixed SAMs of MUA and MUD and DNA aptamer probes	Sandwich assay using antibodies for Tau protein – SPR	Plasma	NR	1.0×10^{-5}	[227]
Tau protein	Prism coupler modified with gold and antibodies	Sandwich immunoassay with antibodies	aCSF	0.13– 1.0	0.13	[228]

		coupled with MWCNTs - SPR				
Tau protein	Gold surfaces modified with a MU SAM and antibodies	1) Direct and 2) sandwich immunoassays: 2.1) antibody and 2.2) tubulin - QCM	aCSF	1) 0 – 250 2.1) 0 – 250 2.2) 0 – 500	1) 42 2.1) 10 2.2) 10	[229]

*Value was expressed in $\mu\text{g mL}^{-1}/\text{ng mL}^{-1}/\text{pg mL}^{-1}$ and converted to nM.

aCSF, Artificial cerebrospinal fluid; AuE, Gold electrode; AuNPs, Gold nanoparticles; CS, Cysteamine; CSF, Cerebrospinal fluid; DDA, 4,4'-dithiodibutyric acid; DPV, Differential pulse voltammetry; DTSSP, 3,3'-dithiobis(sulfosuccinimidyl propionate); EDTA, Ethylenediamine tetraacetic acid; EIS, Electrochemical impedance spectroscopy; LSPR, Localized surface plasmon resonance; MES, 2-(N-Morpholino) ethanesulfonic acid; MU, 11-mercapto-1-undecanoic acid; MUA, 11-mercaptopundecanoic acid; MUD, 11-mercaptopundecanol; MWCNTs, Multi-walled carbon nanotubes; NHS, N-hydroxysuccinimide; NR, Non-reported; p-Tau, Phosphorylated tau; QCM, Quartz crystal balance; SPCE, Screen-printed carbon electrode; SPR, Surface plasmon resonance; t-Tau, Total tau.

Table 6 - Configuration, analytical performance and application of the developed biosensors for ApoE determination.

Biomarker	Transducer	Detection Method	Sample	Detection Range (nM)	LOD (nM)	References
ApoE	SPCE modified with magnetic beads functionalized with antibodies	Sandwich immunoassay through iridium oxide nanoparticles with secondary antibodies - CA	Buffer and Human plasma	2.94 – 29.4 *	2.0 *	[230]
ApoE DNA	GCE modified with graphene-mesoporous silica hybrids coupled with methylene blue, ferrocenecarboxylic and DNA probe	DPV ratiometric signal detecting target DNA	Buffer	$1.0 \times 10^{-5} - 10$	1.0×10^{-5}	[231]
ApoE	Graphite ink (microfluidic platform) modified with magnetic beads functionalized with antibodies	Sandwich immunoassay with biotinylated antibodies and streptavidin/QD conjugate -	Buffer and Diluted human plasma	0.29 – 5.9 *	0.37 *	[232]

					Square Wave Anodic Stripping Voltammetry				
ApoE4	ITO	electrode	modified	with	Sandwich	Buffer	0.0290 – 290 *	8.8×10^{-3} *	[233]
		polyelectrolytes, electrodeposited fractal gold nanostructures and antibodies			immunoassay with HRP-labeled antibodies - CA				
ApoE4 DNA	ITO	modified	with	graphene-QD conjugates, electropolymerized curcumin and DNA probe	DPV and Fluorescence detection	Buffer and Human blood plasma	DPV) 20 – 400 ** Fluorescence detection) 45 – 400 **	DPV) 2.18 ** Fluorescence detection) 12.4 **	[234]
ApoE DNA	ITO	modified	with	AuNPs and oligonucleotides	LSPR and EIS	Buffer	NR	LSPR) 512 EIS) 286	[235]
ApoE	ITO	modified	with	APTMS,	Sandwich assay	Buffer and	2.90×10^{-5} – 0.290	1.2×10^{-5} *	[236]

glutaraldehyde, ionic liquid (BMIMBF ₄)- chitosan, AuNPs and antibodies	using Au-TiO ₂ , GOx, and anti-ApoE – Colorimetric detection using glucose	Serum	*
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*Value was expressed in $\mu\text{g mL}^{-1}$ / ng mL^{-1} / pg mL^{-1} and converted to nM.

** The results were not possible to be converted to molar units thus being displayed in pg mL^{-1} .

APTMS, 3-aminopropyltrimethoxysilane; AuNPs, Gold nanoparticles; BMIMBF₄, 1-butyl-3-methyl-imidazolium tetrafluoroborate; CA, Chronoamperometry; DNA, Deoxyribonucleic acid; DPV, Differential pulse voltammetry; EIS, Electrochemical impedance spectroscopy; GCE, Glassy carbon electrode; GOx, Glucose oxidase; HRP, Horseradish peroxidase; ITO, Indium tin oxide; LSPR, Localized surface plasmon resonance; NR, Non-reported; QD, Quantum dots; SPCE, Screen-printed carbon electrode; SPR, Surface plasmon resonance.

Table 7 – Configuration, analytical performance and application of the developed biosensors for multiplexed detection of AD biomarkers.

Biomarker	Transducer	Detection Method	Sample	Detection Range (nM)	LOD (nM)	References
A β 40 A β 42	AuE modified with MPA SAM and antibodies which were later conjugated with A β (1–16)-heme-AuNPs	Competitive assay - CV	Buffer and aCSF	0.0200 – 150	0.010	[237]
A β 42 t-A β	AuE modified with MPA SAM, antibodies and biotinylated A β /streptavidin-conjugated ALP	Competitive amperometric detection using p-aminophenol redox cycling	Buffer and aCSF	A β 42) 0.50 – 50 t-A β) 0.050 – 5.0	5.0×10^{-3}	[238]
A β 40/A β 42	GCE modified with MWCNTs, AuNPs and Gelsolin	Sandwich Immunoassay using AuNPs modified with gelsolin and HRP for DPV signal amplification	Buffer, CSF and brain tissue of rats	0.10 – 50	0.028	[239]
A β 40	Triple barrel carbon fiber microelectrodes	SWV –	Mice CSF	A β 40) 20-50	20	[240]

A β 42	functionalized with antibodies through EDC/NHSS coupling	Oxidation of A β through a Tyrosine residue		A β 42) 20-140		
A β 40	Microelectrode modified with conductive	Linear Sweep	Buffer and	A β 40) 2.1×10^{-3} –	A β 40)	[241]
A β 42	silk fibroin based immunoparticles through one-step electrophoresis/electropolymerization	Voltammetry	Serum	0.52 *	1.53×10^{-3} *	
				A β 42) 4.9×10^{-3} –		
				0.25 *	A β 42)	
					8.8×10^{-4} *	
A β 40/A β 42	Au films coated onto glass slides functionalized with mixed SAM composed of carboxyl- and hydroxyl-terminated PEG molecules, streptavidin and biotinylated antibodies	Sandwich Immunoassay through the conjugate preformed between biotinylated A β (1-16) antibody and streptavidin - SPR	Buffer and CSF	0.020 – 1.0	0.020	[242]

Aβ40/Aβ42	AgNPs modified with antibodies against Aβ40/Aβ42	Colorimetric detection through coupling of modified AgNPs with Cu ²⁺	Buffer and Human blood serum	0.250 – 150	0.086	[243]
Aβ40 Aβ42 Tau Protein	APTES-coated glass modified with immuno-PEG-AuNPs functionalized with antibodies through EDC/NHS	LSPR	Dulbecco’s PBS mixed with human plasma samples	1.00×10 ⁻⁵ – 100	Aβ40) 3.5×10 ⁻⁵ ; Aβ42) 2.6×10 ⁻⁵ ; Tau protein) 2.4×10 ⁻⁵	[244]
Aβ ApoE	SPCE modified with porous magnetic microspheres functionalized with antibodies through EDC/NHS, antigen and AuNPs functionalized with antibodies through adsorption due to the presence of cysteine	Sandwich immunoassay - CA	Buffer, CSF, serum and plasma samples of AD patients	Aβ) 4.4×10 ⁻³ – 2.8 * ApoE) 2.9×10 ⁻³ – 0.37 *	Aβ) 4.2×10 ⁻³ * ApoE) 2.4×10 ⁻³ *	[245]

1) H ₂ O ₂ ; A β	Glass microscope slides coated with sol-gel derived optical array modified with:	Fluorescent detection	3-bis [tris (hydroxy methyl) methylamino] propane buffer and Human serum	1) H ₂ O ₂) 100 – 10 ⁷ ; A β) 2.2 – 22200 *	1) 1.57; 0.63	[246]
2) Glutamate; Acetylcholine	1) Amplex red and FITC-dextran with HRP for H ₂ O ₂ and A β detection 2) Glutamate dehydrogenase and acetylcholinesterase combined with carboxy SNARF-1-dextran for glutamate and acetylcholine detection			2) Glutamate) 5000 – 5 $\times$ 10 ⁶ ; Acetylcholine) 5000 – 9 $\times$ 10 ⁶	2) 530; 1020	

*Value was expressed in $\mu\text{g mL}^{-1}/\text{ng mL}^{-1}/\text{pg mL}^{-1}$ and converted to nM.

aCSF, Artificial cerebrospinal fluid; AD, Alzheimer's disease; AgNPs, Silver nanoparticles; ALP, Alkaline phosphatase; APTES, 3-aminopropyltriethoxysilane; AuE, Gold electrode; AuNPs, Gold nanoparticles; CA, Chronoamperometry; CSF, Cerebrospinal fluid; CV, Cyclic voltammetry; DPV, Differential pulse voltammetry; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EIS, Electrochemical impedance spectroscopy; FITC, Fluorescein isothiocyanate; GCE, Glassy carbon electrode; HRP, Horseradish peroxidase; LSPR, Localized surface plasmon resonance; MPA, Mercaptopropionic acid; MWCNTs, Multi-walled carbon nanotubes; NHS, N-hydroxysuccinimide; NHSS, N-hydroxysulfosuccinimide; PBS, Phosphate buffered saline; PEG, Polyethylene glycol; SAM, Self-assembled monolayer; SPCE, Screen-printed carbon electrodes; SPR, Surface plasmon resonance; SWV, Square-wave voltammetry; t-A β , Total A β .

4.1.2.1. A β and related variants

A lot of studies focus their efforts in A β detection (Table 4) as this peptide is considered to be the main pathological feature of AD. A β 42 is the sequence more prone to aggregate thus being the peptide with more expression in the amyloid plaques making it a very interesting and important biomarker to study AD onset and progression. From Table 4, it is possible to conclude that a large number of the developed biosensors were able to successfully quantify A β 42 below the cut-off value of 110-155 pM (500-800 pg mL⁻¹) in CSF [162, 206], but colorimetric [207] and fluorescent [205] detection systems were not able to attain limits of detection (LODs) as low as the other systems. In addition, it is important to point out that five works were able to attain extremely low LODs achieving values lower than dozens of fM (Table 4) [197, 203, 204, 208, 216]. Three of these studies [197, 203, 216] determined trace level concentrations of A β 42 through analysis of impedance variation.

Yoo et al. [197] developed a biosensor based on a microfluidic platform with interdigitated microelectrodes that comprised an ion concentration polarization preconcentration element that increased the local concentration of analytes and consequently improved the determination of A β 42. The monoclonal antibodies were immobilized on the polyvinyl pyrrolidone-aldehyde and glutaraldehyde modified surface via interaction with aldehyde groups. Through changes in the impedance signal, this group was able to measure concentration levels as low as 2.2×10^{-3} – 221 pM achieving a LOD of 8.15 fM in buffer. Despite achieving very interesting detection levels for AD diagnosis, the performance of this biosensor in terms of stability and specificity was not evaluated. Wu et al. [203] attained a LOD of 2.2 fM for A β 42 determination via EIS using a nanostructured label-free biosensing platform based on an AAO substrate modified with uniformly deposited AuNPs and 11-mercaptopundecanoic acid self-assembled monolayer (SAM) that was further functionalized with antibodies via EDC/NHS interaction [203]. The high surface-to-volume ratio of the AuNPs enabled the immobilization of more antibodies onto the electrode. For detection of A β 42, this team [203] used a 30 μ L sample volume with a detection period of 30 min attaining a linear detection range between 0.22–2200 pM in buffer. Despite achieving an extremely low LOD, the stability of the biosensor over time as well as its performance in real samples were not reported. Another very sensitive electrochemical biosensor for A β oligomers determination was designed by Qin et al. [216]. In this work, the

developed biosensor was based on an AuE modified with an electrically conductive polypyrrole containing carboxyl groups for linking of the biorecognition agent, an amine modified PrP^C while also promoting the electron transfer for the electrochemical analysis. Applying the developed biosensor for A β oligomers detection, a 0.1 fM LOD was reached through EIS, while differentiating A β oligomers from monomers and fibrils, in addition to providing reliable performance in brain samples of AD infected mice [216]. Moreover, the developed electrochemical immunosensor presented no significant changes in signal after one month storage at 4 °C. In a different electrochemical approach, Zhao et al. [204] developed a dual quenching electrochemiluminescence (ECL) immunosensor which was based on a Ru(bpy)₃²⁺ chromophore coupled with zinc oxalate metal-organic frameworks (MOFs) and a second MOFs with a nanocube structure composed of AuNPs-NiFe that acted as the dual quencher for sensitive detection of A β 42 (Figure 37). In this work, the Ru(bpy)₃²⁺ cations were entrapped in a zinc oxalate MOFs with a 3D cage structure with the goal of increasing the energy transfer between Ru(bpy)₃²⁺ units while shielding the electronic structure of the chromophore which led to high-energy Ru emission efficiency [204]. This way, for the immunosensor development Ru(bpy)₃²⁺/zinc oxalate MOFs were conjugated with antibodies via EDC/NHS interaction being the bioconjugate further immobilized on the surface of a GCE. After the A β 42 biorecognition process another bioconjugate composed of AuNPs-NiFe MOFs was added to the modified transducer to complete the sandwich immunoassay. The AuNPs-NiFe acted as the dual quencher as the UV-Vis absorption spectra of this composite overlapped with the ECL emission spectra of the Ru(bpy)₃²⁺/zinc oxalate MOFs which would trigger the resonance energy transfer behavior achieving a dual quenching effect of the encapsulated Ru(bpy)₃²⁺ and significantly increasing the sensitivity of the immunosensor [204]. With the developed strategy A β 42 was detected in the 0.022 – 1110 pM range while achieving a 3.0 fM LOD in buffer in addition to successfully analyzing the immunosensor in artificial CSF. Nonetheless, the stability of the immunosensor over time should be further tested. At last, Kang et al. [208] developed a vertically configured electrical detection system based on a different technique, namely STM, to evaluate a sandwich immunoassay for A β 42 determination [208]. A biosurface was fabricated containing monoclonal antibody fragments, target protein and AuNPs-antibody conjugates for ultra-sensitive detection of A β 42. The electrical tunneling current between the STM tip and the formed immunocomplexes exhibited a peak-like pulse, the frequency of which depended on the

density of the bound complexes on the surface which enable the detection of levels as low as 2.2 fM in buffer. Nevertheless, this highly sensitive biosensor needs further optimizations related with stability and specificity in order to assess its viability for clinical application [208].

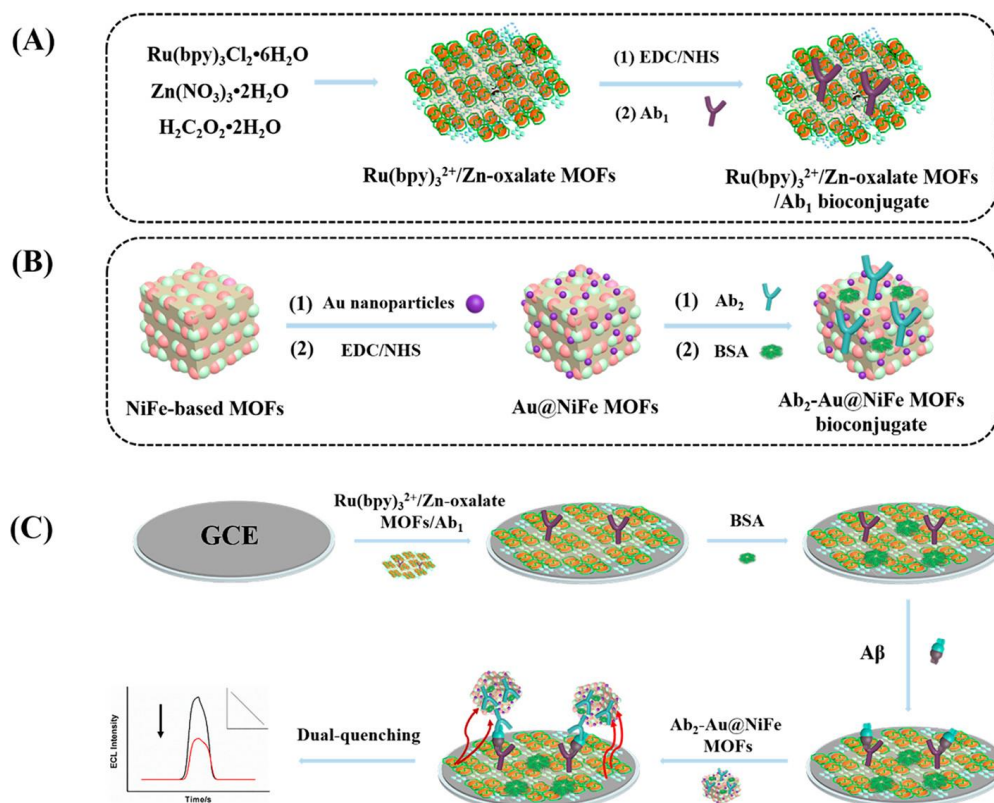


Figure 37 – (A) Preparation of Ru(bpy)₃²⁺/Zinc Oxalate MOFs/Ab₁ Bioconjugate and (B) Ab₂-Au@NiFe MOFs Bioconjugate; (C) Construction Process of the Proposed Dual-Quenching ECL Immunosensor [204].

Biosensors based on optical determination, particularly LSPR [209] and SPR [210], have also been applied to detect and measure Aβ₄₂ levels in biological fluids. Using LSPR, Kang et al. [209] performed Aβ₄₂ quantification under physiological conditions. The developed system was a label-free biosensor based on AuNPs and ApoE4-mediated Aβ aggregation, where AuNPs acted as a catalytic activator and optical reporter while ApoE4 acted as a chaperon to monitor Aβ₄₂ aggregation (Figure 38), attaining a LOD of 1.5 pM [209]. Moreover, this work also demonstrated that ApoE4 induces Aβ₄₂ aggregation more specifically and rapidly than that of Aβ₄₀. In another work for Aβ₄₂ quantification via SPR, Lee et al. [210] developed a biosensor based on a waveguide-coupled bimetallic chip (WcBiM) modified with antibodies via EDC/NHS covalent

binding. The line width of the reflectance for the WcBiM chip was narrower than the line width of the conventional Au chip resulting in a steeper gradient to maximize the resolution in the intensity detection mode. Application of this biosensor led to A β 42 determination in the 22.2 – 443.1 pM range, although presenting limitations for detecting trace levels concentrations. In addition, both these studies did not evaluate the biosensors performance after exposure to real samples despite the first study conducting the entire work in mimicked CSF.

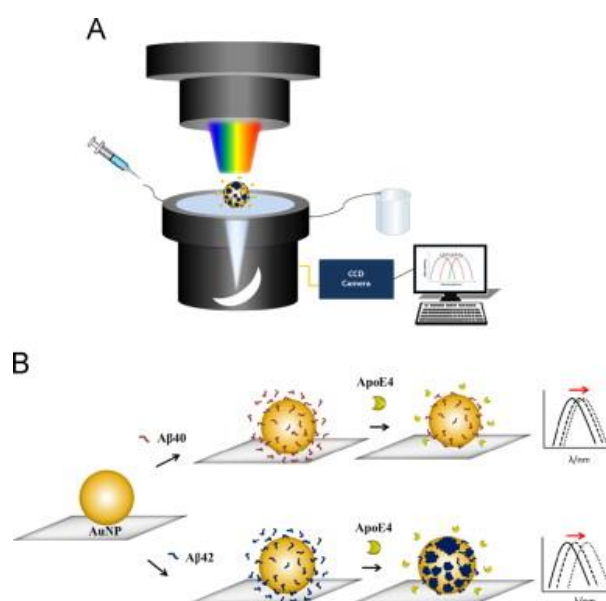


Figure 38 - (A) Schematic of the overall process describing the system setup by LSPR. (B) Experimental process for binding affinity [209].

In recent studies, with the goal of overcoming some limitations related with the use of antibodies towards detection of biomarkers particularly in complex samples such as CSF and blood, biosensors were developed based on electrochemical determination towards A β detection without biological recognition elements. This way, Zhu et al. [255] developed an antibody-mimetic self-assembling peptoid nanosheet containing surface exposed A β 42 recognizing loops which enabled a sensitive and label-free detection of AD in serum. In a different work, Qin et al. [256] prepared a ferrocene-encapsulated Zn zeolitic imidazole framework (ZIF-8) by self-assembly of Zn ions and 2-methylimidazole for detection of A β oligomers. Ferrocene, an optically and electrochemical active signal, was successfully encapsulated inside of the ZIF-8 and released by the competitive coordination between Zn ions and A β oligomers [256].

4.1.2.2. Tau Protein

Electrochemical biosensors have also been the most applied for determination of tau protein [219-224] (Table 5). On the other hand, five works displayed in Table 5 were based on different techniques namely, LSPR [225], SPR [226-228] and QCM [229]. Clinical application of tau protein can be very challenging as its cut-off value in CSF is 4.3 pM [221, 225, 257]; of the reported eleven studies, five biosensors (two electrochemical [221, 223] and three optical [225-227]) fulfilled this requirement.

Applying EIS for determination of full-length 2N4R tau protein, Wang et al. [221] were able to measure values in the fM range exhibiting better performance when compared to enzyme-linked immunosorbent assays (ELISA) by achieving a 30 fM LOD with a detection period of 25 min (Figure 39). The developed biosensor was based on four gold microband electrodes modified with a layer of 3,3'-dithiobis(sulfosuccinimidyl propionate) and protein G for immobilization of antibodies with proper orientation [221]. In another electrochemical approach for tau protein-381 detection in human serum, Shui et al. [223] developed a sandwich assay based on the interaction between tau protein and respective antibodies and aptamers, performing detection in the 0.5 - 100 pM range and attaining a LOD of 0.42 pM with a 45 min incubation period at room temperature. Antibodies were immobilized on a mercaptopropionic acid SAM modified gold electrode while the aptamers were conjugated with gold nanoparticles to complete the sandwich assay. AuNPs played a dual role of enhancing the electrochemical behavior of the biosensor, for a more sensitive determination of tau protein, while offering a suitable matrix for the immobilization of aptamers. Both these biosensors were successfully tested in human serum spiked with tau protein but to confirm their applicability to clinical analysis, their performance over time should be evaluated.

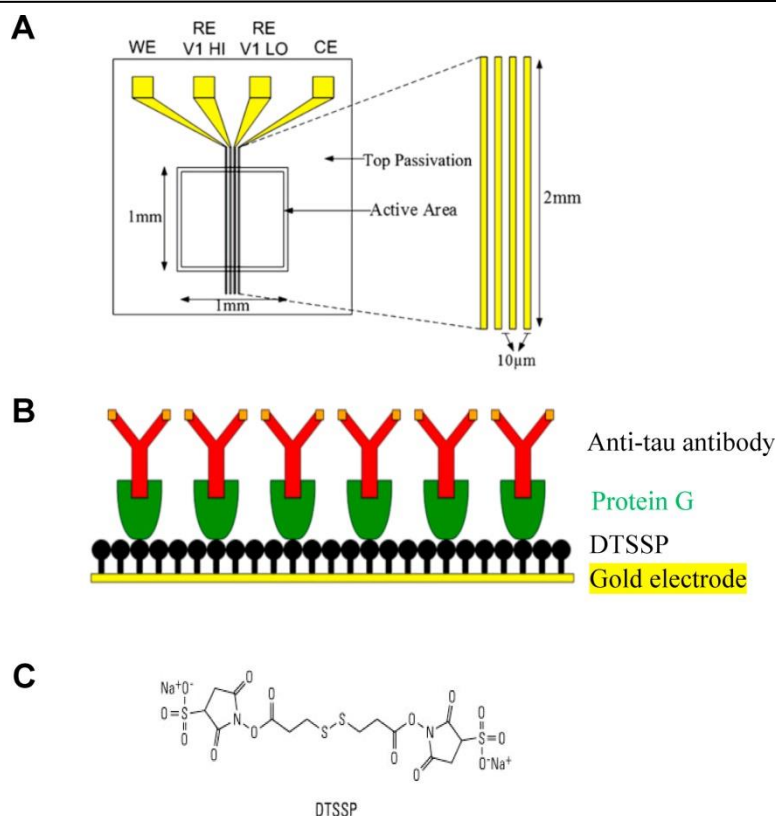


Figure 39 - (A) Details of the purchased gold microband electrodes. It shows the 1×1 mm active sensor area and the connections to the impedance analyser. (B) Schematic side view of the biosensor. From bottom to top: gold microband electrode, SAM layer, protein G layer and antibodies. (C) Chemical structure of DTSSP which contains an amine-reactive *N*-hydroxysulfosuccinimide (sulfo-NHS) ester at each end and forms the SAM layer [221].

Three biosensors based on LSPR and SPR exhibited detection limits lower than tau protein physiological level [225-227]. A method centered on the conjugation of affinity-based biosensing and LSPR multi-spot nanoparticle chip was employed for the detection of tau protein at room temperature [225]. In this work, Vestergaard et al. [225] modified a glass chip with a chromium and gold layer that was further functionalized with a 4,4-dithiodibutyric acid SAM, amino-surface modified silica nanoparticles, another gold layer to cap the nanoparticles and finally protein G to promote the antibodies immobilization with proper orientation. With this method, the authors were able to attain a 0.17 pM LOD in buffer. Despite achieving a low detection limit, this work involved longer incubation periods and the biosensor performance was not evaluated in real samples. In a more recent study, Thi Vu Nu et al. [226] presented an immunoassay for the detection of both total tau and phosphorylated tau protein based on a gold coated fiber core functionalized with an 11-mercaptoundecanoic acid SAM, where the carboxylic groups were activated via EDC/NHS interaction to promote

immobilization of the respective antibodies. Using SPR, detection limits of 0.53 fM for total tau and 1.3 pM for phosphorylated tau were achieved in addition to determining its levels in human blood. Moreover, this study concluded that the concentration of total tau protein in AD groups was six times higher than that of controls while phosphorylated tau was three times higher [226]. This difference of concentration between the two types of tau protein was attributed to the fact that unphosphorylated tau is more likely to be produced in blood than the phosphorylated protein [226]. In another work based on a multichannel SPR platform, Kim et al. [227] introduced a DNA aptamer/antibody pairing sandwich assay specific for tau-381 and applied it for detection of human Tau in undiluted human plasma quantifying levels within the range 2 – 80 pM and attaining a detection limit of femtomolar concentration. The DNA aptamer was immobilized on a gold chip modified with a mixed monolayer of 11-mercaptopundecanoic acid and 11-mercaptopundecanol through EDC/NHSS covalent linking. After tau protein specific adsorption, antibodies were flowed through the modified gold chip surface leading to the formation of a sandwich immunocomplex which increased the SPR detection signal. Both these studies [226, 227] did not address the period of stability of the developed biosensors, which is a crucial parameter to ensure that a biosensor can perform with *ca.* the same efficiency over time.

4.1.2.3. ApoE

ApoE is another target biomarker for which mostly electrochemical biosensors have been developed; only one work was based on a different technique (colorimetric detection) (Table 6). Three of the electrochemical biosensors determined ApoE through evaluation of DNA hybridization at a specific point mutation in the ApoE gene [231, 234, 235] and two of these works used two techniques to study this event. Mars et al. [234] used DPV and fluorescent detection while Cheng et al. [235] performed its evaluation through EIS and LSPR. In both cases, the biosensors performance was better when evaluated through electrochemical techniques demonstrating once again their high sensitivity. Beyond DPV and EIS, other electrochemical techniques have been efficient for determination of biomarkers such as square wave anodic stripping voltammetry, which was applied by Medina-Sánchez et al. [232] for ApoE detection. The developed biosensor consisted on a microfluidic system with integrated SPEs where the immunocomplex was performed using magnetic beads for a preconcentration step and

QDs as labeling carriers [232]. QDs are semiconductor nanoparticles which have been gaining increasing relevance in the biosensing field, mainly applied with a labelling function, due to their optical properties and stability in addition to presenting interesting electrochemical properties [53, 59, 232]. Therefore, magnetic beads were coupled with antibodies and immobilized on the electrode surface by using a neodymium magnet placed underneath the substrate. Then, biotinylated ApoE was flushed through the magnetic beads followed by QDs conjugated with streptavidin to complete the magneto immunocomplex. Applying an incubation period of 10 min, this system attained a LOD of 0.37 nM with a linear range from 0.29–5.9 nM through measurement of the QDs signal, while also demonstrating high accuracy in diluted human plasma [232]. Stability assays should be further carried out as this is a crucial parameter.

The only work that did not perform ApoE determination through electrochemical techniques was reported by Ren et al. [236] that presented a colorimetric immunosensor with one of the lowest LODs (12 fM) being its performance evaluated in buffer and human serum (Table 6). The biosensor was based on a sandwich assay and was developed in a layer-by-layer approach [236]. Firstly, the ITO surface was modified with a 3-aminopropyltrimethoxysilane and glutaraldehyde conjugate, which exhibited a brick-red color that faded when oxidized by H_2O_2 . Then, a second conjugate composed of an ionic liquid (BMIMBF_4) and chitosan was incubated in the surface to stabilize the first layer. Lastly, to promote the immobilization of the antibodies through amine binding, AuNPs were added to the modified ITO surface. To complete the sandwich assay, a nanocomposite of AuNPs/ TiO_2 modified with antibodies and glucose oxidase was used to perform the biomarker quantification [236]. As a final step, glucose was dropped on the immunosensor surface being catalyzed by the glucose oxidase to produce H_2O_2 promoting the respective color fading that was further evaluated through an image processing software by performing quantification of its mean gray value. Despite displaying good analytical behavior, further assays related to reproducibility and stability parameters need to be carried out.

4.1.2.4. Multiplexed detection

As already referred, AD is affected by multifactorial pathways that lead to changes at biomolecular levels as the disease progresses [2, 163, 171]. Thus, an accurate diagnosis of AD should arise from the evaluation of a combination of core biomarkers

such as the quantification of A β 42 and A β 40 and respective assessment of their concentration ratio [163], in conjunction with determination of tau protein or ApoE or even both [163]. In this context, seven works (Table 7) reported the development of biosensors for the determination of A β 40 and A β 42; as for the individual biomarker analysis, electrochemical detection [237-241] was preferred over other strategies [242, 243]. All of the seven works described in Table 7 for A β 40/A β 42, with exception for one [240], were able to attain LODs in the pM range; in addition, five works also evaluated the performance of the developed biosensors in real samples [239-243]. From the seven biosensors, three performed determination of A β 40/A β 42 through a competitive assay [237, 238, 241], two via a sandwich assay [239, 242] and two accomplished direct determination of the analytes [240, 243].

A competitive immunoassay using *p*-aminophenol (*p*-AP) redox cycling was described by Liu et al. [238] for detection of both A β 42 and total A β . Specifically, the interaction between biotinylated A β peptides already captured by the antibodies immobilized on the mercaptopropionic acid modified gold electrodes and the conjugate streptavidin- modified alkaline phosphatase (SA-ALP) induced the production of electrochemically active *p*-AP from the *p*-aminophenyl phosphate (*p*-APP) substrate (Figure 40) [238]. The proposed electrochemical immunosensor was able to detect levels of 5 pM for both A β 42 and total A β . Moreover, the authors [238] concluded that artificial cerebrospinal fluid (aCSF) had no interference in the analysis. Yu et al. [239] by means of a sandwich-type biosensor array was able to simultaneously quantify A β 40/A β 42, developing a transducer platform based on a GCE modified with MWCNTs, AuNPs and using gelsolin as the biorecognition element, which is a secretory protein with high specificity towards A β monomers. MWCNTs and AuNPs due to their excellent conductivity and electrochemical properties enhanced the electrochemical behavior of the biosensor by increasing the charging currents in addition to providing an appropriate environment for the immobilization of gelsolin. AuNPs were also used for signal amplification by coupling them with gelsolin and horse-radish peroxidase (HRP) to complete the sandwich immunoassay. Electrochemical signal was amplified via enormous loading of HRP on the AuNPs which could catalyze the substrate 3,3',5,5'-tetramethylbenzidine in the presence of H₂O₂ to produce measurable redox currents [239]. Using the proposed biosensor, the research team [239] was able to attain a 28 pM LOD in spiked buffer in addition to successfully determine A β 40/A β 42 in CSF and in three different brain tissues of rats,

observing a clear decline in the concentrations of soluble A β 40/A β 42 of AD rats as a result of A β aggregation. Despite exhibiting sensitive and specific determination of A β 40/A β 42, the proposed biosensor requires long incubation periods while displaying only two weeks stability when stored at 4 °C. In a different approach, Liu et al. [241] developed a hierarchical immunoelectrochemical interface based on a microelectrode nanostructured with conductive silk fibroin (SF)-based immunoparticles via an one-step electrophoresis/electropolymerization. Antibody conjugation was promoted through the interactions with the hydrophilic N-terminal sites on the surface. This nanostructured conductive interface favored penetration of water-soluble biomolecules and catalyzed a redox reaction of Met(35) providing detection limits of 0.88 pM and 1.53 pM for A β 42 and A β 40, respectively. Moreover, the developed biosensor was successfully tested in real serum samples while only displaying two weeks stability when stored at 4 °C [241].

Low levels of A β 40/A β 42 were also detected by Xia et al. [242] via SPR. This work had the advantage of simultaneously, but separately, quantifying both peptides from CSF of AD patients by pre-immobilizing the respective antibodies onto two separate fluidic channels minimizing, this way, the non-specific determination. Signal amplification was achieved by the formation of a conjugate between streptavidin and an antibody selective to the common N-terminus of A β peptides that were previously captured, leading to the measurement of concentrations as low as 20 pM. Furthermore, this study [242] revealed that the ratio between the concentrations of A β 40 and A β 42 in CSF samples from AD patients is almost twice as high as that from healthy persons. Comparing to more traditional methods such as ELISA, this method offered significant advantages by presenting faster analysis time, obviation of an enzyme-linked antibody for signal detection and regeneration of the biosensor surface for replicate measurements [242].

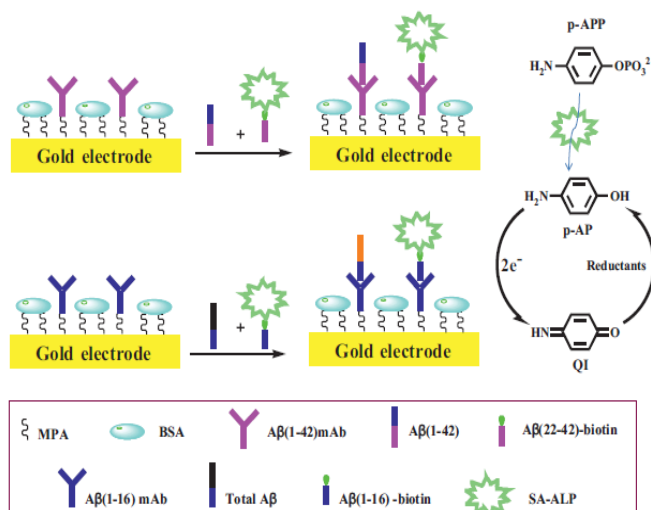


Figure 40 - Schematic representation for the detection of A β (1–42) and total A β using p-amino phenol redox cycling by chemical reductants [238].

Despite exhibiting excellent performance through detection of low levels of A β , some of the works displayed [237-239] in Table 7 present the common disadvantage of lack of specificity of the biological recognition elements. Half of the designed biosensors for both A β 40 and A β 42 detection used antibodies capable of recognizing a broad range of amyloid beta peptides. Thus, the development of biorecognition elements capable of distinguishing different variants of a protein is a topic that requires more focus as it will contribute to better and more accurate diagnostic procedures.

For quantification of the AD core biomarkers, Kim et al. [244] using LSPR presented a highly selective shape-code biosensor based on a single platform modified with AuNPs of different shapes (Figure 41). These AuNPs were previously modified with polyethylene glycol (PEG) that through EDC/NHS interaction promoted stable antibody immobilization. AuNPs of different shapes and sizes, such as spheres (diameter of 50 nm), short rods (aspect ratio of 1.6) and long rods (aspect ratio of 3.6), exhibit different optical behaviors which could be identified and differentiated through LSPR as if a bar-code was being scanned. Applying a 1 hour incubation period and under physiological conditions, this research team [244] reported a detection limit of 34.9 fM for A β 40, 26 fM for A β 42 and 23.6 fM for tau protein. A different conjugation of AD biomarkers was determined by de la Escosura-Muñiz et al. [245] that developed a biosensor based on a magnetosandwich-immunoassay for A β and ApoE determination in CSF, plasma and serum. In this work, porous magnetic microspheres were used for the antibody immobilization, which due to their inherent characteristics, allowed for a

more efficient capture of the analyte while also providing a high active area for enhanced catalytic activity of the conjugate detection-antibody/AuNPs [245]. AD biomarkers were added to the microspheres modified with the capture antibodies, being the detection performed through the conjugate detection antibody/AuNPs. The magnetic conjugate suspension was placed on the surface of a SPCE and analyzed through chronoamperometry presenting a LOD of 4.2 pM for A β and 2.4 pM for ApoE. The detection process involved two immunoreaction stages that were carried out for 20 min at 25 °C each.

The possibility of performing detection of multiple biomarkers in a single platform brings the proposed biosensors closer to real clinical diagnosis being the focus of recent studies [258]. Moreover, despite centering primarily on the main biomarkers of AD such as A β 42, A β 40 and t-tau and p-tau, other studies have also performed detection of multiple analytes that extend beyond this main core of biomarkers such as A β and phosphorylated A β [259], tau protein and alpha-1-antitrypsin [260, 261], A β , glutamate and acetylcholine [246].

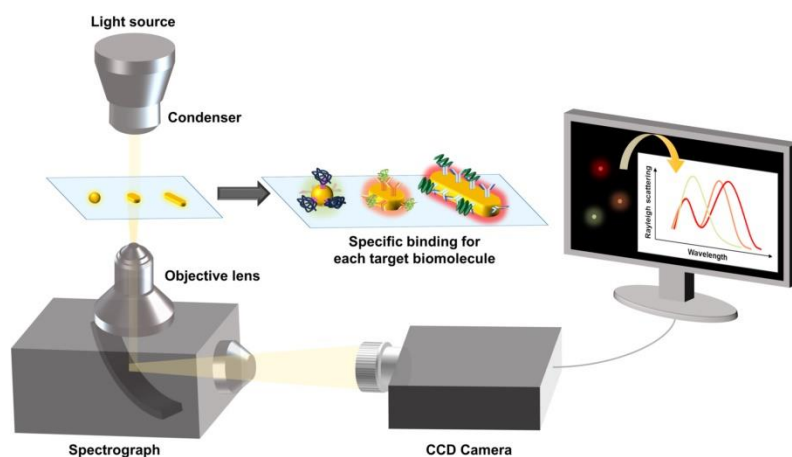


Figure 41 - Schematic illustration of shape-code plasmonic biosensor for multiple Alzheimer's disease biomarkers [244].

4.2. An antifouling matrix for affinity-based electrochemical biosensing of A β 42

As reported in the previous chapter, AD is the most common neurodegenerative disease with a prevalence expected to keep increasing in the upcoming decades as a result of the continuous aging of the world population. The pathogenesis of AD involves a cascade of interacting events, the core of which include the aggregation of the peptide A β 42 leading to the formation of extracellular deposits and the formation of

intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau protein. Recently, substantial progress has been made in identifying PET and CSF biomarkers for AD, but their use is limited by invasiveness of procedures and high costs, restricting their use as front-line diagnostic tools [262]. On the other hand, longitudinal and cross-sectional studies have stated that the levels of A β 42, phosphorylated tau protein, and total tau protein start to change almost 10–15 years before the appearance of AD symptoms. Therefore, it is of crucial importance to develop novel sensing approaches capable of detecting low levels of these biomarkers in more accessible biological fluids such as blood.

From the previous chapter, it is possible to conclude that a large number of surface chemistries have been reported in the scientific literature to address this topic attaining successful analytical behavior towards detection and quantification of low levels of these biomarkers [123]. Nonetheless, the commercialization and application of these biosensors in real clinical settings has been limited by their inability to maintain functionality when exposed to biological fluids [263]. In fact, biofouling can hinder or completely prevent analyte from reaching the sensing surface or can drastically impact the resistive or capacitive properties of the sensing elements, therefore compromising the biosensors analytical performance [264]. Electrochemical biosensors inability to resist non-specific binding and maintain sensing functionality has been a common reported problem. In fact, from the previous chapter is possible to conclude that most biosensors did not evaluate the analytical performance of the developed biosensors in biological samples, while the works that conducted these studies had to dilute the samples to minimize the biofouling effect. Since non-specific binding to surfaces is unavoidable it is important to develop effective strategies to minimize this effect. The most common approaches involve the use of blocking agents (e.g. BSA), surface engineering (e.g. long chain organic layers) or sample pretreatment (i.e. dilution, depletion or enrichment) [265]. Moreover, a common limitation of the first two antifouling strategies is that despite being effective in preventing protein adsorption, they lead to the formation of a high-impedance layer, which prevents faradaic electrochemistry from occurring with the transducer.

In this work, an antifouling matrix was developed to allow affinity biosensing towards detection of A β 42. This way, this chapter is organized in two parts. Firstly, two different approaches were studied in order to evaluate the most efficient and stable coating to minimize the events of non-specific binding while also maintaining surface

functionality. Secondly, an immunosensor was developed based on the optimized antifouling coating for the detection of A β 42. This immunosensor was based on a sandwich detection scheme with colloidal AuNPs acting as immunosensing probes and electrochemical tags to produce the analytical signal specifically related to the concentration of the analyte of interest.

In the first antifouling strategy, the nanostructured SPCE optimized in chapter 3 was further modified with a cysteine monolayer, which is a zwitterionic molecule. Zwitterionic molecules consists of an equal ratio of positive and negative charges in close proximity, and thus preserve overall electrical neutrality, which is essential for non-fouling surfaces [266]. The use of cysteine as a coating material for bioelectronics is particularly desirable because the sulfhydryl group in its structure allows for easy and established thiol chemistry to create a monolayer (Figure 42A) [266, 267]. In addition, this molecule creates an ultrathin layer capable of preventing protein adsorption while having little impact on the resistive properties of the transducers. The second antifouling approach consisted on the formation of a polyethylene glycol (PEG) coating on the surface of SPCEs. Ethylene glycols covalently immobilized on the sensing surfaces provide effective non-fouling coatings by repelling nonspecific proteins due to their high conformational entropy and high exclusion volume [265]. There are several ways to synthesize effective antifouling coatings based on PEG, all of which directly impact their surface characteristics and performance. The self-assembly mechanism allows for simple surface application while the vast range of functional group modifications provides functionality customization [268]. In this approach, a PEG coating functionalized with amine and maleimide groups (Figure 42B) was covalently formed on the surface of SPCEs modified with carboxylated SWCNTs.

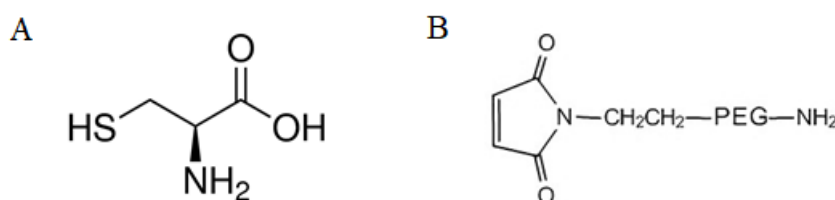


Figure 42 - A) Cysteine molecular structure; B) PEG molecular structure.

Finally, in the second part of this work, an electrochemical immunosensor based on a sandwich assay was developed towards detection and quantification of A β 42 taking advantage of the optimized antifouling matrix. In this approach, novel electrochemical

signal tags were developed based on colloidal AuNPs modified with specific monoclonal antibodies in which sensitivity is amplified through the oxidation of Pb^{2+} ions coupled to the immunosensing probe. Colloidal AuNPs are one of the most common probes applied towards optical and electrochemical sensing strategies as monodisperse suspensions with controllable dimensions of nanoparticles are easily produced through cost-efficient procedures [58]. In addition, they are biocompatible and easily conjugated with biological recognition elements through covalent and non-covalent procedures [58, 269]. With this biosensing strategy, a simple procedure to specifically detect A β 42 was proposed avoiding the need for time consuming and costly enzymatic reactions.

To conclude, considering the clinical relevant levels of A β 42 that consistently exhibited decreased concentrations in CSF (< 500 to 800 pg mL^{-1}) [162, 193, 270], analytical criteria of low detection limits and linear range must be taken into consideration for the immunosensor development.

4.2.1. Material and Methods

4.2.1.1. Materials

2-(N-Morpholino)ethanesulfonic acid (MES), human α -synuclein, BSA, di-sodium phosphate dihydrate, dimethylformamide (DMF), gold(III) chloride solution, *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), potassium carbonate, potassium hexacyanoferrate (III) ($\text{K}_3\text{Fe}(\text{CN})_6$), potassium hexacyanoferrate (II) trihydrated ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$), potassium phosphate monobasic (KH_2PO_4), SWCNTs carboxylic acid functionalized and L-cysteine were purchased from Sigma-Aldrich (Steinheim, Germany). Lead nitrate, potassium chloride, sodium chloride, tannic acid and tri-sodium citrate 2-hydrate were purchased from Riedel-de Haën (Germany). Sodium acetate 3-hydrate was purchased from Panreac (Spain). Acetic acid glacial was acquired from Carlo Erba Reagents. Hydrogen tetrachloroaurate (III) trihydrate, ACS, 99.99% (metal basis), Au 49.0% was purchased from Alfa Aesar. PEG functionalized with maleimide and amine groups (Mal-PEG-NH₂, MW 2000) was acquired from Nanocs Inc. (Boston, MA, USA). Rabbit monoclonal antibody to β -Amyloid (1-42) (ab180956) and mouse monoclonal antibody to β -Amyloid (ab11132) were purchased from Abcam (UK). Human β -Amyloid (1-42) and β -Amyloid (1-40) were purchased from GenScript

(USA). The electrochemical transducers used were SPCEs (DRP-110) acquired from Metrohm-Dropsens. Ultrapure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$ resistivity) was produced by a Milli-Q Simplicity 185 system.

4.2.1.2. Electrochemical assay

Autolab electrochemical system (Eco Chemie, The Netherlands) equipped with PGSTAT-30 and NOVA software were used to perform all electrochemical studies. SPCEs were employed throughout all experimental work. Solution of 2.5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 2.5 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ prepared in 10 mM PBS, pH 7.4 were used as the electroactive indicator and supporting electrolyte, respectively. SPCEs were used throughout this work with an electrochemical cell composed of carbon working electrode (4.0 mm diameter), carbon auxiliary electrode and a silver reference electrode.

CV and SWV measurements were performed by varying the potential from -0.5 to 0.7 V and -0.2 to 0.6 V, respectively, with a 0.05 V s^{-1} scan rate. EIS measurements were accomplished using a set potential of 0.2 V and a frequency range from 10^{-1} to 10^5 Hz with an amplitude perturbation of 10 mV.

For the electrochemical detection of A β 42, SWV current intensity was measured by scanning the SPCE from -1.0 to -0.2 V in 0.2 M sodium acetate/acetic acid buffer (HAc/NaAc) (pH 4.5) to record the electrochemical responses produced by the immunosensing probe.

4.2.1.3. Fabrication of antifouling layers

4.2.1.3.1. Zwitterionic antifouling matrix

A suspension of SWCNTs (1.0 mg mL^{-1}) was prepared in DMF and ultrasonicated until a homogeneous suspension was obtained. 3.0 μL of a 0.10 mg mL^{-1} SWCNTs suspension diluted in ultra-pure water was drop-casted on the surface of the SPCE and set to dry at RT.

AuNPs were electrodeposited on the surface of the modified SPCEs by chronoamperometry in a 0.1 M KNO_3 solution containing 3 mM HAuCl_4 under a -0.2 V potential for 150 s.

Finally, a SAM of the zwitterionic molecule L-Cysteine was formed by drop-casting 10 mM of L-cysteine on the AuNPs/SWCNTs modified SPCE during an overnight incubation at RT in a water saturated environment.

4.2.1.3.2. Antifouling matrix with PEG

A suspension of SWCNTs (1.0 mg mL^{-1}) was prepared in DMF and ultrasonicated until a homogeneous suspension was obtained. $3.0 \text{ }\mu\text{L}$ of a 0.10 mg mL^{-1} SWCNTs suspension diluted in ultra-pure water was drop-casted on the surface of the SPCE and set to dry at RT. Mal-PEG-NH₂ was immobilized on the SWCNTs SPCE surface via the classical EDC/NHS conjugation reaction between the -COOH groups on the SWCNTs and the amino groups on the mPEG. Specifically, the SWCNTs modified SPCE was activated with a 20 mM EDC and 50 mM NHS solution prepared in 50 mM MES buffer (pH 6.0) during 15-20 min at RT followed by incubation with Mal-PEG-NH₂ solution prepared in 10 mM PBS (pH 8.0). Different concentrations of Mal-PEG-NH₂ (0.10, 1.0 and 10 mg mL^{-1}) were evaluated during an overnight incubation at RT in a water saturated environment.

4.2.1.3.3. Determination of protein resistance

CV, EIS and SWV were used to ascertain the ability of the developed platforms to prevent nonspecific protein adsorption using a redox probe solution of 10 mM PBS (pH 7.4) containing 2.5 mM K₃(Fe(CN)₆ and 2.5 mM K₄(Fe(CN)₆). This study was conducted by exposing the developed surfaces to a 1% BSA solution for 1 h at RT under a water saturated environment. After 1 h adsorption, the protein solution was removed, PBS buffer was added to the SPCE surfaces to allow a 5–10 min soaking to remove weakly adsorbed proteins from the surfaces. This step is followed by removal of PBS buffer and replaced with Milli Q water to remove the buffer salts from the protein adsorbed surfaces. Then the SPCEs were dried under a gentle flow of nitrogen gas.

4.2.1.3.4. Stability studies

Two different stability studies were conducted to evaluate their resistance and stability. In the first study, both matrixes were subjected to an electrochemical stability study where the functionalized SPCE surfaces were treated with continuous CV scans in a potential range of -1 V to 1 V for 20 cycles in a 0.1 M KCl solution.

In the second study, the functionalized SPCE were stored over time (1, 3 and 6 months) at 4 °C while their electrochemical signal was studied to evaluate the stability of the modification.

4.2.1.4. Immunosensor development

4.2.1.4.1. Gold nanoparticles synthesis and characterization

10-12 nm gold nanoparticles were synthesized following established procedures [271, 272]. A 25 mL of freshly prepared 0.5 mM HAuCl_4 solution was brought to a boiling point under vigorous stirring. While the solution boils the reducing agent prepared from 0.5 mL of a 125 mM trisodium citrate dihydrate solution and 0.106 mL of 1% tannic acid solution was poured rapidly into the boiling solution. The solution immediately turned dark, and with continued heating, the color changed to clear wine red within 5 seconds. The boiling of the solution was continued for another 5 min, after which the solution was cooled under running tap water and then stored at 4 °C.

The mean diameter and size distribution of the prepared AuNPs were evaluated by dynamic light scattering (DLS). The attained data is given in intensity distribution. The zeta potential values of the prepared AuNPs were determined by laser Doppler velocimetry method. The analysis was performed using dielectric constant of water. Both analyses were performed in a Zetasizer Nano ZS (Malvern Instruments, UK) while performing at least three independent measurements.

4.2.1.4.2. Preparation of the immunosensing probes

For the conjugation with antibodies by passive adsorption, the optimal pH and antibody concentration were established by the flocculation stress test. In the first place, 50 mM potassium carbonate solution was added to the colloidal AuNPs solution until pH 7.5 was reached. Next, serial dilutions of both protein solutions (BSA and IgG) were

prepared. 10 μL of each dilution was added to 100 μL of the colloidal gold solution. After 15 min, flocculation was induced by adding 10 μL of 10% NaCl and absorbance was measured at 580 nm [138]. The amount of protein necessary to prevent flocculation was deduced graphically from the concentration at which the absorbance becomes nearly constant.

To synthesize AuNPs-Ab conjugates, 25 $\mu\text{g mL}^{-1}$ of monoclonal antibody to A β 42 was added to the AuNPs solution and left to react at RT for 2 h under stirring. Then, the solution was centrifuged in two stages: firstly, the solution was centrifuged at low speeds (2000 rpm; 10 min) to remove large aggregates that might have been formed; secondly, the supernatant resultant from the first centrifugation was further centrifuged at higher speeds (6000 rpm; 10 min). After centrifugation, BSA 1% was added and the solution was left to react for 1 h at RT under stirring to block any remaining active sites. Then, after a new centrifugation process (6000 rpm; 10 min), a 10 mM $\text{Pb}(\text{NO}_3)_2$ solution was added to the modified AuNPs and left to react overnight at RT under stirring. Finally, the immunosensing probe was centrifuged three times (6000 rpm; 10 min), washed with ultra-pure water and dispersed in 10 mM PBS (pH 7.4) and stored at 4 $^{\circ}\text{C}$ until further use.

Note: Recognizing the toxicity of the Pb element, in this work a small volume of 10 mM $\text{Pb}(\text{NO}_3)_2$ was prepared, which was enough to conduct all of the experimental work.

4.2.1.4.3. Immunoassay development

The monoclonal antibodies were modified through a thiolation process to promote their immobilization on the pegylated SPCE surface [137]. Briefly, a solution containing monoclonal antibody against α -synuclein prepared in PBS (10 mM, pH 7.4), EDTA (2.5 mM) and 2-iminothiolane (20 times molar excess over the amount of antibodies) was prepared and left to react for 1 h at RT [138]. Then, 5.0 μL were drop-casted on the previously modified SPCE followed by 1 h incubation at RT and overnight incubation at 4 $^{\circ}\text{C}$. Different antibody concentrations were evaluated for the SPCE (10, 25, 50 and 100 $\mu\text{g mL}^{-1}$). Finally, the modified SPCE was incubated in BSA for 30 min at RT in order to block the remaining adsorption-reactive sites. Different BSA concentrations were studied (10, 100, 500, 1000 $\mu\text{g mL}^{-1}$).

All biological incubations were carried out in a water saturated environment.

4.2.1.4.4. Electrochemical detection of A β 42

Different concentrations of α -synuclein in the range of 0.10 – 1.0 ng mL⁻¹ prepared in 10 mM PBS (pH 7.4) were drop-casted (5.0 μ L) on the surface of the functionalized SPCE and left to react at RT for 1 h. Then, the prepared immunosensing (5.0 μ L) probe was drop-casted on the SPCE and left to react also for 1 h at RT. Both incubation steps were performed in water saturated environments. After each step the SPCE were washed with 10 mM PBS (pH 7.4) and ultra-pure water.

Finally, SWV current intensity was measured by scanning the SPCE from -1.0 to -0.20 V to record the electrochemical responses for the measurement of A β 42 in 0.2 M acetic acid/sodium acetate buffer (HAc/NaAc), pH 4.5.

All reagents and dilutions used in the electrochemical immunosensing procedure were prepared in PBS 10 mM, pH 7.4. All measurements were carried out in triplicate.

4.2.1.4.5. Specificity studies

The developed biosensor was exposed to solutions of A β 40 (10 ng mL⁻¹) and α -synuclein (1.0 ng mL⁻¹) prepared in 10 mM PBS (pH 7.4) for 1 h at RT.

4.2.2. Results and Discussion

4.2.2.1. Development of the antifouling coatings

The antifouling surfaces developed in this work followed the same design principle adopted in the previous chapter. Both surfaces were developed in a layer-by-layer approach, which enabled to control the properties and functional groups exposed on the SPCE surface in every moment in order to create the appropriate environment for the successful formation of each layer (Figure 43).

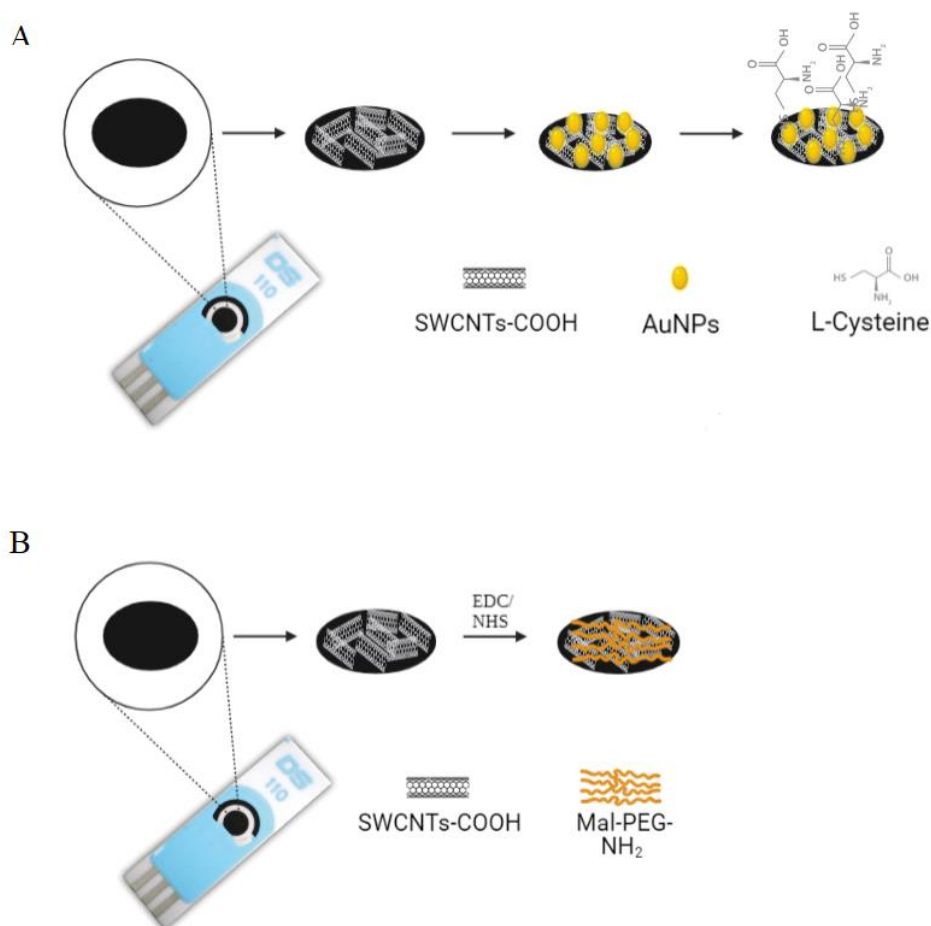


Figure 43 - Layer-by-layer assembly of the antifouling coatings: A) Zwitterionic molecule cysteine; B) Mal-PEG-NH₂ coating functionalized with amino and maleimide groups.

Before evaluating the antifouling effect of both strategies, it is possible to see from Figures 44 and 45 that the modification of the nanostructured SPCE with both antifouling coatings did not cause an increase in the resistance of the surface maintaining their functionality for further electrochemical studies. In fact, from Figure 44 (A to C) it is possible to observe that the signal of the nanostructured SPCE presented no differences following an overnight incubation at RT of the modified SPCE on a 10 mM cysteine solution prepared in 10 mM PBS (pH 7.4).

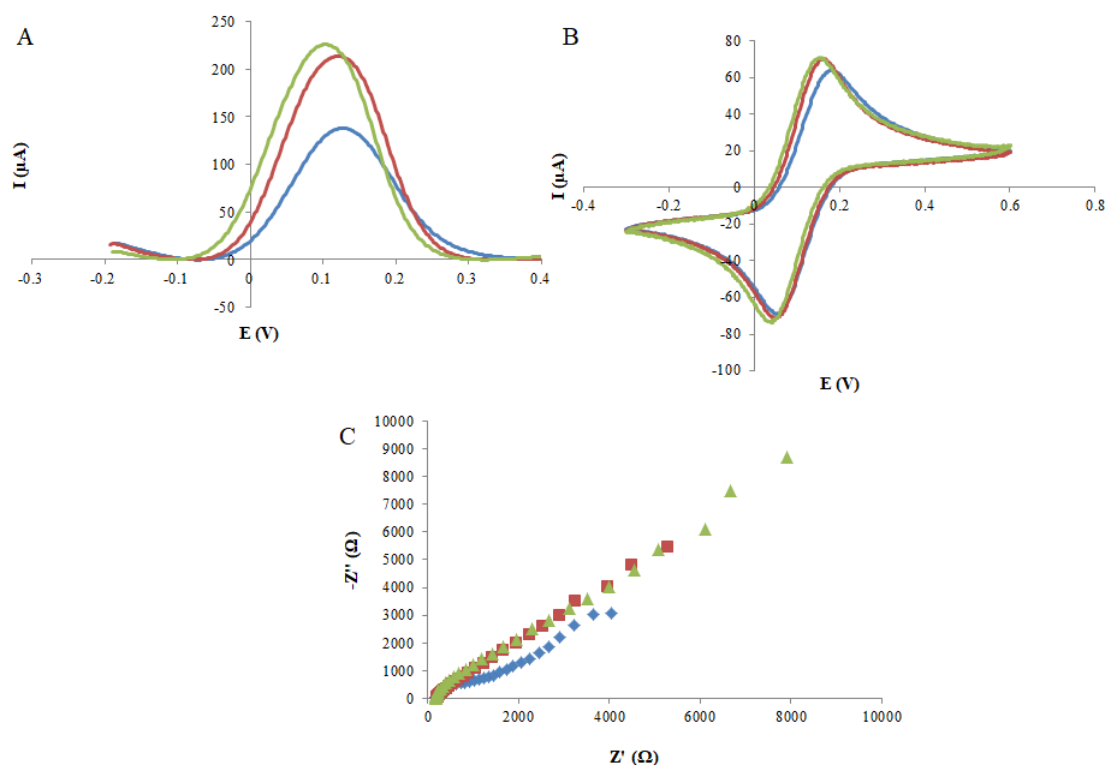


Figure 44 – Layer-by-layer assembly of the antifouling matrix based on the formation of a cysteine monolayer: SPCE/SWCNTs (blue); SPCE/SWCNTs/AuNPs (red) and SPCE/SWCNTs/AuNPs/Cysteine (green). Electrochemical study conducted through A) SWV, B) CV and C) EIS analysis. Measurements were performed in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

Concerning the formation of a PEG layer on the surface of the SWCNTs functionalized SPCE, it is possible to notice from Figure 45A-C that this modification led to more significant differences in the electrochemical signal, but without causing a blockage of the electron transfer. These changes were more evident through SWV and EIS analysis, from which a more clear understanding of the signal changes upon formation of a PEG layer could be perceived (Figure 45A). Specifically, the signal of the SPCE modified with PEG exhibited a lower peak current, approximately 36 μA , in comparison to the nanostructured SPCE with SWCNTs, 143 μA . Nevertheless, despite this difference, the complete signal of the SPCE modified with PEG appeared at higher current ranges, between 280 and 320 μA , while the SWCNTs modified SPCE signal ranged between 10 and 150 μA . This electrochemical profile obtained through SWV analysis may explain the results of more qualitative techniques such as CV and EIS, where a slight signal improvement was observed in CV after the formation of a PEG layer while a reduction of impedance signal was perceived in EIS. These changes on

the electrochemical signal were reproducible over time ensuring the successful formation a PEG coating on the nanostructured SPCE.

From Figures 44 and 45 is possible to conclude that after the assembly of the antifouling matrices there is still significant Faradaic signal of the redox species in solution.

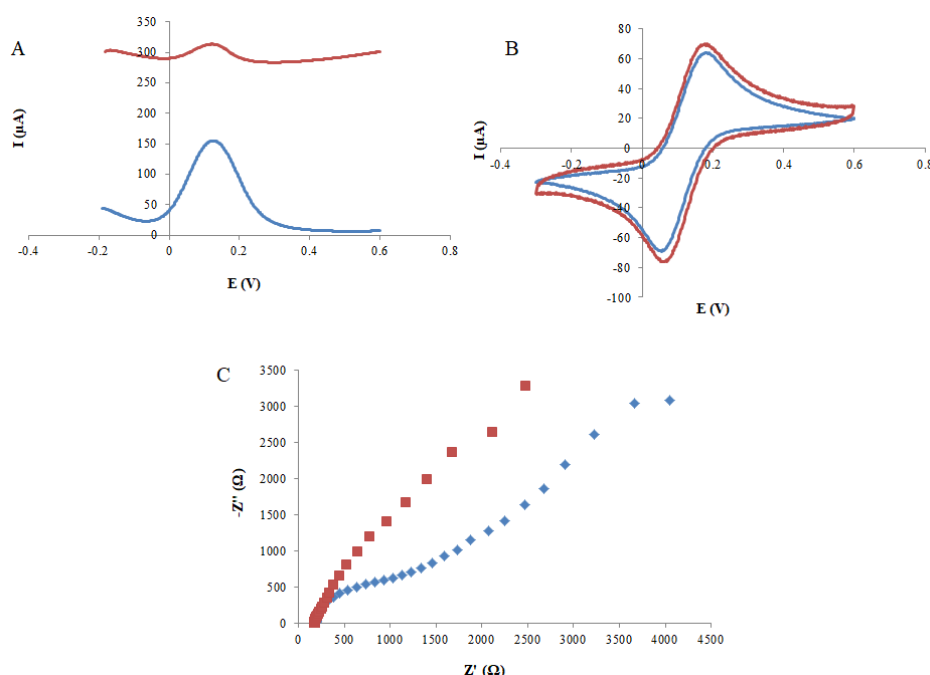


Figure 45 - Layer-by-layer assembly of the antifouling matrix based on the formation of a Mal-PEG-NH₂ coating: SPCE/SWCNTs (blue) and SPCE/SWCNTs/Mal-PEG-NH₂ (red). Electrochemical study conducted through A) SWV, B) CV and C) EIS analysis. Measurements were performed in a 2.5 mM Fe(CN)₆^{3-/4-} solution in 10 mM PBS (pH 7.4).

4.2.2.1.1. Antifouling properties of the sensing interface

The antifouling behavior of the developed surfaces was evaluated through EIS based on the blocking effect and charge transfer resistance after exposure of the modified SPCEs to a 1.0% BSA solution for 1 h at RT. From Figures 46 and 47 it is possible to observe the excellent antifouling properties of the developed coatings. In fact, in the absence of these antifouling elements there was a substantial increase in the impedance signal of the nanostructured SPCEs after exposure to BSA. More specifically, there was a clear enlargement of the semi-circles diameters as a result of the formation of an insulating protein layer which covered the surface of the nanomaterials resulting in an increase of electron transfer resistance (Figures 46A and 47A). On the other hand, in the SPCEs

modified with antifouling coatings it is possible to observe that the EIS signal suffered almost no change after exposure to BSA, ensuring the successful protective properties towards non-specific binding of proteins (Figure 46B and 47B).

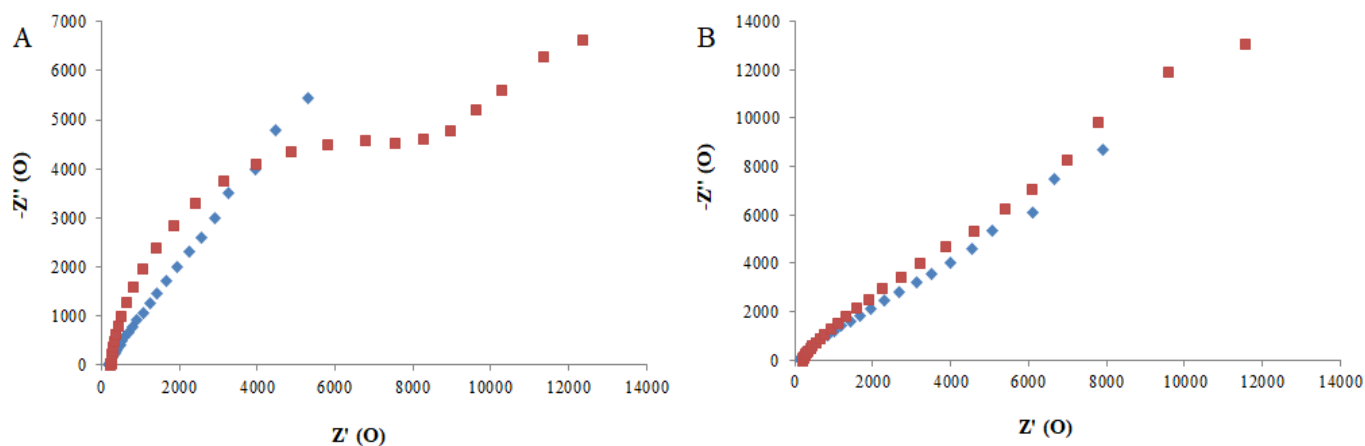


Figure 46 – Examination of the antifouling properties of the SPCE modified with SWCNTs and AuNPs in the absence (A) and presence (B) of the monolayer cysteine before (blue) and after (red) exposure to 1% BSA for 1 h. Measurements were performed using EIS in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

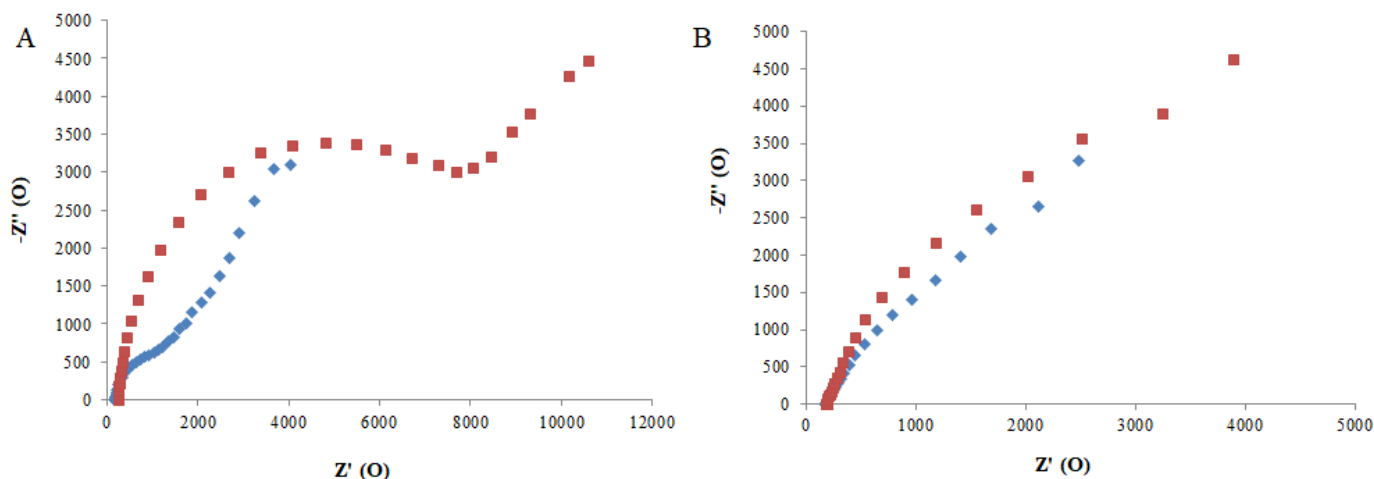


Figure 47 - Examination of the antifouling properties of the SPCE modified with SWCNTs in the absence (A) and presence (B) of the Mal-PEG-NH₂ coating before (blue) and after (red) exposure to 1% BSA. Measurements were performed using EIS in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

Moreover, in order to evaluate the stability of these coatings the modified SPCEs were submitted to an electrochemical stability test. The surfaces were treated with continuous CV scans in a potential range of -1.0 V to 1.0 V for 20

cycles in a 0.1 M KCl aqueous solution. After this electrochemical treatment the electrodes were again exposed to 1% BSA for 1h at RT, being that the proposed coatings still exhibited protective properties (Figure 48). Nonetheless, the monolayer formed from the zwitterionic molecule cysteine may have become less organized or suffered some defects since the response after exposure to BSA was no longer linear as reported in Figure 46. Even so, the EIS signal was still substantially inferior when compared to the signal of the nanostructured SPCE after exposure to BSA, confirming that the antifouling properties of this coating remained functional (Figure 48A). On the other hand, the coating that resulted from the covalent bonding of PEG molecules to the SWCNTs modified SPCE presented better stability by registering almost no differences in response before and after the electrochemical stability test (Figure 48B). In fact, it is possible to conclude from Figures 47B and 48B that the EIS signal of the PEG coating before and after exposure to BSA suffered almost no changes. Nonetheless, after exposure to BSA the signal pattern became more pronounced. This may be indicative of some protein adsorption although with no significant impact to increase the resistive properties of the surface.

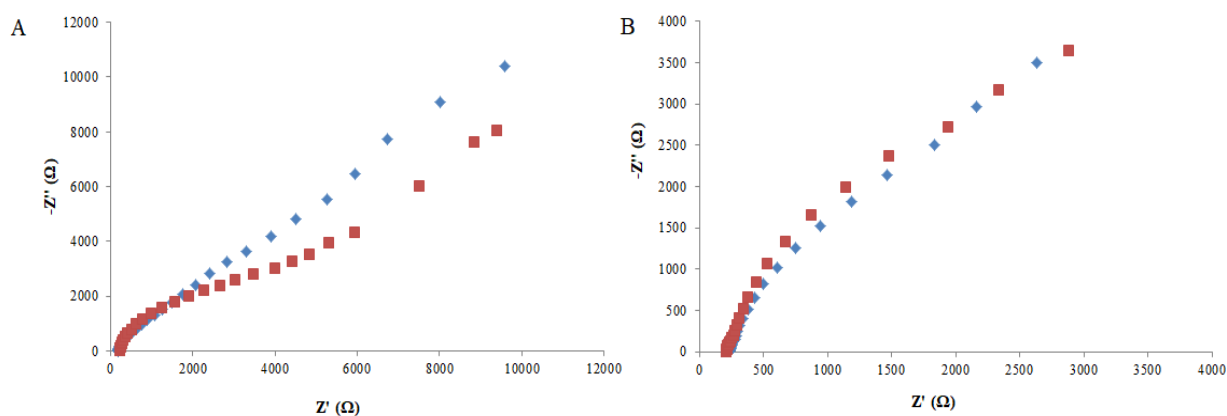


Figure 48 - Examination of the antifouling properties of the proposed coatings before (blue) and after (red) exposure to 1% BSA for 1 h: monolayer of cysteine (A) and covalent attachment of PEG (B), after the electrochemical stability test. Measurements were performed using EIS in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

In Table 8, it is possible to find a summary of the EIS analysis performed to evaluate the antifouling properties of the proposed coatings. It is possible to conclude, that the matrix that resulted from the covalent immobilization of PEG to the SPCE presented superior antifouling properties while exhibiting excellent stability.

Table 8 - Summary of the antifouling studies performed for the proposed coatings based on EIS analysis (Incubation in 1% BSA for 1 h at RT).

	R _{ct} (Ω)			
	Before Electrochemical stability study		After Electrochemical stability study	
	Before	After 1% BSA	Before	After 1% BSA
SPCE/SWCNTs/AuNPs	NR	12314.9	-	-
SPCE/SWCNTs/AuNPs/Cysteine	NR	NR	NR	7326.5
SPCE/SWCNTs	1053.3	8366.8	-	-
SPCE/SWCNTs/Mal-PEG-NH ₂	NR	NR	NR	NR

* The charge-transfer resistance R_{ct} values were obtained by fitting the Nyquist plot with a Randles equivalent circuit. AuNPs, Gold nanoparticles, Mal-PEG-NH₂; Polyethylene glycol functionalized with amine and maleimide groups; NR, Non-reported; SPCE, Screen-printed carbon electrode; SWCNTs, Single-walled carbon nanotubes.

Finally, the stability of the proposed coatings was studied by incubating the modified SPCEs at 4 °C over time and using SWV for the electrochemical analysis. It is possible to see from Figure 49 that the coating based on the cysteine monolayer presented lower stability since the electrochemical signal starts reducing after one month, eventually leading to a signal loss of almost 50% after 6 months. On the other hand, the PEG coating presented remarkable stability over time exhibiting negligible differences in the electrochemical signal over a 6 month period. Concluding, despite the excellent antifouling properties exhibited by the proposed coatings, the modification that resulted from the covalent bonding of Mal-PEG-NH₂ to the SPCE modified with carboxylated SWCNTs exhibited an overall better behavior particularly in the stability studies. In addition, the possibility of exposing maleimide functional groups on the SPCE surface will enable the covalent attachment of thiolated antibodies on the transducer surface, a protocol previously adopted.

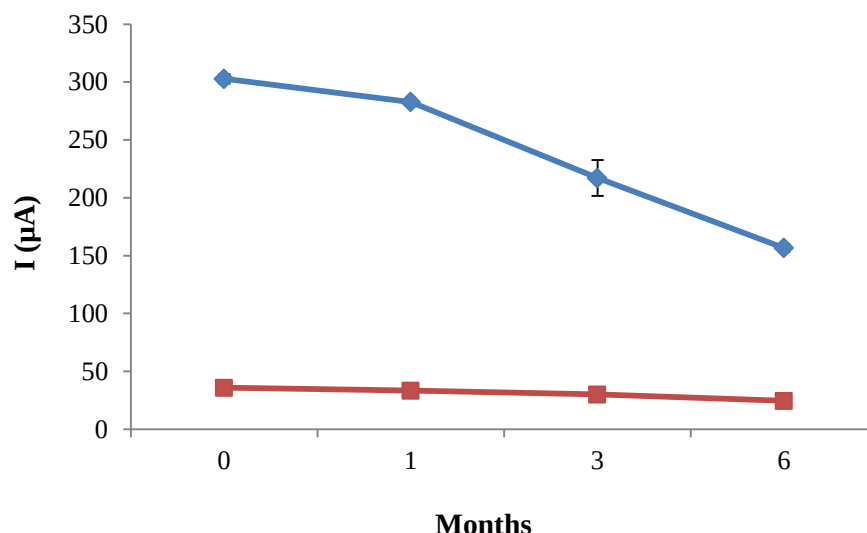


Figure 49 – Stability studies of the proposed antifouling coatings: SPCE nanostructured with SWCNTs and AuNPs and further modified with a monolayer of cysteine (blue); SPCE modified with SWCNTs and Mal-PEG-NH₂ (red). Measurements were performed using SWV in a 2.5 mM Fe(CN)₆^{3-/4-} solution in 10 mM PBS (pH 7.4).

All of the results presented in this study concerning the development of antifouling coatings were promoted through overnight incubation at RT and with higher concentrations of the antifouling elements. This way, in order to simplify the experimental procedure towards development of the antifouling coating based on the covalent bonding of Mal-PEG-NH₂, different incubation periods and concentrations were studied. In the first place, three different concentrations of PEG (0.1, 1.0 and 10 mg mL⁻¹) were evaluated after an overnight incubation at RT, being their SWV (Figure 50) signal and antifouling properties (Figure 51) compared. From this study, it is possible to conclude that at the lowest studied concentration (0.1 mg mL⁻¹) of PEG coating was not successfully formed. In fact, from the analysis of SWV results (Figure 50) it is possible to observe that the signal pattern for the lowest PEG concentration was similar to the signal of SWCNTs modified SPCE (Figure 45A). In addition, from the protein adsorption studies, it is possible to conclude that the immobilization of low concentrations of PEG led to the formation of an antifouling matrix that it is not able to completely prevent protein adsorption. On the other hand, with the concentrations of 1.0 and 10 mg mL⁻¹ similar SWV and EIS behaviors were obtained (Figures 50 and 51). Nonetheless, it is possible to see from the EIS results that the difference in signal before and after exposure to 1% BSA was inferior at a concentration of 10 mg mL⁻¹ where the signals practically overlap (Figure 51C). This way, the concentration of 10 mg mL⁻¹ was chosen to develop this antifouling matrix as it proved to be most effective in preventing

non-specific binding events. Finally, shorter incubation times were studied in order to simplify the experimental procedure by making it less time-consuming. Incubation periods of 1 and 2 h at RT were studied. Similar SWV signals were obtained with a 2 h and overnight incubation with peak currents of 34.3 μA and 35.2 μA and a standard deviation (SD) of 2.6 and 1.5, respectively. On the other hand, for 1 h incubation the SWV peak current almost doubled presenting values of the order of 60.9 μA with a 10.1 SD, which may result in less stable and less reproducible antifouling coatings. This way, the final conditions for the formation of a PEG antifouling coating were chosen at a concentration of 10 mg mL^{-1} and an incubation period of 2 h at RT.

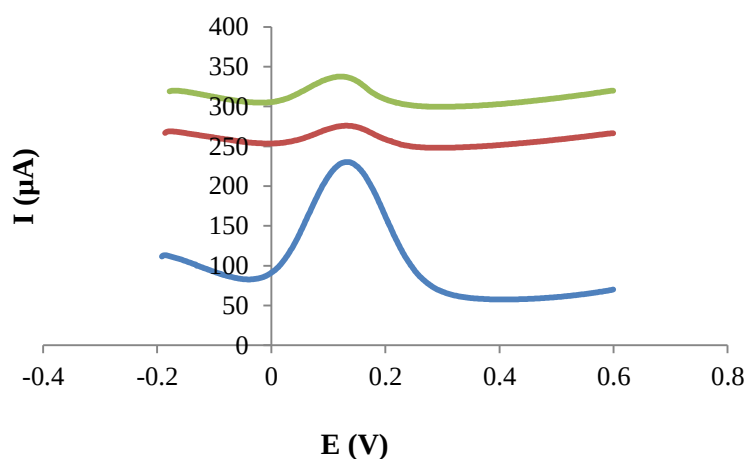


Figure 50 – SWV signal after overnight incubation of different concentrations of PEG: 0.10 (blue), 1.0 (red) and 10 (green) mg mL^{-1} . Measurements were performed in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

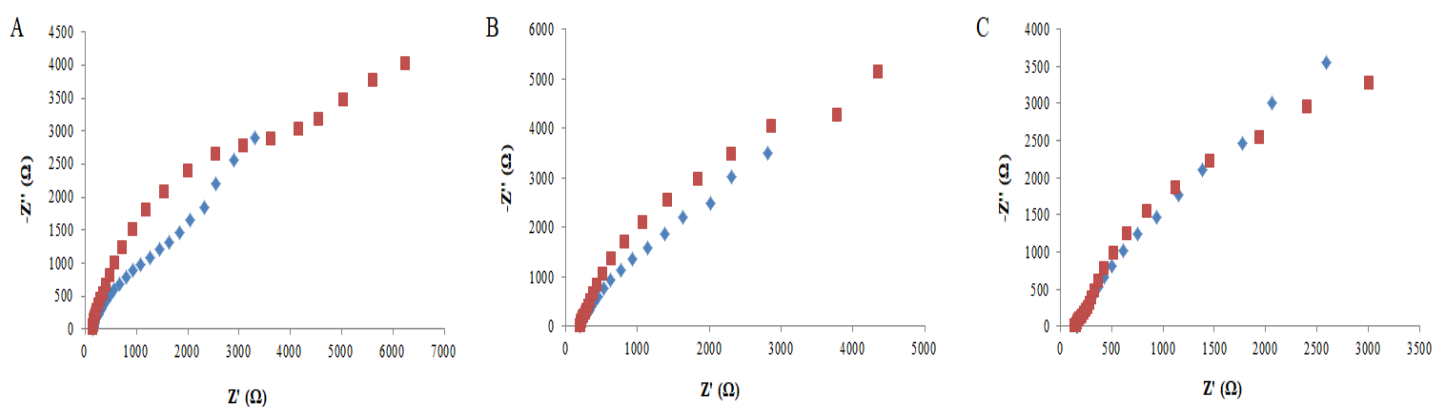


Figure 51 – Study of different concentrations of Mal-PEG-NH₂ coating before (blue) and after (red) exposure to 1% BSA for 1h: 0.10 (A); 1.0 (B) and 10 (C) mg mL^{-1} . Measurements were performed in a 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution in 10 mM PBS (pH 7.4).

4.2.2.2. Immunosensor development

The different steps involved in the preparation and functioning of the proposed immunosensor based on the optimized antifouling matrix is schematically illustrated in Figure 52. The A β 42 detection was promoted via a sandwich detection scheme with colloidal AuNPs acting as immunosensing probes labeled with Pb²⁺ ions to produce the analytical signal. The capture antibodies were immobilized on the transducers surface by enabling the covalent coupling of thiolated monoclonal antibodies (anti-A β) with the chemically reactive end-group of the maleimide functionalized PEG. This stage was followed by a blocking step with low concentrations of BSA in order to deactivate residual reagents from the thiolation process, namely 2-iminothiolane that could lead to non-specific binding events. Finally, for the development of the immunosensing probe the conjugation of monoclonal antibodies specific to A β 42 was realized via physical adsorption. Despite several limitations inherent to this approach (weak attachment and random orientation of antibodies upon immobilization) the simplicity and fast conjugation procedures make this approach one of the most commonly applied techniques for the preparation of immunosensing probes based on colloidal AuNPs. In fact, proteins spontaneously adhere to the surface of colloidal AuNPs through several noncovalent interactions. Therefore, the direct adsorption of antibodies leads to the formation of protein layer on the surface of the AuNPs that significantly contributes to stabilize and protect them from aggregation. In addition, the gold nanoparticles were further blocked with BSA in order to minimize the occurrence of non-specific binding events. Finally, after the biorecognition events conducted by AuNPs bearing the immunoconjugates, the electrochemical detection of the immunosensing probe was conducted in HAc/NaAc buffer (pH 4.5) producing a signal related to the presence of Pb²⁺ ions that appeared around -0.8 V.

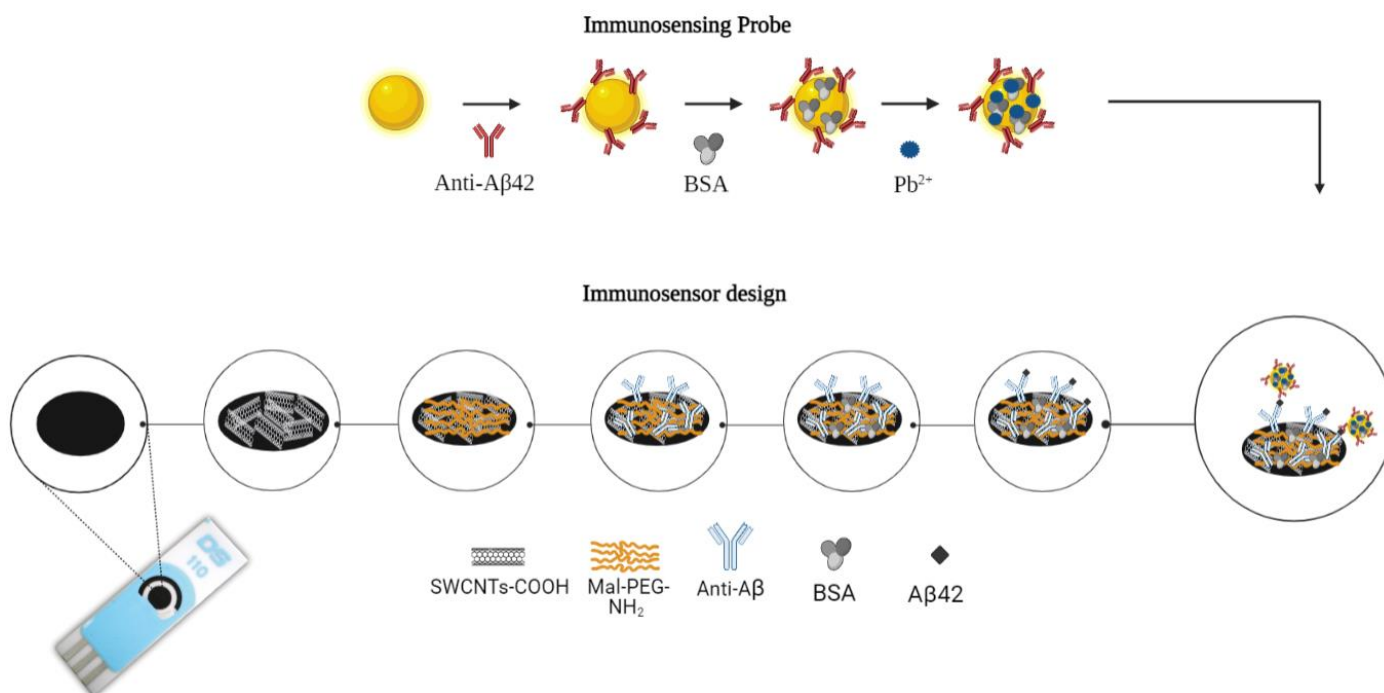


Figure 52 – Schematic illustration of the different steps involved in the preparation of the electrochemical immunosensor for A β 42.

4.2.2.2.1. Optimization of the experimental variables involved in the preparation of the immunosensor

The effect of the different variables involved in the preparation of the A β 42 immunosensor on the corresponding analytical responses was studied. In the first place, the physicochemical properties of the synthesized colloidal AuNPs were evaluated exhibiting a mean diameter of $11.9 \text{ nm} \pm 0.2 \text{ nm}$ and zeta potential of $-11.3 \pm 2.6 \text{ mV}$, presenting a negative charge in compliance with colloidal stability. In the next step, the concentrations for the successful conjugation of anti-A β 42 antibody and BSA by passive adsorption on the colloidal AuNPs were optimized by the flocculation stress test. The amount of antibody and BSA necessary to prevent flocculation was deduced graphically from the concentration at which the absorbance becomes nearly constant. From Figure 53 it is possible to see that the absorbance values became constant at a concentration of $50 \text{ } \mu\text{g mL}^{-1}$ while $25 \text{ } \mu\text{g mL}^{-1}$ of BSA were sufficient to stabilize the AuNPs (Figure 54). Nonetheless, it was demonstrated by Byzova N. *et al.* [273] that the immobilization of a high density of antibodies on the surface of AuNPs can lead to negative effects, like shielding the binding site of antibodies or preventing antigens from interacting with immobilized antibodies. In fact, this was a problem faced in this

work since it was not possible to differentiate analytical signals from blank assays with immunosensing probes prepared with an antibody concentration of $50 \mu\text{g mL}^{-1}$. This way, the evaluated concentration immediately below the stabilizing value ($25 \mu\text{g mL}^{-1}$) was tested to develop the immunosensing probe, attaining an overall better analytical behavior. All of these optimizations were performed at pH 7.5 since recent studies have shown that under these conditions the immobilization of antibodies with better orientation is favored [274].

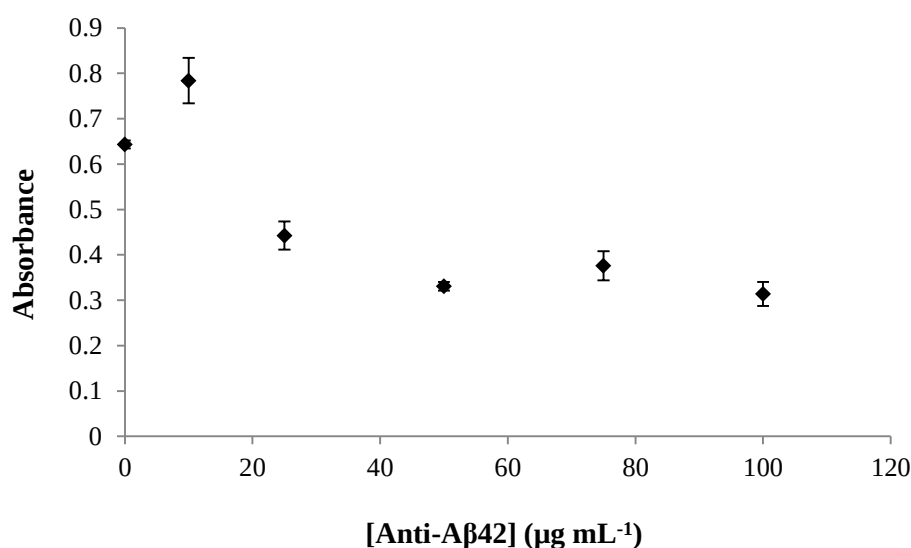


Figure 53 – Flocculation curve of the monoclonal antibodies immobilization on the AuNPs surface through passive adsorption. Antibody concentrations influence on the AuNPs absorbance measured at 580 nm in the presence of excess salt (10% NaCl). Measurements were carried out in triplicate.

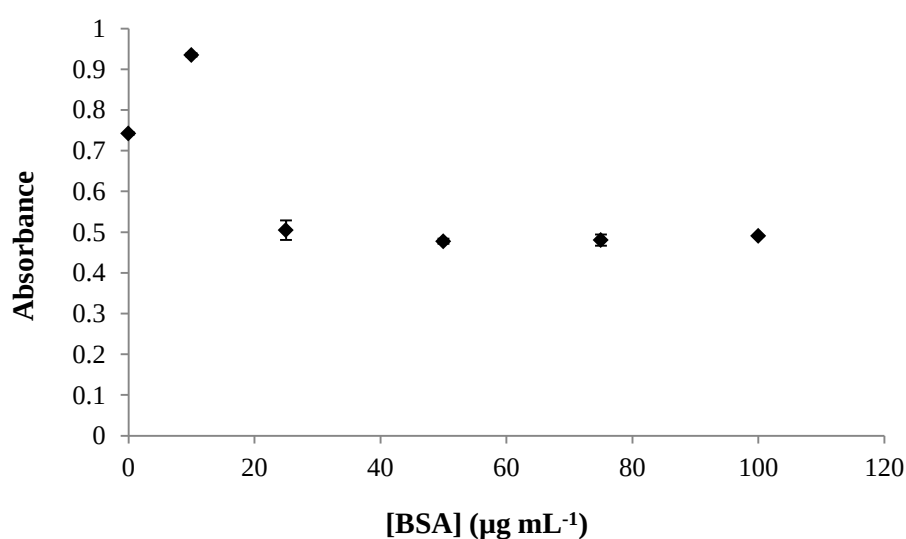


Figure 54 - Flocculation curve of the immobilization of BSA on the AuNPs surface through passive adsorption. BSA concentrations influence on the AuNPs absorbance measured at 580 nm in the presence of excess salt (10% NaCl). Measurements were carried out in triplicate.

In the next stage of the immunosensor development and using the optimized conditions of the immunosensing probe, the concentration of capture antibodies and blocking agent were optimized by measuring the specific and unspecific responses with antibody concentrations ranging from 10 to 100 $\mu\text{g mL}^{-1}$ (Figure 55). The antibody concentration of 50 $\mu\text{g mL}^{-1}$ produced the largest specific to unspecific current ratio (Figure 55A). Nevertheless, the current response of unspecific assays presented high values across the range of antibody concentrations analyzed. This way, a blocking step was promoted to minimize this effect, being that a 100 $\mu\text{g mL}^{-1}$ BSA concentration exhibited the largest specific to unspecific current responses (Figure 54B). Higher concentrations of BSA produced a significant decrease of the specific response most likely due to hindering the electrochemical reaction in the presence of a large biomolecule loading.

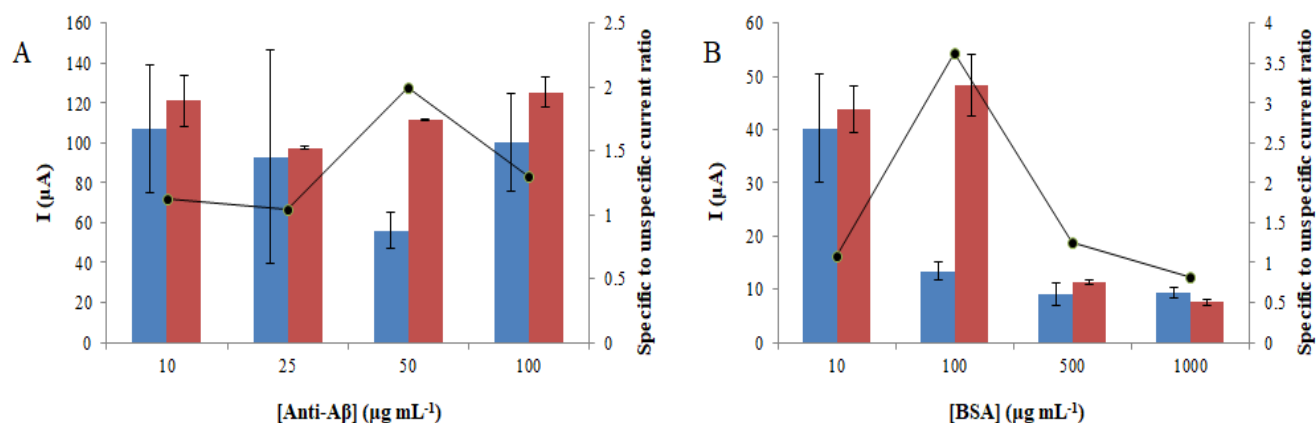


Figure 55 - Effect of the anti-A β loading (10 – 100 $\mu\text{g mL}^{-1}$) (A) and the blocking agent concentration (10 – 1000 $\mu\text{g mL}^{-1}$) (B) on the SWV response obtained with the A β 42 immunosensor: 1.0 ng mL $^{-1}$ A β 42 (red) and without A β 42 (blue) ($n=3$). Measurements were performed in a 0.2 M HAc/NaAc buffer (pH 4.5).

4.2.2.2.2. Analytical characteristics of the immunosensor

The calibration curve for A β 42 constructed with the developed immunosensor under the optimized working conditions is depicted in Figure 56. The SWV signal increases as a result of the alterations to the interface properties of the biosensor due to the detection of increasing A β 42 concentrations by the immunosensing probe labeled with Pb $^{2+}$ ions. Error bars were calculated from measurements carried out with three different immunosensors in each case. The SWV current vs. logarithm of A β 42 concentration followed the adjusted equation $I(\mu\text{A}) = 21.26 \log C(\text{ng mL}^{-1}) + 47.72$ ($r=0.997$), with a range of linearity extending between 0.1 and 1.0 ng mL $^{-1}$ A β 42. This

linear range covers clinical relevant levels of A β 42 to be studied in CSF. Based on the analytical data, LOD and LOQ were calculated to be 36.2 pg mL⁻¹ (8.02 pM) and 77.6 pg mL⁻¹ (17.2 pM), respectively.

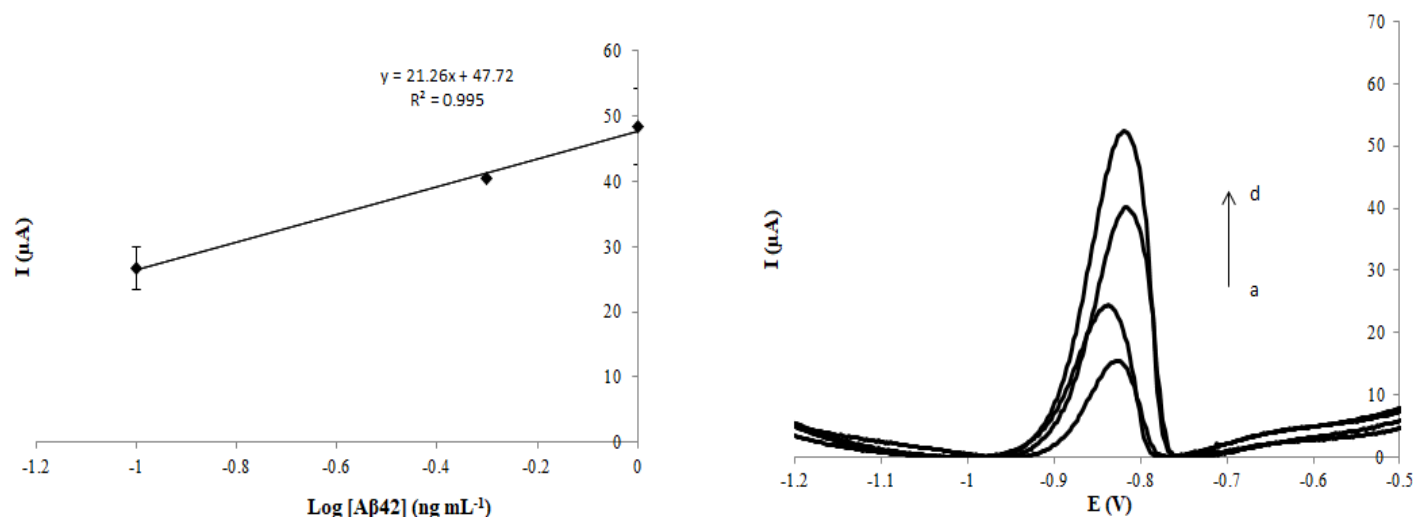


Figure 56 - A) SWV response of the immunosensor to different A β 42 concentrations (from a to d: 0, 0.10, 0.50, and 1.0 ng mL^{-1}); B) Calibration curve based in the SWV signal reduction after detection of A β 42 concentrations (0.10, 0.50, and 1.0 ng mL^{-1}) ($n=3$). Measurements were performed in a 0.2 M HAc/NaAc buffer (pH 4.5).

Despite presenting promising results towards detection of low levels of A β 42, the developed immunosensor exhibited major problems concerning the reproducibility of the developed immunosensing probe. Therefore, the development of a calibration curve with a wider concentration range as well as the validation of its analytical properties was severely compromised. The main advantages of the developed immunosensing probe were related to the simplicity of procedures that would enable the loading of metal ions on the AuNPs to amplify the electrochemical signal. This way, more expensive and time consuming amplification procedures based on enzymatic reactions would be avoided. Finally, the Pb^{2+} signal could be directly detected through SWV without the need for metal dissolution or pre-concentration steps, presenting a very simple and straight forward amplification strategy. Nonetheless, the problems of reproducibility in the immunosensing probe synthesis as well as its poor stability overtime compromised the immunosensors application for detection of A β 42. Figure 57 displays the reported problems in a clear manner. From Figure 57A it is possible to see the clear difference in the signal obtained towards detection of 1.0 ng mL^{-1} of A β 42

using immunosensing probes synthesized in different days. The second assay reported a SWV signal approximately 12 to 15 μA higher towards detection of the same level of $\text{A}\beta_{42}$. In Figure 57B the stability problems of the immunosensing probe are demonstrated. In this study, after one week stored at 4 °C the immunosensing probe produced an analytical signal 24 times inferior, while maintaining the ability to successfully distinguish the analyte from blank assays.

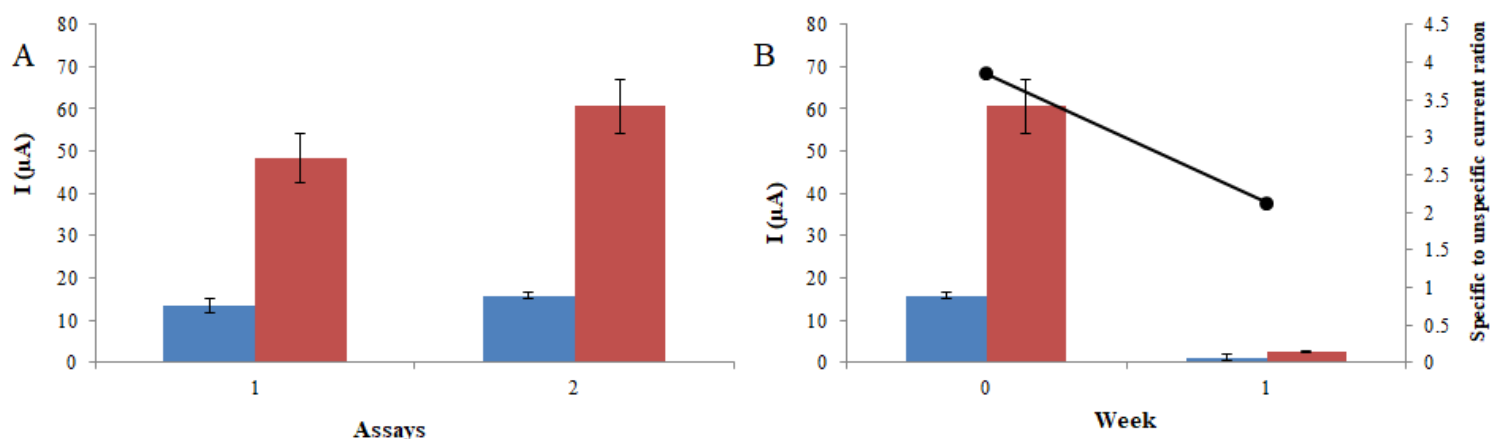


Figure 57 - Reproducibility (A) and stability studies (B) of the immunosensing probe based on the SWV response obtained with the $\text{A}\beta_{42}$ immunosensor: 1.0 ng mL^{-1} $\text{A}\beta_{42}$ (red) and without $\text{A}\beta_{42}$ (blue) ($n=3$). Measurements were performed in a 0.2 M HAc/NaAc buffer (pH 4.5).

Finally, in the last study performed to characterize the analytical performance of the developed immunosensor, specificity studies were conducted with a newly prepared immunosensing probe (Figure 58). From this study is possible to conclude that the proposed immunosensor could specifically detect $\text{A}\beta_{42}$ while presenting no analytical response after exposure to 1.0 ng mL^{-1} of α -synuclein and 10 ng mL^{-1} of $\text{A}\beta_{40}$ prepared in 10 mM PBS (pH 7.4).

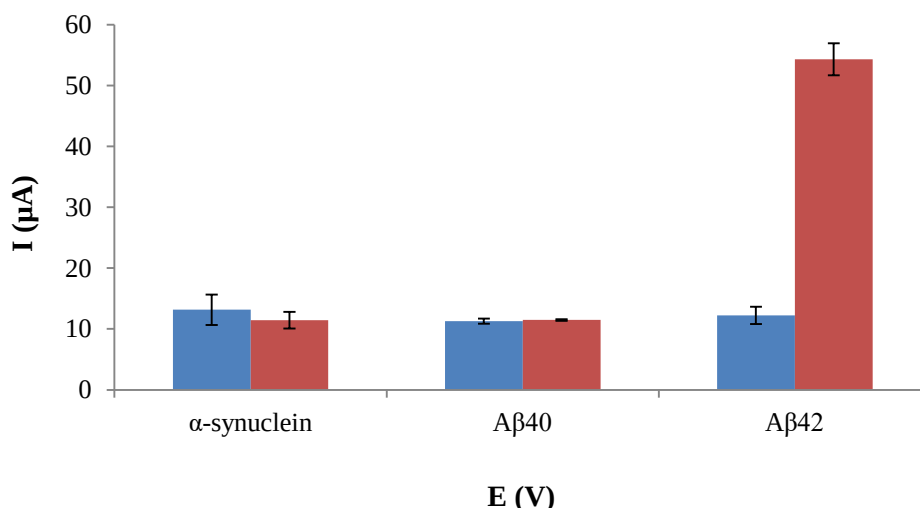


Figure 58 – Specificity study based on the SWV response obtained with Aβ42 immunosensor with (red) and without (blue) the analytes: 1.0 ng mL⁻¹ of α-synuclein, 10 ng mL⁻¹ of Aβ40 and 1.0 ng mL⁻¹ of Aβ42 prepared in 10 mM PBS (pH 7.4). Measurements were performed in a 0.2 M HAc/NaAc buffer (pH 4.5).

In order to overcome the presented limitations and taking advantage of the developed antifouling coating a more classical amplification strategy is being pursued by developing an immunosensor based on an indirect detection with enzymatic amplification. In addition, an interesting approach could be to develop more stable AuNPs by functionalize them with groups such as carboxylic acid. This way the antibodies would have to be immobilized on the AuNPs surface via covalent coupling. This type of approach usually requires lower antibody concentrations, there is lower risk of AuNPs aggregating during conjugation, the binding is not dependent on pH and ionic strength of the buffer while different cross-linking strategies can be applied. In addition, the use of different labels, like enzymes, fluorophores or electrochemiluminescent tags would be of extreme usefulness to overcome the limitations of the developed immunosensing probe.

4.2.3. Conclusions

The main objectives of this chapter were to develop an antifouling matrix capable of resisting to non-specific protein adsorption while maintaining the transducers functionality with the goal of promoting the electrochemical detection of AD main biomarker, Aβ42.

The proposed antifouling coatings were based on modifications that did not passivate the transducers surface while presenting excellent ability to prevent protein

adsorption. The first approach was based on the optimized nanostructured SPCE with SWCNTs and electrodeposited AuNPs that was further modified through the formation of a monolayer of the zwitterionic molecule, cysteine. The second approach consisted of a SPCE modified with drop-casted carboxylated SWCNTs, which increased the surface area and conductive properties of the transducer followed by covalent bonding of PEG functionalized with amine and maleimide groups. The second approach presented excellent properties to resist protein adsorption while exhibiting excellent stability that may result from the covalent attachment between the carboxylic acid groups on the SWCNTs and the amine groups on the functionalized PEG.

Subsequently, an electrochemical immunosensor based on a sandwich assay was developed towards detection and quantification of A β 42. The immunosensor design was built from the optimized antifouling coating while a novel amplification strategy based on colloidal AuNPs was developed. The immunosensing probes consisted of AuNPs modified with monoclonal antibodies specific towards A β 42 and labeled with Pb²⁺ ions in order to produce an electrochemical signal specifically related to the concentration of the analyte of interest. In this immunosensor design, the optimized antifouling coating was also used as the platform to promote the covalent bonding of thiolated monoclonal antibodies by taking advantage of the functional groups maleimide. This way, the developed antifouling coating exhibited excellent properties to interface affinity binding events with electrochemical sensing.

The combination of all these steps promoted a sensitive and specific quantification of A β 42 within clinical relevant levels, exhibiting low LOD and LOQ, through the use of simple, fast and relatively inexpensive instrumentation. Nonetheless, despite presenting some positive results towards detection of A β 42, the developed immunosensing probe presented major problems related with lack of reproducibility and stability. Consequently, the development of a calibration curve with a wider concentration range or the possibility of validating it was severely hampered by the inconsistencies demonstrated by the immunosensing probe.

To conclude, taking advantage of the optimized antifouling coating that exhibited high tolerance towards high-concentration of nonspecific protein adsorption coupled with stellar stability and excellent properties to interface biorecognition events, a more traditional detection scheme with enzymatic amplification is being pursued to overcome the limitations demonstrated by the developed immunosensing probe.

Chapter 5. Conclusions and Future directions

5. Conclusions and Future perspectives

5.1. Conclusions

The prevalence of neurodegenerative diseases is expected to keep increasing in the upcoming decades as a result of the continuous aging of the world population. AD and PD are the most common neurodegenerative diseases. AD is associated with most cases of dementia while PD is defined as a progressive movement disorder. Timely and accurate diagnosis of neurodegenerative diseases in clinical practice is a great need, not only to provide patients with diagnostic and prognostic information but also to optimize treatment strategies, providing appropriate support and care while giving patients opportunities to join clinical trials. Therefore, there is urgency in the development of diagnostic tools capable of providing insightful information concerning biomarkers levels in order to help medical teams assess diagnosis, disease progression and effect of treatment strategies.

The biosensing technology is diligently developing towards the production of POC technology capable of performing accurate and sensitive determination of key AD and PD biomarkers in biological fluids. In fact, some of the reviewed works in the state of the art exhibited remarkable sensitivity quantifying concentrations below sub-picomolar levels. Nonetheless, these biosensors were based on highly complex biosensing schemes with time consuming and labor intensive procedures. Another common limitation of the reported biosensors is associated with the biofouling effect upon exposure of the sensing surface to biological samples. In fact, most of the reported studies had to dilute the biological samples, with a high dilution factor, to avoid matrix interferences. These are particularly relevant factors that can compromise the applicability of these laboratory based biosensors in real clinical settings.

The goal of this work was to develop novel electrochemical immunosensors for detection of clinical relevant levels of AD and PD main biomarkers. In addition, with the proposed biosensors, some problems associated with biosensing were addressed by developing biosensors based on fairly simple detection schemes that exhibited sensitivity while maintaining its electrochemical and analytical behavior in complex samples.

In chapter 3, a nanostructured label-free electrochemical immunosensor for detection of α -synuclein was developed. In this work, two different carbon based electrochemical transducers (GCE and SPCE) were studied being their

electrochemical and analytical behavior compared. The transducers were modified in a layer-by-layer approach, which enabled to control the surface properties in each moment favoring the assembly of different nanomaterials. The optimal conditions for the transducers functionalization resulted from the combination of nanomaterials with excellent electrical properties such as SWCNTs and AuNPs, which promoted a synergistic effect to significantly increase the electrochemical behavior of the transducers. In addition, the combination of both nanomaterials increased the stability of the nanostructuration while also providing an appropriate matrix for the immobilization of antibodies. Therefore, a sensitive and specific quantification of clinical relevant levels of α -synuclein, with low LOD and LOQ were attained with both transducers. Moreover, the immunosensor developed with SPCE exhibited a significantly better analytical behavior attaining detection levels compatible with analysis in more accessible biological fluid, such as blood. In addition, SPCE also exhibited a more reproducible behavior while only requiring small volumes to conduct the immunosensor development and biorecognition events. These are essential characteristic for integration into POC devices.

The main objective of chapter 4 was to develop an electrochemical immunosensor based on a sandwich detection scheme towards detection of A β 42. With this work, two main biosensing problems were addressed: development of an antifouling matrix capable of resisting non-specific protein adsorption and development of an immunosensing probe for signal amplification through simpler and more cost-effective procedures.

The proposed antifouling coatings were based on modifications that did not passivate the transducers surface while presenting excellent ability to prevent protein adsorption. From this study, it was possible to conclude that the antifouling coating that resulted from the covalent bonding of PEG functionalized with amine and maleimide groups to the SPCE functionalized with carboxylated SWCNTs exhibited an overall better behavior. In fact, this coating coupled excellent properties to resist protein adsorption with a high stability to different stimuli. In addition, the functional groups on the PEG molecule make this coating ideal to interface biorecognition events by promoting covalent immobilization of thiolated antibodies.

To complete the sandwich detection scheme, a novel amplification strategy based on colloidal AuNPs was developed. The immunosensing probe consisted of AuNPs

modified with monoclonal antibodies specific towards A β 42 and labeled with Pb²⁺ ions in order to produce an electrochemical signal specifically related to the concentration of the analyte of interest. Therefore, with the optimized immunosensor, it was possible to specifically quantify clinical relevant levels of A β 42 while exhibiting low LOD and LOQ. All of these indicators were achieved through the use of simple experimental procedures. Nonetheless, despite presenting some positive results towards detection of A β 42, the developed immunosensing probe presented major problems related with lack of reproducibility and stability. Consequently, the development of a calibration curve with a wide concentration range or the possibility of validating its analytical parameters was severely hampered by the highly variable electrochemical and analytical behavior demonstrated by the immunosensing probe. Therefore, in order to overcome this problem a more traditional detection approach is being developed based on an indirect detection scheme with enzymatic amplification.

The developed electrochemical immunosensors presented interesting characteristics to be applied in the detection of clinical relevant levels of the analytes of interest. They addressed common biosensing problems by achieving the detection of low levels of AD and PD biomarkers with simple, low-cost and fast detection processes. Nonetheless, there are experimental procedures that need further studies to minimize the exhibited inconsistencies.

5.2. Future perspectives

Future studies should be focused on the development of technology capable of bridging the gap between studies and clinical application. Therefore, as result of the multifactorial nature of AD and PD, it is imperative that future work focuses on the development of immunosensors capable of simultaneously detect more than one biomarker. In fact, the development of single platforms capable of performing multiplexed analysis with excellent sensitivity, specificity and stability, while achieving all these characteristics through the use of lower sample volumes and with shorter detection periods, holds enormous potential for early detection and personalized therapy.

The goal of multiplexed detection can be accomplished with two different strategies: using multi-electrode arrays where each immunoreagent is attached to each

electrode, or by means of barcode configurations involving a single electrode platform and different electroactive labels with dissimilar electrochemical properties for each analyte.

In the case of multi-electrode arrays, SPEs hold great promise since they can be printed in a wide range of materials and formats and, therefore adapted towards detection of a wide range of biomarkers. On the other hand, for the successful determination of multiple analytes through a barcode configuration, sensitive and stable labels need to be developed. Magnetic, metallic or liposomal particles containing enzymes, fluorophores or electrochemiluminescent tags can be excellent options to accomplish this goal. Moreover, the use of magnetic particles can minimize non-specific binding events by separating specifically bound particles by the application of larger magnetic or fluid-shear forces that will further test the strength of the biochemical bonds.

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