TALLINN UNIVERSITY OF TECHNOLOGY
SCHOOL OF ENGINEERING
Department of Materials and Environmental Technology

BRAIN DERIVED NEUROTROPHIC FACTOR-IMPRINTED POLYMER ON SCREEN-PRINTED ELECTRODES: TOWARDS ANALYSIS OF CLINICAL SAMPLES

MOLEKULAARSELT JÄLJENDATUD POLÜMEERIGA MODIFITSEERITUD ELEKTROODID AJU-PÄRITOLUGA NEUROTROOFSE TEGURI MÄÄRAMISEKS KLIINILISTES PROOVIDES

MASTER THESIS

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Thesis main objectives:

1. Electrosynthesis of poly(m-phenylenediamine) film on the working surfaces of array of 96 SPE (96xSPE) in the presence of the template molecule, Brain-derived Neurotrophic Factor (BDNF) protein that was covalently immobilised to the surface of SPE.
2. Generation of BDNF imprints in the polymer by cleavage and washing out the BDNF from the polymer matrix using selected reagents aiming to form BDNF molecularly imprinted polymer (BDNF-MIP).
3. Electrochemical characterization of the formation of BDNF-MIP on 96xSPE using CV and EIS to confirm polymer electrodeposition and template removal from the matrix.
4. Study of the binding performance of BDNF-MIP/96xSPE sensor towards BDNF in plasma.

Thesis tasks and time schedule:

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1.	Electrosynthesis of poly(m-phenylenediamine) film on the working surfaces of 96-well screen printed electrodes (SPE) in the presence of the template molecule, Brain-derived Neurotrophic Factor (BDNF) protein that was covalently immobilised to the surface of SPE.	Aug 2022
2.	Generation of BDNF imprints in the polymer by cleavage and washing out the templates from the polymer matrix using selected reagents.	Sep 2022
3.	Electrochemical characterization of the formation of BDNF imprinted polymer (BDNF-MIP) on 96xSPE using CV and EIS to confirm polymer electrodeposition and template removal from the matrix.	Oct 2022
4.	Study of the sensors' performance in the analysis of blind plasma samples.	Nov 2022

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CONTENTS

Preface

This research was carried out at the Biofunctional Materials laboratory, Department of Materials and Environmental Technology, Tallinn University of Technology. This thesis has been written to fulfil the graduation requirements of the university.

This thesis was supervised by Dr. Vitali Sõritski and it was co-supervised by Dr. Akinrinade George Ayankojo. I want to sincerely appreciate Dr. Akinrinade George Ayankojo for taking me through every process, detailed explanation and mentorship and Dr. Vitali Sõritski for their support and patience all through the stages of writing this thesis. Also, I would like to appreciate Dr. Roman Borozнjak for his help, support and friendliness all through the experimental process.

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Ayomide Deborah Olumide

Keywords: Molecularly imprinted polymers; brain-derived neurotrophic factor; screen-printed electrodes; electrochemical sensor; master thesis.

List of abbreviations

96xSPE	Array of 96 screen-printed electrodes
AA	Acetic Acid
AD	Alzheimer's Disease
AE	Auxiliary Electrode
ATP	4- aminothiophenol
BDNF	Brain-derived Neurotrophic Factor
BDNF-MIP	Brain-derived neurotrophic factor- Molecularly Imprinted Polymer
BDNF-MIP/96xSPE	Brain-derived Neurotrophic Factor-Molecularly Imprinted Polymer /96x Screen Printed Electrode
BDNF-MIP/SPE	Brain-derived Neurotrophic Factor-Molecularly Imprinted Polymer /Screen Printed Electrode
CDNF	Cerebral Dopamine Neurotrophic Factor
CV	Cyclic Voltammetry
DPV	Differential Pulse Voltammetry
DTSSP	3,3'-dithiobis(sulfosuccinimidyl propionate)
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme-linked Immunosorbent Assay
HSA	Human Serum Albumin
ME/EtOH	2-mercaptoethanol in ethanol
MIP	Molecularly Imprinted Polymers
mPD	meta-Phenylenediamine
ND	Neurotrophic Disorder
NF	Neurotrophic Factor
PmPD	Poly(meta-Phenylenediamine)
RE	Reference Electrode
SPE	Screen Printed Electrode
WE	Working Electrode

INTRODUCTION

Neurodegenerative diseases (NDs) are a group of progressive disorders that result in the gradual loss of nerve structure and function. This process occurs naturally with ageing but recent research has indicated that even younger populations are not immune from the disease as they may also be at risk in certain circumstances [1, 2]. NDs, including Alzheimer's, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis, and multiple sclerosis are becoming prevalent in clinical practice [3, 4]. Given the precarious nature of the diseases, early diagnosis combined with the current treatment regime can delay hospitalisation [5]. This is especially essential since clinical diagnosis following the appearance of symptoms usually delay the treatment process. In this regard, detection of biomarkers, which are distinctive molecules in biological fluids, involved in neurodegeneration processes, has the potential to allow early diagnosis of neurodegenerative diseases.

Brain-derived neurotrophic factor (BDNF) probably plays a significant role in the pathological state of ND. As a result, BDNF has received attention as one of the potential biomarkers for ND and has undergone extensive research. In fact, BDNF levels in the blood decrease in ND patients [6, 7]. However, some investigations have reported contradictory results regarding the levels of circulating BDNF in ND patients [7, 8], which raises concerns about its usefulness as a blood biomarker [9].

The identification and quantification of NF proteins in blood can now be done using a variety of analytical techniques, such as the enzyme-linked immunosorbent assay (ELISA), liquid chromatography-mass spectrometry, fluorescence spectroscopy, size-exclusion chromatography, and protein immunostaining [10, 11]. All of these techniques, despite being renowned for their extremely accurate analytical measurements, have a number of drawbacks, such as the need for costly analytical grade solvents and unique analytical conditions, the perception that they are generally too slow when a quick response is required, and the fact that the equipment typically requires a highly skilled and experienced operator [12, 13].

The potential of biosensors and chemical sensors to provide fast and reliable detection of biochemical markers in human biofluids, such as interstitial fluid, sweat, tears, and human serum, is generating interest in the field of diagnostic applications [10, 14]. A biosensor is an analytical device consisting of a biological or biologically derived sensing element that is either integrated with or directly connected to a physicochemical transducer [15]. Devices that use optical, piezoelectric, and electrochemical transducers to detect analytes have advanced dramatically during the past 50 years [16]. The

development of biosensors is arguably one of the most promising approaches to provide an accurate, quick, and affordable measurement tool for clinical diagnostics [17].

Early biosensors were catalytic systems that combined enzymes in particular with transducers that convert biological responses into electronic signals [18]. The subsequent generation of biosensors, known as affinity biosensors, benefited from various biological receptors , including antibodies and nucleic acids [19].

According to a clinical scientist, the ideal analytical tool for the quantitative detection of protein should be straightforward, call for a limited number of processes, and be resistant to environmental changes [20]. Therefore, modern sensor research aims to create synthetic receptors that could offer the molecular recognition ability by mimicking the interactions between antibodies and antigens while capable of withstanding the harsh environmental conditions [21]. By building a polymer network around a target molecule of choice that serves as a template, molecularly imprinted technology is utilised to produce synthetic receptors known as molecularly imprinted polymers (MIP) [20, 22, 23]. Due to their selectivity, specificity, biological stability, and adaptability (e.g., surface chemistry modifications and/or signalling functionalities), MIPs are relatively inert materials that can be used as inexpensive artificial receptors for biological sensor purposes. They also have exceptional physicochemical properties and a long shelf life [24, 25]. Employing these material characteristics would enable more stable synthetic receptors for biomarker detection as compared to biological receptors. MIPs have previously been studied for several target analytes, including proteins (such as immunoglobulins) [26], enzymes [27], and small molecules (such as antibiotics) [27]. Kidakova et al., researched on BDNF-imprinted polymer (BDNF-MIP) on MIP-based synthetic receptor for BDNF prepared by photopolymerization and integrated with SPE sensor for rapid Point of Care (POC) medical diagnostic purposes [22]. According to the study's findings, the BDNF-MIP/SPE electrochemical sensor was able to distinguish between BDNF and its structural counterparts, the neurotrophic factors CDNF and MANF, down to 6 pg/mL in the presence of the interfering HSA protein [29, 30].

There have been several studies on the effectiveness of protein-MIPs when integrated into several label-free sensors such as screen-printed electrodes (SPEs) [16], surface plasmon resonance [17], and quartz crystal microbalance [31]. However, one of the most often used MIP-based sensors is the electrochemical sensor. Due to their outstanding performance, portability, ease of use, and low cost, electrochemical sensors are becoming used in a range of analytical, medical diagnostic, and screening applications. A well-established technology for developing chemical and biosensors is screen printing. Screen-printing technology is a tool for generating electronic sensors that are quick, affordable, on-site, real-time, and cheap, for use in healthcare,

environmental monitoring, industrial monitoring, and agricultural monitoring [32, 33]. The technology is compatible with mass production and enables the fabrication of arrays of SPEs, thus providing an opportunity for multiplexed detection of analytes [34]. Till date, MIPs for biosensing have mostly concentrated on imprinting single species and then optimising, assessing selectivity in the presence of structurally homologous species, and improving performance. However, the majority of early disease detection techniques rely on simultaneously identifying a number of biomarkers that are present in the same media. The therapeutic value of MIP-biosensors can be increased by the design and development of methods for the creation of multiplexed sensing platforms or arrays of disease-relevant biomarkers [35].

In this thesis, the focus is to fabricate a sensor having BDNF-MIP layers integrated with 96xSPE for detecting BDNF in human plasma. The electrosynthesis technique makes it possible to successfully integrate BDNF-MIP films onto the SPE using an electro polymerizable functional monomer. The clinically relevant neurotrophic protein used in this thesis, BDNF, was used as a target molecule for molecularly imprinting to generate BDNF-MIP. The ability of the BDNF-MIP to bind to the target protein was assessed by electrochemical techniques such as cyclic and differential pulse voltammetry. In order to accomplish the aim of this thesis, the following four objectives must be fulfilled: electrosynthesis of poly(m-phenylenediamine) film on the working surfaces of 96-well SPEs in the presence of the template molecule - BDNF protein covalently immobilised on SPE; generation of BDNF imprints in the polymer by cleavage and washing out the BDNF from the polymer matrix using selected reagents aiming to form BDNF molecularly imprinted polymer (BDNF-MIP); electrochemical characterization of the formation of BDNF-MIP on 96xSPE using CV and EIS to confirm polymer electrodeposition and template removal from the matrix; study of the binding performance of BDNF-MIP/96xSPE sensors towards BDNF in human plasma samples.

1. THEORY AND LITERATURE REVIEW

1.1 Brain-Derived Neurotrophic Factor (BDNF)

1.1.1 BDNF as a neurotrophin

Neurotrophins belong to a family of neurotrophic factor (NF) proteins that cause survival, development and improve functions of neurons [36]. Neurotrophins can also be seen as a class of growth factors that secretes proteins that are capable of signalling particular cells in the body. They have a crucial role in the growth and survival of neurons, as well as in the development of the mature nervous system [37]. They also keep synaptic plasticity intact and aid in the development of selective memories. According to the most recent research, NFs are linked to the emergence of multiple neurodegenerative conditions, including Parkinson's, Alzheimer's, and a number of other mental diseases [36].

Nerve growth factor (NGF), the first isolated neurotrophic factor in its purest form, was found before Brain-derived neurotrophic factor (BDNF) [38]. The NF family also comprises numerous additional proteins, including mesencephalic astrocyte-derived neurotrophic factor (MANF), cerebral dopamine neurotrophic factor (CDNF), ciliary neurotrophic factor (CNTF), and glial cell-derived neurotrophic factor (GDNF) [4, 37].

BDNF is a neurotrophin that supports the development, survival, and activities of neurons [40]. By boosting neurotransmission across synapses, stimulating neurogenesis and synaptic development, and regulating synaptic plasticity, BDNF also affects cognition and memory. In humans, BDNF is a protein that is a part of growth factors which are related to the nerves growth. It is found in the brain and peripheral nervous system. The motor neurons, kidneys, retina, saliva, and skeletal muscle, blood are all found to contain BDNF protein [38, 39]. BDNF is also found in the nerves controlling muscles, kidney, prostrate and saliva [7]. It was discovered that neurodegenerative diseases like Huntington's [43], Parkinson's [44], and Alzheimer's disease [45] related to the level of BDNF in the patient's serum [13]. Both early-onset and late-onset Alzheimer's disease were associated with considerable declines in serum levels of BDNF [44].

BDNF is an oval-shaped protein with dimensions 59x31 Å with a molecular weight of 27 kDa and an isoelectric point (Ip) of 9.43 [46] (Fig. 1.1). Six tyrosines, nine serines, thirteen threonines, and two aspartic acid residues [46] are present in BDNF and can interact noncovalently with the amine groups of a functional monomer to form a complex [47]. In exchange, the asparagine, glutamine, and glutamic acid residues on the surface of BDNF can bind noncovalently with the hydroxyl groups, carboxylic oxygens, and amino

groups of a functional monomer [48]. BDNF has around 50% amino acid similarity with NGF, NT-3, and NT-⅘. Each neurotrophin is a noncovalently 1-linked homodimer that includes an N-linked glycosylation site and a signal peptide that comes after the initiation codon [37].

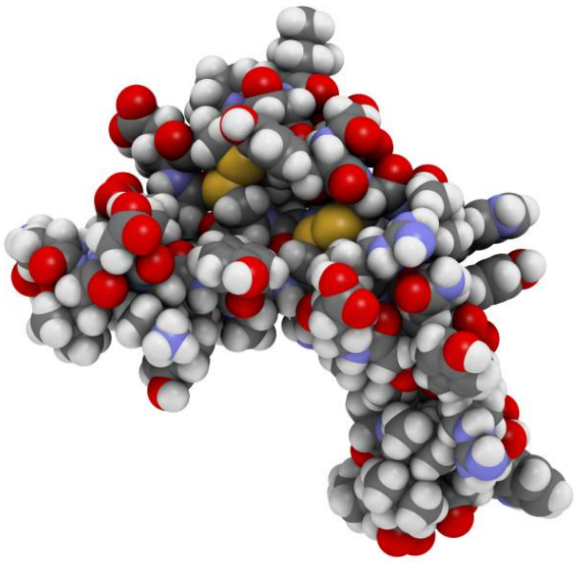

Figure 1.1 3D structure of BDNF(1B8M.pdb)

1.1.2 BDNF as a biomarker

NF proteins are possible biomarkers for early diagnosis and subsequent neuroprotective treatment because changes in the amount of NF concentration in the serum and cerebrospinal fluid may be linked to the phases of development of neurological illnesses [49]. Numerous studies have linked BDNF levels to a variety of ailments that influence how the brain functions, such as depression and neurodegeneration (Alzheimer's and Parkinson's diseases) [50]. In numerous neurodegenerative conditions, including Parkinson's disease [44], Huntington's disease [43] and multiple sclerosis (MS) [51], the levels of BDNF are decreased. As one of the potential biomarkers for AD, BDNF has garnered interest and has undergone extensive research. Mori’s group studied patients with mild cognitive impairment caused by AD to see if serum BDNF could be a viable blood biomarker for AD [45]. The results show that a possible biomarker for the early detection of AD is decreased serum BDNF. In addition, BDNF level correlates with amyloid beta peptides aggregation in the brain, which invariably means that it may be employed as a biomarker for early AD diagnosis in clinical practice[52]. However, due to methodological flaws, certain research [6, 10] have shown contradictory results on circulating BDNF levels in AD patients.

Furthermore, a research carried out revealed that the neurobiological changes that may otherwise result in schizophrenia or depression appear to be significantly influenced by

BDNF [40, 41]. The results demonstrated that serum BDNF levels were found to be strongly linked with the progression of severe schizophrenia and depression disorders. In addition, it is suggested that blood-based BDNF levels could be used as a potential biomarker to detect the progress or prognosis of Repetitive Transcranial Magnetic Stimulation (rTMS) treatment (schizophrenia and depression) [50, 51].

In a subsequent study, BDNF significantly influences the development of the cardiovascular system. According to recent research, BDNF negatively affects subclinical cardiac remodelling in people who have risk factors for cardiovascular disease [48]. Result from this research shows a significant molecular linkage between the serum BDNF level and cardiovascular function, hence, BDNF could be used as a potential biomarker clinical diagnostics. Recent studies show that the mean plasma BDNF level was found to be ~92.5 pg/ml (8.0 – 927.0 pg/ml) and seen to be higher in women and lower as people got older, regardless of gender [55]. Previous research also shows that BDNF levels in plasma may be utilised as a biomarker to identify autism in its early stages [56].

In this thesis the fabricated BDNF-MIP sensor was used to detect BDNF in human plasma. Therefore, a short description of human plasma function and composition is provided in the next paragraph.

The blood is made up of red blood cells which is about 45% and the remaining 55% of the blood is made up of liquid. This liquid part is called the plasma. Plasma is made up of several vital proteins such as anti-hemophilic factor, albumin, gamma, and globulin [57]. These proteins make up 7% of the plasma while 1% of the plasma contains hormones, vitamins, sugar and mineral salt. 92% of the plasma is also made up of water [58]. Plasma plays a vital role in the body by maintaining blood volume and pressure, it carries electrolytes such as potassium and sodium to the muscles and also bicarbonate calcium and chloride helps in the maintenance of a stable pH balance in the body [12], [59]. Meanwhile plasma comprises critical proteins such as coagulants for blood clotting and immunity [60].

1.2 The need for sensor generation

Early diagnosis of neurodegenerative diseases is very essential as it enhances the optimization of treatment strategies and provision for adequate support and care because biomarkers help to ease the process of monitoring and development of productive disease-modifying treatments in neurodegenerative diseases [37, 58]. In order to diagnose diseases at an earlier stage, biological compounds known as biomarkers can be utilised to detect the presence or beginning of many disorders [47, 59].

The common drawbacks of the methodology, such as the quality of the purchased antibodies, the dependability of the immobilisation techniques, the orientation of the antibodies on the transducer, difficulties in obtaining results in biological matrices, and the lifespan and storage of manufactured devices, cause many of these to exhibit reduced sensitivity, selectivity, or reproducibility [50, 61]. Since many of these issues are caused by the usage of biological receptors such as antibodies, efforts have been made to replace them with artificial recognition components known as Molecularly Imprinted Polymers (MIPs). When compared to the usage of biological recognition components, these polymers have a number of potential advantages, including greater chemical and temperature stability, the flexibility to cheaper production and easier to customise the synthesis to the target molecule [22, 50].

Currently, traditional techniques such as western blotting and ELISA are used to measure the quantity of neurotrophins in serum. Likewise, at the moment, only commercially available ELISA can quantify BDNF in plasma, necessitating blood collection in a clinic, processing, specific tools, and technical expertise for analysis. These multi-step and costly analytical techniques require highly skilled analysts and specialised work environments [60, 61]. Therefore, it is necessary to create a straightforward alternative technique for the quick measurement of BDNF in tiny amounts of peripheral blood without the use of expensive specialist equipment or high-level technical expertise. Herein, 96xSPE interfaced with a MIP-based recognition element could serve as a promising alternative to ELISA-based method detecting BDNF in peripheral blood samples.

1.3 Molecularly imprinted polymer (MIP)

Molecular imprinting is one of the most effective techniques for producing materials with antibodies-like properties for binding and discriminating between molecules [65]. This technique involves generating molecular recognition in a synthetic polymer matrix, resulting in an artificial receptor called a MIP. Dickey published the first English report on MIPs in 1949 [66], introducing some concepts still in use in MIP development, such as identifying the target molecule as a "template." Mosbach's group introduced non-covalent imprinting in the 1980s, which is more commonly seen in current research [65]. MIPs has well built inter-linked polymers which have a biological-like structure that recognises the presence of unique sites that are supportive to a target molecule [67]. The primary advantages of MIPs stem from their synthetic nature, which includes exceptional chemical and thermal stability along with their repeatable and affordable manufacture [67]. Currently, this strategy is frequently employed as a quick and flexible method for creating synthetic receptors for both big biological macromolecules and tiny chemical substances [4, 30]. However, some MIP drawbacks, such as insufficient

template removal, high non-specific adsorption, low affinity and poor conductivity, may restrict their use in the sensor industry [24, 67].

MIPs have been implemented in a variety of shapes, including membranes, thin layers, nanoparticles, regularly spherical particles, irregularly ground particles, composites, etc. MIPs are therefore used in a wide range of applications, including as sensors, chromatography, catalysis, sample pre-treatment, purification, and medication delivery [69]. Numerous studies have been conducted on the use of MIP-based sensors for the detection of various proteins.

1.3.1 Principle of molecular imprinting

The formation of a MIP generally involves the combination of functional monomers with a template molecule, leading to the formation of covalent or non-covalent interactions between the functional monomers and the template in a pre-polymerization complex [70]. When it comes to creating synthetic polymers, functional and cross-linking monomers are copolymerized to create a tightly cross-linked polymer lattice surrounding a target molecule that serves as a molecular template [68]. The subsequent extraction or removal of the template from the polymer matrix leaves behind designated binding cavities that are identical to the template in size, shape and arrangement of functional groups (Figure 1.2).

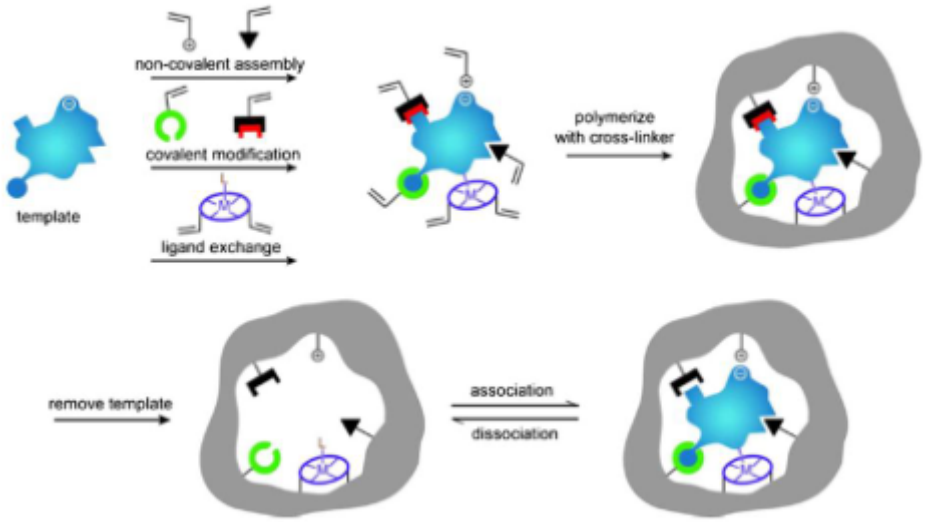

Figure 1.2. Schematic representation of the general principle of molecular imprinting process [72].

Covalent, electrostatic, Van der Waals, hydrophobic, and metal-coordination interactions are only a few examples of the interactions that might occur during affinity creation at the template active site [73]. Covalent and non-covalent interactions are two different methods of molecular imprinting.

Covalent imprinting

The use of templates that are covalently linked to one or more functional groups of the monomer distinguishes covalent imprinting from other types of imprinting. Following polymerization, the template is removed by chemical cleavage of the reversible covalent bond in order to create the imprinted cavities in the polymer. During subsequent rebinding the functionality that is present in the binding site reestablishes the covalent link with the target molecule [69, 70]. With this strategy, the functional groups are only linked to the template molecule, which is advantageous. This method is not appropriate for applications needing quick binding kinetics because of the slow kinetics of template rebinding [74] [75]. However, this method can only imprint a small subset of substances, including alcohols (diols), aldehydes, ketones, amines, and carboxylic acids.

Non-covalent imprinting

The non-covalent approach's simplicity is its main draw because non-covalent forces including H-bonding, ion pairing, and dipole-dipole interactions are used to produce the template monomer complex. This approach relies on several weak interactions between the templates and functional monomers [72, 73]. This approach simplified the removal procedure compared to covalent imprinting since no chemical connections were formed and then broken. However, the disadvantage is an inescapable inhomogeneity of the discovered binding sites [66, 74]. This is due to the fact that many complexes between the functional monomers and template were formed during the early stages of polymerization.

In non-covalent MIP synthesis, the functional monomer should be chosen based on the interactions created by the template due to the functional groups in the chosen template [72, 75]. The multi-interaction between the template molecule and functional monomers has been enhanced by the combination of two or more functional monomers that are complementary to various areas of the template. The kind and quantity of functional groups that are present on the surface of the protein further take part in the formation of a complex with functional monomers that are of significant relevance when using BDNF as a template molecule for molecular imprinting [76, 77].

The target molecule that the MIP is intended to detect specifically influences the choice of templates for molecular imprinting. The templates can be small organic or inorganic compounds like metal ions, insecticides, antibiotics, or biological molecules like proteins, enzymes, amino acids, antibodies, bacteria, or viruses[82]. Functional monomers, crosslinkers, and initiators are the three fundamental components used in the synthesis of MIPs. The choice of a functional monomer is essential for creating extremely selective cavities for the template molecule [83]. The strongest interactions between the

functional groups of monomers and a template molecule are taken into account while choosing the best monomers for a noncovalent MIP synthesis [84]. To guarantee a high binding capacity for the target molecule, a sufficient number of functional monomers is needed [73, 81]. A more non-specific interaction site typically results from an excessive use of monomers. The composition of the template/analyte and its functional monomer will determine how the template and functional monomer interact [74, 82].

The cross-linker aids in controlling the MIP matrix's shape. As a MIP reagent, the cross-linker plays a crucial part in the creation of MIP. The function of the cross-linker's in MIP is to contribute to the development of the physical properties of polymers [78, 79].

1.3.2 Synthesis methods

Several synthesis techniques, including the sol-gel method [87], surface imprinting [88], and solid-phase synthesis [89], have been developed to manufacture MIPs. Spin coating, surface-initiated polymerization, and direct electrodeposition can all be used to create MIP films on planar surfaces [15, 90]. Electropolymerization is suitable for electrically conductive surfaces such as gold, silver or graphite. Since a polymer can be directly deposited on the sensor transducer and the thickness of the polymer layer can be easily controlled by the amount of electrical charge passed through the electrode, electropolymerization has grown in popularity as a method for the synthesis of MIP films, particularly for sensing applications [77, 91]. By carefully choosing the experimental parameters, such as the quantity of electric charge transmitted, the solution pH, and the kind of electrolyte, the characteristics of the polymer coating, such as porosity and morphology, may be simply regulated [92]. An original synthesis strategy for molecular imprinting of macromolecules such as protein, i.e protein-MIPs, was developed by Syritski’s group [26] strategy is based on the surface imprinting approach and utilises the covalent immobilisation of a target protein on the transducer surface via a cleavable linker, the electrodeposition of a thin polymer film and subsequent removal of the protein by destruction of the cleavable linker [93]. In this strategy it is crucial to control the electrodeposited polymer thickness to prevent the protein from becoming trapped in the polymer in order to effectively convert the binding event of the protein to the resulting MIP into useful analytical signals, [73, 90]. In this study, MIP films with selective recognition cavities for BDNF (BDNF-MIP) were created according to this surface-imprinting strategy by electropolymerization of m-phenylenediamine (mPD) as a functional monomer.

1.4 MIP-based sensors

The MIPs can be connected to a sensor "reporter" system to provide a tool for analyte detection or monitoring the environment [95]. A crucial component of the design of a MIP-based sensor is the robust integration of a MIP with a sensor platform capable of responding upon interaction between a MIP and a binding analyte [76, 92]. Surface plasmon resonance, surface acoustic wave, voltammetry, impedance spectrometry, fluorescence, and chemiluminescence spectroscopy are just a few of the transduction techniques that can be used in combination with MIP films as recognition elements [15, 93]. Based on the physical characteristics of the sensor transducer, MIP sensors can be categorised into a wide range of subtypes, including electrochemical, optical, piezo gravimetric and thermal sensors [74, 94]. It is critical to establish the perfect interaction between the MIP film as a recognition element and the sensor transducer when creating a MIP for a chemosensor. MIP films can presumably be synthesised using the surface-initiated polymerization process, which also aids in the reliable adherence of the polymer film [72, 95] to the sensor surface. Pereira et al. [81] has investigated MIP receptors to selectively bind amyloid peptides, a biomarker for Alzheimer's disease. In addition, it has been shown that MIP-modified sensors can detect cardiovascular disease biomarkers (myoglobin) [96, 97] and cardiac troponin T as [102] as well as cancer biomarkers (such as prostate specific antigen [103], epithelial ovarian cancer antigen [95], and carcinoembryonic antigen [104].

An enhanced sensing device for the identification of biomarkers linked to NDs was created by combining the MIP with a transducer. The development of MIP-based sensors for the detection of NF proteins, such as BDNF [28] and CDNF, was studied recently [28]. Additionally, MIP-based sensors have been studied for the detection of viral proteins as the gp51 glycoprotein of the bovine leukemia virus, the NS1 protein of the dengue virus, and the glycoprotein of HIV type 1 [87, 88]. In a previous research, surface imprinting and nanotechnology were used to create an electrochemical sensor that is sensitive and selective for the detection of prostate specific antigen [95]. In addition, based on a novel plastic antibody with charged binding sites to improve protein binding a potentiometric biosensor for the detection of prostate specific antigen was developed [94]. Silva’s group [96] developed a unique electrochemical sensor for the measurement of trimethoprim by pyrrole electropolymerization and MIP, which were produced onto a glassy carbon electrode using cyclic voltammetry. Research was carried out to develop molecularly imprinted electrochemical sensors for phenobarbital measurement on the basis of electrodes enhanced with nickel nanoparticles [14].

1.4.1 Electrochemical sensors

In MIP-based electrochemical sensors a MIP film is generated on the surface of the working electrode, which is used in an electrochemical cell containing also the counter and reference electrodes. Rebinding analyte molecules to the MIP film influences the charge exchange between a test solution and MIP-modified working electrode resulting in the change of electrical signals (usually currents) [15]. Thus, an analyte molecule concentration can be correlated with the current magnitude. There are several electrochemical techniques which are used to assess the permeation of redox active markers, these techniques are CV, EIS, and DPV [85]. The electrochemical sensors offer several advantages over other sensors that have been utilised to create MIP-based sensors, including portability, high sensitivity, low cost, ease of usage [29, 30].

In this study, a MIP layer was combined with screen-printed electrodes (SPE), a low-cost electrochemical transducer, and DPV was used to measure the sensor response.

Screen-printed electrodes

SPE is an electrochemical device composed of a substrate made of a chemically inert material, such as plastic or ceramic, and three electrodes: a working electrode (WE), a reference electrode RE), and a counter electrode (CE) Figure 1.4 [105]. Silver ink and carbon ink are the two pastes that are most frequently used for printing SPEs. While other materials, such as gold, platinum inks etc., can also be used to manufacture SPEs based on requirements and applications. When working with enzymes, proteins, etc. gold electrodes SPEs are preferable [30]. Due to SPE's flexibility, it is possible to modify its sensing (working) electrode in a variety of ways including the attachment of biological molecules or deposition of MIPs [22, 23].

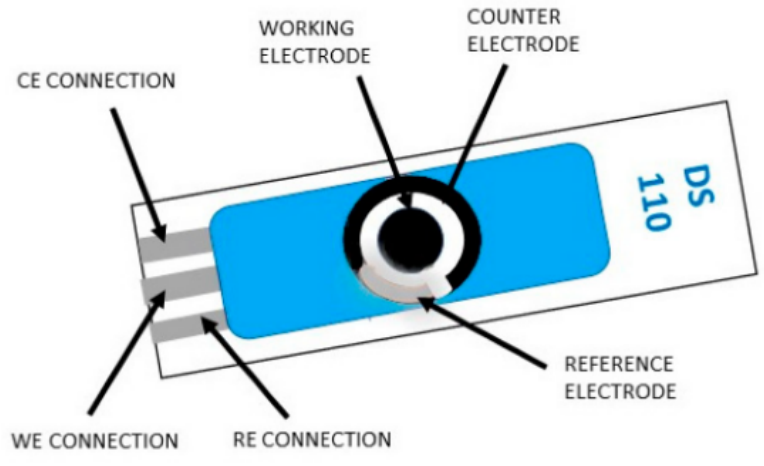

Figure 1.4. Typical screen-printed electrode [105]

SPEs are disposable, inexpensive, portable [97], and allow to reduce sample volume to microliters as well as the overall size of the diagnostic device. Thus, SPEs are ideal for designing portable devices that provide quick response and are suitable for on-site analysis [32]. SPEs are greatly compatible with multiplexing, when an array of SPEs is used, large-scale manufacturing and are easy to combine with microfluidic systems. Applications of an array of SPEs allow to simultaneously detect different analytes. An example of an array with 96 SPEs (96X SPEs, DROPSENS) used for this research is shown in Fig. 1.5.

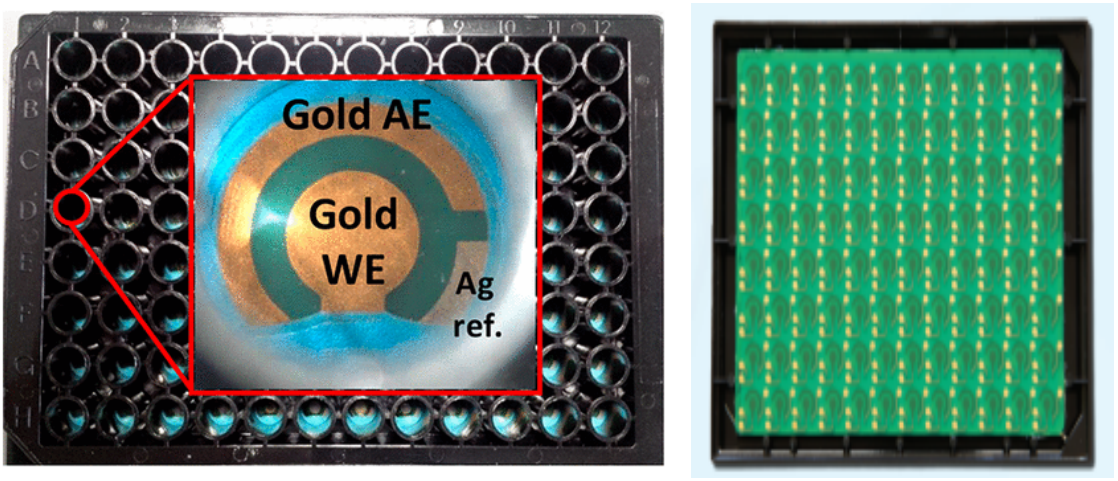

Figure 1.5. 96XScreen-Printed Gold Electrode, DROPSENS [106].

1.5 Experimental methods

1.5.1 Cyclic voltammetry

Cyclic voltammetry (CV) is an effective electrochemical technique frequently used to study the reduction and oxidation processes of molecular species. The study of chemical reactions involving electron transfer, such as catalysis, is greatly aided by CV [107]. It is an efficient technique for quickly determining the thermodynamics of redox reactions. [63]. In this technique, the working electrode's potential is linearly ramped up and down cyclically versus a reference electrode while the current between the working and auxiliary electrodes is being measured [108].

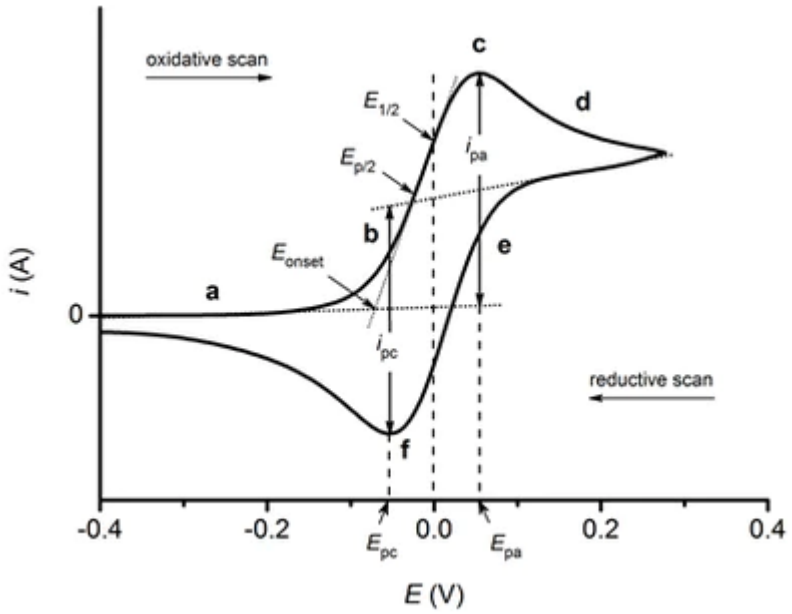

Figure 1.5. (A-F): Initial potential applied (A), Reduction peak (C), Higher potential limit (D), Oxidation peak (F), extracted from [107].

An important relationship that is used to calculate reaction equilibrium constants and concentration potentials is the Nernst equation. The Nernst equation describes the equilibrium that develops between the oxidised (Ox) and reduced (Red) electrodes [109]. The relationship between the potential (E) of an electrochemical cell and the standard electrode potential (E°), as well as the relative behaviour of the oxidised (Ox) and reduced (Red) analytes in the system at equilibrium.

$$E=E^0 + (RT/nF)\ln(Ox/Red) = E^0 + 2.3026(RT/nF)\log(Ox/Red) \quad (1.1)$$

where E° is the electrode's standard reduction potential (for a 1M solution at 298 K); E is the electrode's potential (V); R is the gas constant, which is 8.314 J/mol K, T is temperature, in (K), n is the reaction's stoichiometric number of electrons, and F is the Faraday constant, which is 96 485 Coulombs/mol.

1.5.2 Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is a potent characterization tool that is frequently employed in industries as diverse as energy, electrocatalysis, and medicine. Like other typical electrochemical techniques, EIS examines the relationship between the applied potential difference and the current, but in the frequency domain. At the electrode interface, electrochemical characteristics are measured. This measurement reveals the relationship between the magnitude of the property measured and the concentration of a particular chemical species.

EIS uses ohms to evaluate the impedance of a circuit (as resistance unit). EIS has a number of benefits due to its steady-state nature, tiny signal analysis, and ability to investigate signal relaxations across a very broad range of applied frequency, from less than 1 mHz to higher than 1 MHz, utilising commercially accessible electrochemical working stations (potentiostat). In general, electrochemical impedance is computed by providing an AC potential, measuring the system's current, and then calculating the impedance Z=E/I using a unit of Z in Ω. As a result, the impedance is described in terms of a magnitude, Z_0, and a phase shift Φ. the impedance of the system may determined by using an equation analogous to Ohm's Law:

$$Z = E_t / I_t = E_0 \sin(\Phi t) / I_0 \sin(\Phi t + \Phi) = Z_0 \sin(\Phi t) / \sin(\Phi t + \Phi) \quad (1.2)$$

where E_0 is the signal's amplitude, E_t is the potential at time t, and ω is its radial frequency.

A complex number is used to represent the impedance. There are real and imaginary parts to the equation for Z(ω). The resistors are represented by the real part of the equation, while the input of the capacitors and/or inductors are represented by the imaginary part. If the real part is displayed on the X-axis and the imaginary part is displayed on the Y-axis of a chart, we have a "Nyquist Plot" (Fig. 1.6.).

$$Z(\varphi) = E/I = Z_0 \exp(j\varphi) = Z_0 (\cos\varphi + j\sin\varphi) \quad (1.3)$$

Including:

$$Z = Z' + jZ'' = R - jX$$

$$X = 1/\omega C$$

$$j = \sqrt{-1}$$

where X is the reactance, ω is the applied angular fr, R is the resistance (Ω), and C is the capacitance (F). Two processes can be seen in this graph; the first is represented by a semicircle that represents a charge-transfer-controlled process, where values on the Z' axis give Re and Rct values; the second is a line with a slope of 1, since extrapolating Zw to the X-axis enables the measurement of the Warburg coefficient (σ), which enables estimating the electroactive species diffusion coefficients. The resistances and capacitances in this circuit typically combine in series and/or parallel to represent the various physicochemical and electrochemical characteristics of the system being studied.

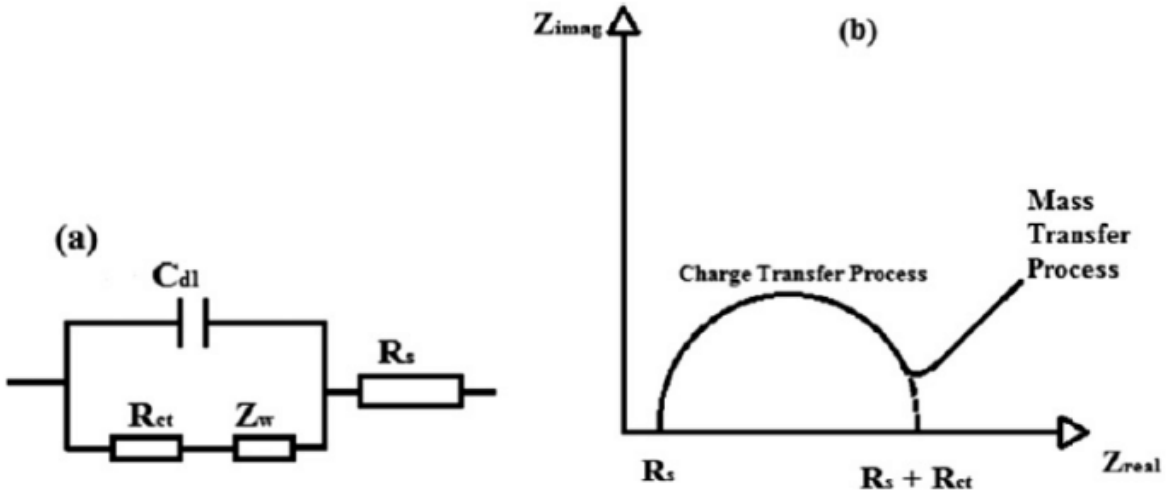

Figure 1.6. (a) Randle 's equivalent circuit. (b) Nyquist plot for the equivalent circuit [110].

The most commonly cited equivalent circuit for interpreting electrical interpretations of EIS experimental data is Randle's equivalent circuit [111], as shown in Figure 1.6 (a). It is made up of charge transfer resistance Rct in series with Warburg impedance (Zw) that reflects the mass transport of electroactive species, double layer capacitance Cdl, and solution resistance Rs connected in series to each other. Re represents the electrolyte resistance at the electrode-electrolyte interface between the working and reference electrodes, Cdl represents the double-layer capacitance, and Zf represents the Faradaic impedance brought about by the charge-transfer process there [112, 113].

1.5.3 Differential pulse voltammetry

Differential Pulse Voltammetry (DPV) is a sensitive pulse technique that enables investigation at the nanoscale while minimising background effects. With this technique, the electrode receives potential pulses after the initial potential has been held for a certain amount of time. The difference between the current readings taken before and after the application of the pulse is immediately recorded. Consequently, the DPV is a graph of the variations between measured currents and applied potentials (Fig.1.7). DPV are more sensitive than linear sweep methods because the capacitive current is minimised.

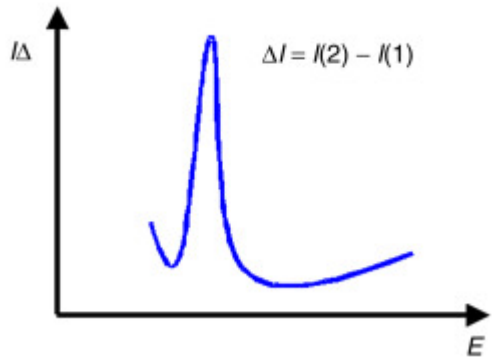

Figure 1.7. The typical response of current from applied pulse voltammogram adapted from [114].

1.5.4 Characterization of MIPs

The choice of functional monomers, the imprint method, the polymerization conditions etc. can all be used to modify the properties of MIPs [115]. To determine whether a MIP matrix indeed consists of molecular imprints of a target analyte, a corresponding non-imprinted polymer is employed as a reference. Non imprinted polymer is prepared and processed in a very same manner as the corresponding MIP, but its synthesis is carried out in the absence of a template molecule. Thus, if molecular imprints have been formed, the chemical and physical differences can be displayed more apparently by various characterization methods [115, 116]. Additionally, MIP has to demonstrate elevated affinity toward a target analyte as compared to non imprinted polymer. Such a characterization of MIP films can be assessed by various bio- and chemo-sensors, where these films are placed to serve as a selective layer. Studying the sensor responses (Q) upon an analyte binding to MIP modified sensor surfaces, it can be concluded whether a MIP indeed prevails in terms of selectivity to a designated analyte. With the aid of theoretical models of binding kinetics, the response obtained from the rebind experiment can be examined for this purpose. The value of the sensor response under equilibrium conditions (Qeq), as well as the reaction rate constant, can be calculated using kinetics models of pseudo-first (Eq.1.4) or pseudo-second order reactions (Eq.1.5) [117].

$$Q=Q_{eq}(1-e-k_1*t) \quad (1.4)$$

$$Q=(Q_{eq2}*k_2*t)/(1+Q_{eq}*k_2*t) \quad (1.5)$$

Qeq is the response upon template rebinding at equilibrium, Q is the response upon template rebinding at time t, k_1 and k_2 are pseudo-first order and pseudo-second order rate constants, respectively. The rate at which adsorption sites are occupied is proportional to the number of available active sites, and the adsorbate binds to only one active site on the adsorbent surface [116]. This is in accordance with the pseudo-first

order kinetics model. The rebinding of the analyte on MIP surfaces is investigated, and the adsorption isotherms are displayed, which is used to compute an imprinting factor [116, 117]. A large value of the imprinting factor (IF) indicates that there are more binding sites with high affinity available in the resultant polymer, which is why it is a common metric used to quantify the imprinting effect.

An adsorption isotherm describes the relationship between the amount of analyte adsorbed on the MIP surface and its volume concentration under equilibrium conditions at a constant temperature and pressure. The adsorption isotherms are fitted to mathematical models such as the Langmuir, Freundlich, and combined Langmuir-Freundlich (LF) models in order to extract the binding characteristics such as the maximum quantity of adsorbed analyte and dissociation constant (K_D).

The Langmuir model is demonstrated as:

$$Q=Q_{max}C/(K_D+C) \quad (1.6)$$

K_D is the equilibrium dissociation constant, while Q and Q_{max} are the responses upon adsorbate rebinding at concentration C and its saturation value, respectively. The Langmuir-Freundlich (LF) isotherm model (Eq. 1.7) is frequently used to explain a heterogeneous adsorption system [115].

$$Q= Q_{max}C^m/(K_D+C^m) \quad (1.7)$$

The LF isotherm, which combines the homogeneous Langmuir and heterogeneous Freundlich models, enables the analysis of several systems across a wide range of concentrations. The heterogeneity index, m, can vary from 0 to 1, and if it is more than 1, the material is heterogeneous [115].

1.6 Summary of the literature review and study objectives

Neurodegenerative disorders are among the most prevalent medical conditions affecting a vast number of people globally. As a result, related pathological conditions like dementia, depression, brain damage, stroke, Alzheimer's, and Parkinson's diseases are frequently seen in neuropsychiatric practice. Blood proteins have been demonstrated to be biologically significant macromolecules since their discovery, and their presence in serum may be a key biomarker for an organism's pathological condition. In particular, the serum levels of NF proteins, which are connected to neural activities, have frequently been related to a number of neurological and psychiatric diseases. For instance, a shift in the concentration of BDNF, a neurotrophin frequently connected to neuronal protection and development, has been linked to the onset of AD [5] and reduced cognitive function in schizophrenia.

The current detection techniques for the identification and quantification of analytes in a sample or a combination of samples require the use of expensive, specialised, and high-grade analytical equipment which can be challenging to maintain. Therefore, it is urgently necessary to develop easy, cheap and reliable analytical tools for the quick detection of biomarkers of these neurological illnesses in order to improve early stage diagnosis and expedite treatments.

A potential analytical tool for protein detection might be a sensor consisting of synthetic receptors acting as recognition elements because they are less expensive, simpler to create, and more stable than biological receptors. Molecular imprinting is widely acknowledged as a promising technique for creating such unique recognition elements, so called molecularly imprinted polymers (MIPs). Target-directed high affinity, reproducibility, ease of manufacture, remarkable chemical and thermal durability, and low cost make MIPs advantageous recognition layers. Using a surface imprinting technique, the MIP can be electrochemically produced as a thin film directly on the conducting surface of a sensor transducer. The electrochemical approach offers a direct, easy, and quick alternative for producing homogeneous polymer films on conducting electrode surfaces at room temperature among the several techniques available for synthesising and integrating MIPs onto sensor platforms. Additionally, electrosynthesis offers good control over the polymer thickness, a crucial aspect in protein-MIP research. This is highly important since a protein-MIP based sensor's analytical performance and repeatability could both be significantly impacted by the thickness of a polymer layer. Furthermore, the use of an array of 96 SPEs in a single plate allows for a multiplex sensor preparation as well as the simultaneous analysis of different samples, thereby saving time and reducing errors from batch to batch.

By harnessing the advantages of a synthetic receptor such as MIP, the multiplexing capabilities of 96xSPE, as well as the convenience of electropolymerization to integrate thin MIP films with electrodes in a fast and homogeneous approach; the combination of MIPs and 96xSPE provides a unique opportunity for a quick fabrication of sensors for diagnosing diseases or monitoring disease progression. Thus, the aim of this thesis is to fabricate a sensor consisting of thin film of BDNF-MIP electrochemically integrated with a multiwell SPEs, for detecting BDNF, a potential biomarker for the clinical diagnosis of NDs. The main objectives of this thesis are listed below:

1. Electrosynthesis of poly(m-phenylenediamine) (PmPD) film on the working surfaces of 96xSPE in the presence of the template molecule, BDNF, covalently immobilised to the sensing surface of SPE.

2. Generation of BDNF imprints in the PmPD film by cleavage and washing out the BDNF from the polymer matrix using selected reagents aiming to form BDNF-MIP.
3. Electrochemical characterization of the formation of BDNF-MIP on 96xSPE using CV and EIS to confirm polymer electrodeposition and the subsequent template removal from the polymer matrix.
4. Study of the performance of BDNF-MIP/96xSPE sensor towards binding BDNF in human plasma samples.

2. EXPERIMENTAL

2.1 Chemicals and materials

4-aminothiophenol 97% (ATP), meta-phenylenediamine (mPD), 2-mercaptoethanol 99%, human serum albumin (HSA, 66.5 kDa), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$) and acetic acid 99.8% were purchased from Sigma-Aldrich. Ethanol 96% was purchased from Estonian Spirit OÜ (Estonia), $MgCl_2$ and KCl were supplied by Lach-ner. 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP) was obtained from ThermoFisher Scientific Inc, and human recombinant BDNF (13.5 kDa, pI 9.43) was obtained from Icosagen AS (Tartu, Estonia). Ultrapure water Milli-Q (MQ) (resistivity 18.2 MΩ cm at 25 °C, Merck KGaA, Darmstadt, Germany) was used to prepare all aqueous solutions. Phosphate buffered saline (PBS) solution (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 137 mM, KCl 2.7 mM) with molar concentration 0.01 M and pH value 7.4 was used to prepare the analyte solutions. DropSens provided gold-coated 96xSPE - a plate consisting of an array of gold WE (3 mm diameter), a silver RE, and a gold AE. The 96xSPE plate's reverse side has independent gold-plated contact pathways to the respective WEs, REs and AEs.

Human plasma samples were provided by Dr Sirje Rüütel Boudinot from the Department of Chemistry and Biotechnology of TalTech. The plasma samples were stored at -80 °C after separation from human venous blood by subjecting to centrifugation at 800 g for 30 minutes at room temperature (RT; 22-23 °C) in a swinging-bucket rotor (Centrifuge 5804R, Eppendorf, Germany) without using break. All plasma dilutions were obtained by making appropriate dilution in PBS.

2.2 Preparation of BDNF-MIP film

The BDNF-MIP film was prepared from a functional monomer, m-phenylenediamine (mPD). The surface imprinting approach that had previously been developed in the laboratory was used for this [26]. The imprinting approach involves the modification of the surface of the cleaned electrode by 4-ATP layer. In order to create the self-assembled monolayer of 4-ATP, the cleaned electrode was submerged in an ethanolic solution of 0.1 M 4-ATP for 1 hour. The electrode was then thoroughly rinsed with ethanol to remove any remaining unbound thiols, and dried under a nitrogen flow. Dropping a 10 mM DTSSP solution inPBS for 30 minutes caused DTSSP linkers to covalently bond to the 4-ATP layer. The ATP/DTSSP functionalized electrode was then submerged for 30 minutes in a 0.025 M BDNF solution in PBS to immobilise BDNF on the electrode surface, and washed with PBS afterward.

In a setup that included 96xSPE connected with an electrochemical connector (DRP-96-Well Plate Connector, Dropsens Technologies, Spain), and an electrochemical workstation (Reference 600TM, Gamry Instruments, USA), PmPD was synthesised on the BDNF-modified WE from a 10 mM solution of mPD in PBS at a constant potential of 0.6 V vs Ag/AgCl/KCl. Synthesis was set to occur after an optimal charge limit of 2 mC/cm^2 was reached. Control of the amount of electrical charge passed through the WE helps to achieve consistency in the thickness of BDNF-MIP ensuring the creation of replicable films at all times.

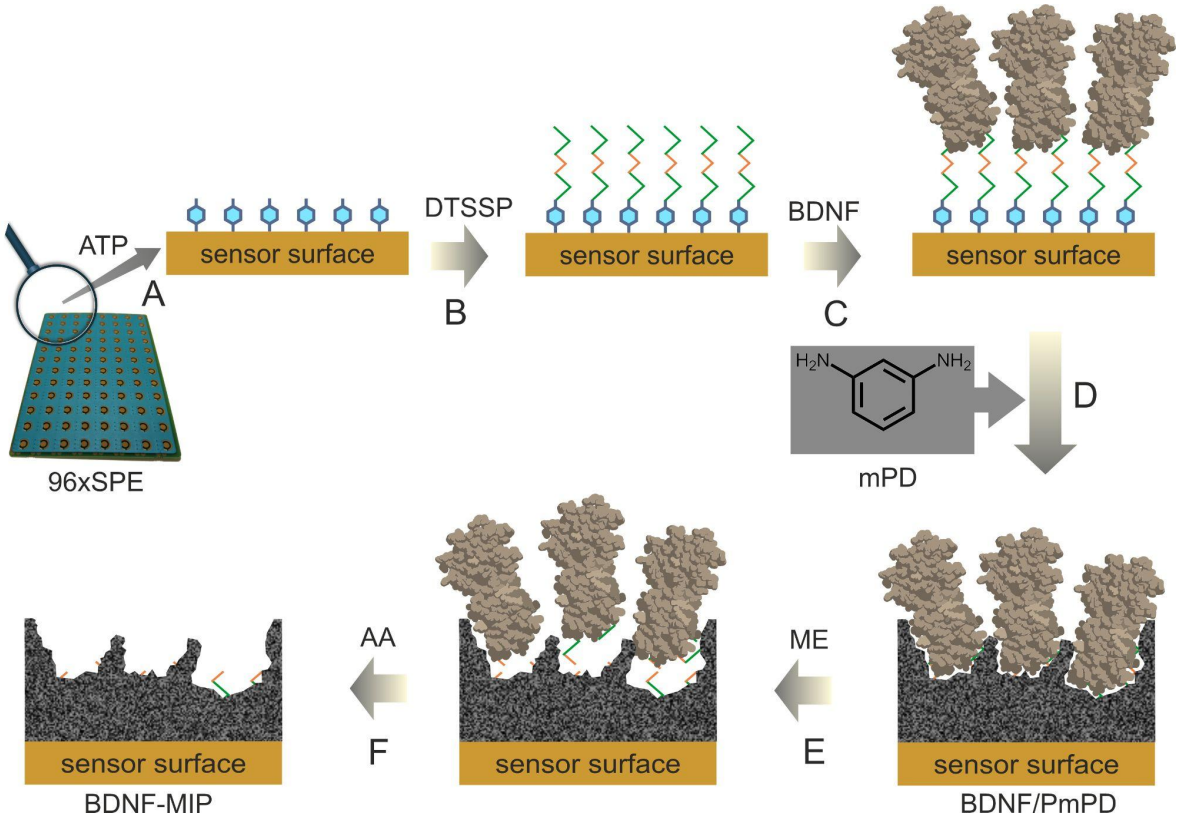

Figure 2.1. BDNF-MIP sensor synthesis routes: (a-c) functionalization of the bare Au surface of 96xSPE (with 4-ATP, DTSSP, and BDNF respectively) (d) PmPD electrosynthesis (e) breakage of disulfide bond by mercaptoethanol (ME) and (f) washing out of BDNF molecules by acetic acid (AA) to form BDNF-MIP.

The removal of the template molecules was achieved by treatment in an ethanolic solution of 0.1 M mercaptoethanol for 30 min to cleave the disulfide bond of DTSSP, followed by a further treatment in 10% acetic acid solution for 30 min. This procedure produced the BDNF molecular imprints in the polymer. The BDNF-MIP films that resulted from this process were cleaned with MQ water and dried using nitrogen flow. Figure 2.1 shows the scheme for this preparation protocol.

2.3 Characterization of BDNF-MIP preparation

Each step of the BDNF-MIP formation was characterised by the electrochemical techniques such as CV, and EIS. All electrochemical characterization measurements were carried out in a setup included 96xSPE plate connected with an electrochemical connector (DRP-96-Well Plate Connector, Dropsens Technologies, Spain), and an electrochemical workstation (Reference 600TM, Gamry Instruments, USA). CV was carried out in a 1 M KCl solution containing 4 mM redox probe, $K_3[Fe(CN)_6]/K_4[Fe(CN)_6)$, with scanning potential in the range of 0 to 0.5 V and at a scan rate of 100 mV/s. There were at least three scanning cycles applied to each sensor. EIS was carried out in a 1 M KCl solution containing 4 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6$ at open circuit potential with AC amplitude of 10mV with a frequency range of 10kHz and 0.1Hz. There were at least three scanning cycles applied to each sensor.

2.4 Study of the binding performance of BDNF-MIP

Using the DPV technique, in a 1 M KCl solution containing 4 mM of the redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6$, the ability of the synthesised BDNF-MIP film to specifically adsorb BDNF was evaluated. The BDNF-MIP/96xSPE was incubated for 30 minutes in PBS. Following a number of DPV measurements in the redox probe solution to establish a reliable baseline, the 96xSPE was then incubated in either PBS or diluted plasma with or without different concentrations of BDNF (1 to 100 ng/ml). Plasma samples were serially diluted in PBS for 5, 10, 20, 40, 80, 160, 320 and 640 folds.

Normalised DPV current peak (I_n) was used as a sensor response and correlated with a respective concentration. The normalisation was performed as follows::

$$I_n = (I_0 - I)/I_0 \quad (1.8)$$

where I_0 and I represent the DPV current peaks that were attained following incubation in PBS or diluted plasma without and with the analyte, respectively. However, for the competitive experiments and blind sample analysis, the I_n values obtained were divided by those of optimally diluted samples, to account for small changes from either batch to batch or from one sensor to another.

3. RESULTS AND DISCUSSION

3.1 Preparation of BDNF-MIP sensor

As shown in figure 3.1a, ATP and DTSSP functionalization mildly suppressed both the anodic and cathodic currents with a further suppression after BDNF immobilisation, indicating the introduction of non-conducting layers on the cleaned Au surface. In addition, following PmPD electrodeposition, a disappearance of these current peaks is observed establishing the formation of a far less conducting layer of polymer film. Similarly, EIS spectra (1b) show a continuous increase in the charge transfer resistance to ions of the probe solution in a sequence from ATP to PmPD modifications of the electrode. However, following MIP formation by treatments in ME and AA, a notable reversal of the trend is observed evident by the sudden reduction in the charge transfer resistance. These results indicate the formation of BDNF imprints (binding sites) within the polymer matrix that is useful for subsequent recognition of the analytes. The obtained result is in agreement with previous research from our research groups [22,23,79].

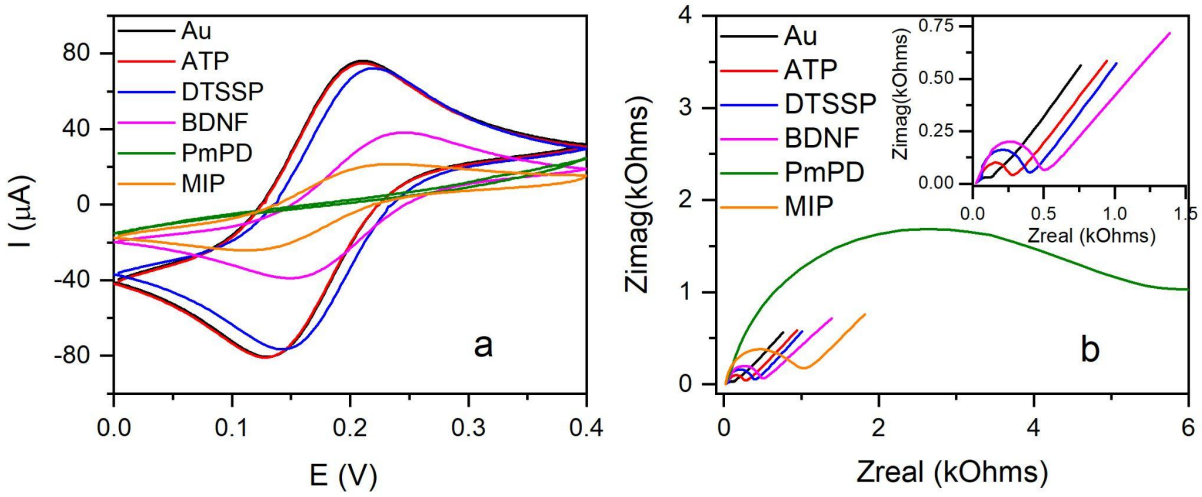

Figure 3.1. (a) CV and (b) EIS spectra of bare, ATP and DTSSP functionalized, BDNF immobilised and PmPD electrodeposited Au electrode in 1 M KCl containing 4 mM of the redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

3.2 Detection principle of BDNF sensor

To evaluate the performance of the sensor, electrochemical detection using DPV was employed. To detect the analyte, the sensor measures changes in charge transfer between $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe and Au surface of SPE. During rebinding, the target is adsorbed in the imprinted cavities previously created in the polymer thereby blocking the charge transfer to the Au electrode of the sensor. Consequently, the current

peak of DPV voltammogram is suppressed and a concentration-dependent signal (as normalized response) can be obtained as demonstrated in Figure 3.2.

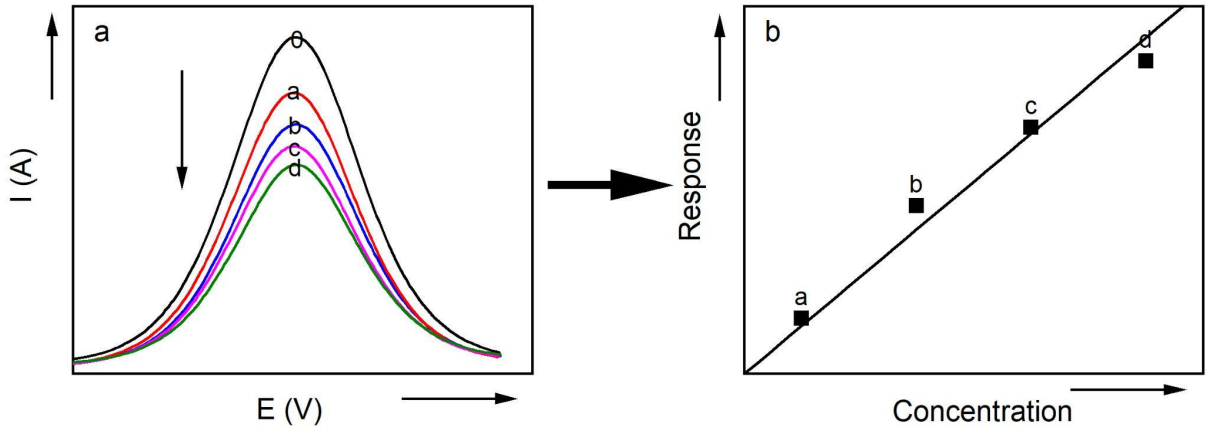

Figure *3.2. Schematic illustration of electrochemical detection of BDNF by (a) measuring DPV voltammograms after adsorption of increasing concentrations (a-d) of the analyte, a line denoted as 0 corresponds to a solution without analyte , (b) response-concentration curve derived from (a) using Eq. 1.8.*

3.3 Evaluation of sensor performance in plasma

3.3.1 Optimization of plasma dilution

Previously, the performance of BDNF sensors has been determined in the PBS [23]. However, since the sensor is intended to be used in clinical samples which by all means contains a wide range of biological molecules chiefly human serum albumin (HSA) and immunoglobulin G (IgG) that can interfere with the sensor performance, this thesis is focused on analysing the sensor performance in real clinical media such as human plasma. This would serve as a measure of validating the suitability of the sensor for practical usage. For this purpose, an initial optimization of the appropriate plasma dilution that would allow accurate analysis is essential. Consequently, human plasma was subjected to a serial dilution in PBS ranging from 5 to 640 fold. This is then followed by measuring and recording the responses of the sensors upon incubating in each diluted sample and using the same to plot an adsorption isotherm (Figure 3.3) vs the concentration factor that is determined as the reciprocal of the dilution. As seen, the sensor response increases with a decreasing dilution of the plasma sample until a dilution of 40 (which corresponds to a concentration factor of ca. 0.03) after which no significant change in the sensor response is observed.

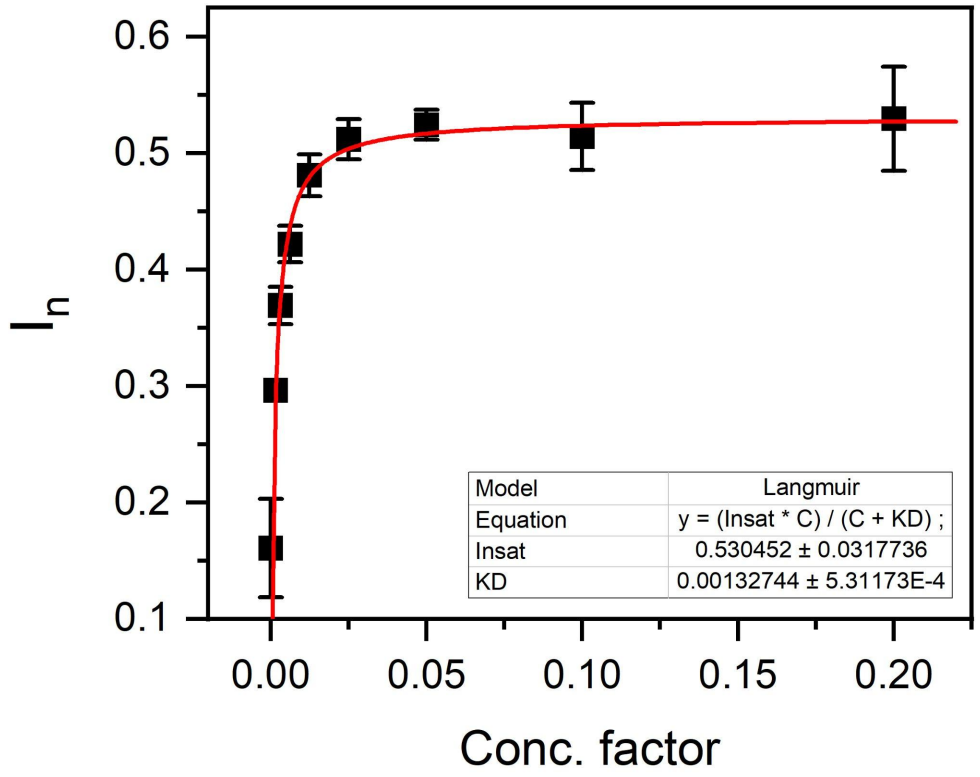

Figure 3.3. Adsorption isotherm on BDNF-MIP modified sensor for different dilutions of plasma. The red line represents a langmuir fit to the experimental data. Measurements were repeated for a minimum of 3 times for each sample.

This clearly indicates that although the adsorption of plasma proteins and other molecules on the sensor are nonspecific, their abundance is strong enough to hamper the accurate determination of the sensor performance in plasma. For a proper estimation of the binding interaction, the isotherm was fitted to the Langmuir equation (Eq. 1.7), and the optimal dilution appropriate for further analysis was approximated as the dilution giving 50% of the maximum adsorption (K_D value) of the plasma constituents on the BDNF-MIP modified sensor [112, 113]. At this dilution the sensor is supposedly most sensitive towards possible target analyte concentration changes. The parameters derived from the fitting is shown in Table 3.1, indicating a derived K_D of ca. 0.001 (concentration factor) which corresponds to 1000-fold dilution. Thus, 1000 is then selected as the optimal plasma dilution that allows accurate analysis of the sensor performance.

Table 3.1. Parameters derived from the Langmuir fit to the binding isotherm.

Parameters	Values
I_{nsat}	0.53 ± 0.03
K_D (a.u)	0.001 ± 5.3E-4
R^2	0.902

3.3.2 Competitive rebinding of BDNF with plasma proteins

For quantitative determination of BDNF in plasma samples, it is essential to derive a calibration of the sensor's response against different concentrations of the analyte. For this purpose, a competitive experiment carried out at a fixed level of the interferent vs increasing concentration of the target is usually conducted. Thus, different concentrations of BDNF were prepared in the optimally diluted plasma and their responses were recorded. Figure 3.4a shows the sensor's response to a competitive rebinding of BDNF while figure 3.4b indicates the linear concentration range of 1 - 1000 ng/mL of undiluted sample (i.e 0.001 - 1 ng/mL after 1000 fold dilution) at which BDNF can be effectively detected by the sensor. The average BDNF level in human serum is 33 ± 8 ng/mL (SD) [120]. This corresponds to about 0.003 ng/mL after subjecting the sample to optimal dilution. Thus, variations in BDNF concentration below or above this level could be effectively determined by the sensor hence, helping to monitor the progression of a neurological disorder.

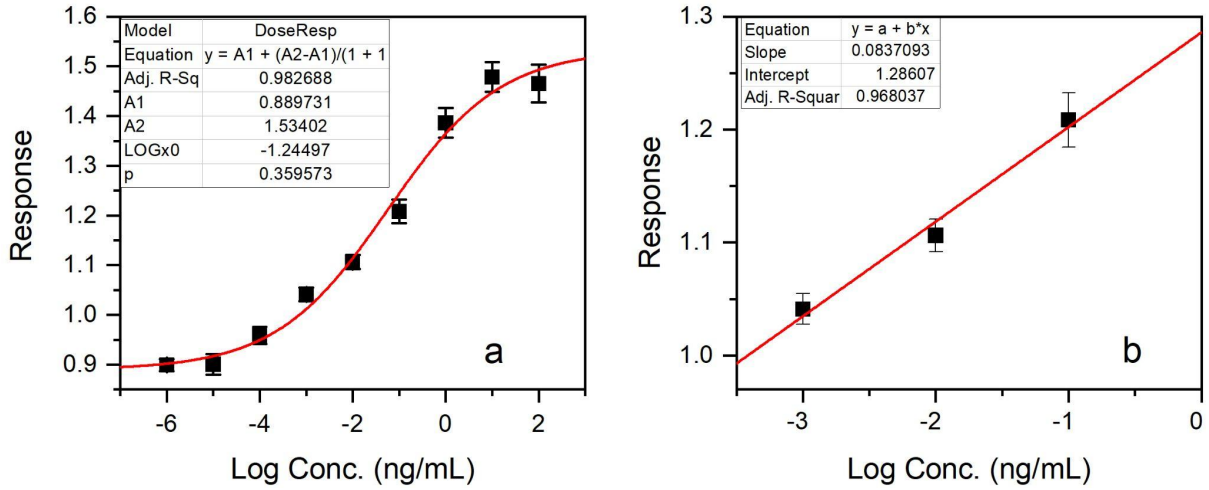

Figure 3.4. The responses of BDNF sensors against BDNF in optimally diluted plasma (a) over a wide range of concentrations, (b) over the linear range of the sensor. Bold lines represent fit to the dose response model or linear fit respectively. A minimum of three repeated measurements were conducted for each sample.

3.3.2 Analysis of blind samples

To further assess the analytical feasibility of the sensor, blind samples whose BDNF concentrations were unknown at the point of measurement, were tested after appropriate dilutions and their induced responses were then used in determining the spiked concentration of BDNF using the calibration graph in figure 3.4(b). Figure 3.5 represents the correlation between the spiked and sensor-determined concentrations with more details shown in Table 3.2.

Table 3.2. Comparison of the actual BDNF concentrations in blind samples with those determined by the sensor.

Sample label	Spiked Conc. (ng/mL)	Conc. determined by sensor	% difference
E	5	6.43	22
D	20	27.32	27
B	30	36.60	18
G	50	44.72	12
F	70	71.29	2
A	90	100.96	11
C	100	124.82	20

As observed in Table 3.2, an almost perfect linear correlation exists between the concentrations spiked and those estimated by the sensor suggesting a range of 5 to 120 ng/mL. Also, Table 3.2 shows that the percentage differences between the determined and actual concentrations is within a range of ca. 2 to 27% which further confirms the capacity of the sensor to analyse BDNF in plasma samples of patients.

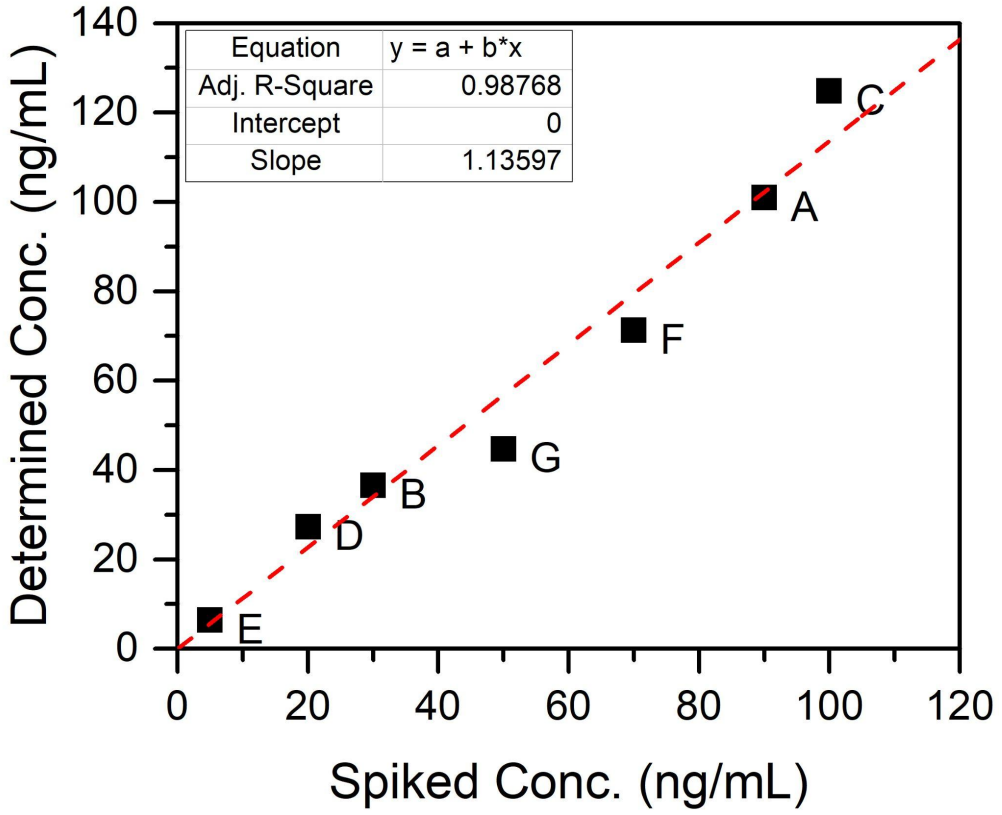

Figure 3.5. The correlation of BDNF-MIP sensor's determined concentration with those originally contained (spiked) in the blind samples.

4. CONCLUSIONS

This thesis demonstrates the preparation of an electrochemical sensor by modifying an 96xSPE with a synthetic receptor - BDNF-MIP for the detection of BDNF in human plasma samples. Using BDNF as the template molecule, MIP films selective towards BDNF (BDNF-MIP) were generated on the working electrode surfaces of 96xSPE by a carefully controlled deposition of electrosynthesized polymer that was then used in the quantitative analysis of BDNF in plasma samples. The following conclusions were drawn from the study:

1. BDNF-MIPs were successfully formed on the array of 96xSPE by applying the surface imprinting strategy that includes the following subsequent steps: the covalent immobilisation of BDNF on the working surfaces of SPEs via DTSSP cleavable linker; electrodeposition of a thin homogeneous poly(m-phenylenediamine) film; removal of BDNF from the film by cleaving the DTSSP linker. The success of each modification step was confirmed by CV and EIS measurements.

2. The dilution of human plasma samples was optimised by measuring the BDNF-MIP/96xSPE responses after incubation in the plasma samples diluted in PBS at different ratios. It was found that the 1000-fold dilution provided the most sensitive conditions for BDNF-MIP/96xSPE operation. Thus, this dilution was used for the subsequent study of the BDNF-MIP/96xSPE performance.

3. The competitive rebinding of BDNF on the BDNF-MIP/96xSPE demonstrated the linearity of their responses in the range of 1 - 1000 ng/mL that spans over the normal level of BDNF in blood samples found in literature (about 33 ng/mL) thus helping to monitor the treatment efficacy or progress of the neurodegenerative diseases

4. The analysis of blind samples (diluted plasma spiked with BDNF at different concentrations) demonstrated a good correlation between the spiked and sensor-determined concentrations with the percentage differences within ca. 2-27 %.

5. The BDNF-MIP/96xSPE enables independent electrochemical measurements in up to 96 wells providing thus multiplexing capability of electrochemical detection of BDNF from the human plasma samples.

The author believes that the result from this research will contribute to further scientific development of the detection of BDNF in human plasma. Future research would concentrate on testing the sensor in real samples and demonstrating capacity for point-of-care testing (PoCT) to further establish its potential usefulness for the intended practical application.

SUMMARY

In clinical practice, neurodegenerative diseases are becoming more common. The clinical diagnosis of these diseases only after the symptoms appear, delaying the treatment process and its success. Detection of biomarkers involved in neurodegeneration processes, has the potential to allow early diagnosis of neurodegenerative diseases. To assess the concentration of neurotrophins in serum, conventional methods like western blotting and ELISA are still utilized. Highly qualified analysts and specific work settings are needed for these time-consuming, expensive analytical approaches. Therefore, it is urgently demanded developing a quick, simple, and inexpensive technique allowing the detection of NF proteins. Molecularly imprinted polymers (MIPs), known for their molecular recognition ability, but synthetic nature providing exceptional chemical and thermal stability along with their repeatable and affordable manufacture, seems to be a great alternative to conventional methods.

In this thesis, an array of 96 screen-printed electrodes (96xSPE) was modified with a synthetic receptor, brain derived neurotrophic factor (BDNF)- selective molecularly imprinted polymer (BDNF-MIP), to create an electrochemical sensor (BDNF-MIP/96xSPE) for the quick detection of BDNF in human plasma samples.

The BDNF-MIP was prepared as a thin film by a previously developed surface imprinting approach that involved at first forming a cleavable linking layer of 4-aminothiophenol (4-ATP) and 3,3’-dithiobis(sulfosuccinimidyl propionate) (DTSSP) monolayer on a working electrode of SPE, followed by BDNF covalent immobilisation to the DTSSP linker, electropolymerization of m-PD and finally the removal of BDNF via linker cleavage (mercaptoethanol) and subsequent treatment in acetic acidic solution. Characterization of BDNF-MIP modification was carried out using CV and EIS. Electrochemical detection of BDNF was performed measuring DPV voltammograms in the redox probe after incubation of BDNF-MIP-modified 96xSPE (BDNF-MIP/96xSPE) in the plasma containing BDNF.

It was discovered that the most sensitive operating conditions for BDNF-MIP/96xSPE required a 1000-fold dilution of human plasma. The BDNF-MIP/96xSPE showed linearity of their responses in the range of 1 - 1000 ng/mL, spanning across the normal level of BDNF in blood samples. The evaluation of blind samples showed a strong correlation between the BDNF-MIP/96xSPE-determined concentrations and spiked values, with the percentage deviations falling between about 2-27%. It is worth noting the high throughput screening capability of the BDNF-MIP/96xSPE allowing for separate electrochemical detection of BDNF from up to 96 probes. Thus, multiplexing capacity of electrochemical detection of BDNF from human plasma samples is provided.

The author believes that the result from this research will contribute to further scientific development of the detection of BDNF in human plasma. Future research would concentrate on testing the sensor in real samples and demonstrating capacity for point-of-care testing (PoCT) to further establish its potential usefulness for the intended practical application.

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