Development of Electrochemical (Bio)sensors for the Detection of Dopamine

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ABSTRACT

The development of dopamine (DA) electrochemical sensors and biosensors is described in this thesis. The aim was to develop (bio)sensors that would continuously and specifically detect DA over a wide concentration range, with high sensitivity and low detection limits. The electrode surfaces used were platinum (Pt), boron-doped-diamond (BDD), and carbon-paste (CP). The first two were used to construct DA sensors, while Pt was shown to be superior for the construction of biosensors. The DA biosensor configurations were based on the use of a Pt electrode as a transducer surface, polyphenol oxidase (PPO) as an enzyme, ferrocene (Fc) as a mediator, and polypyrrole (PPY) and Nafion as membranes. A range of electrochemical techniques were employed in this research.

It was determined that the oxidation of DA on bare Pt is a surface-controlled reaction, occurring at low overpotentials. The reaction is electrochemically reversible, involving the spontaneous adsorption of DA on the electrode surface. The Pt and BDD *sensors* were efficiently used to determine DA in aqueous solutions. In order to increase their resistance to the ascorbic acid (AA) interference, the sensor surfaces were modified by a thin Nafion film. This configuration was shown to selectively detect DA even when AA was present with DA at a 1000-time larger concentration. The lowest DA detection limit was achieved using the unmodified BDD sensor, 50 nM. Nevertheless, both the unmodified and Nafion-modified Pt and BDD sensors were suitable for monitoring of DA at concentration levels typical for urine samples.

It was shown that the sensitivity and detection limit of the developed Pt-based *biosensors* depend on the amount of PPO and Fc incorporated into the PPY membrane, and also on their ratio. The modification of the biosensor by a Nafion membrane offered three benefits: an increase in sensitivity, an improvement in detection limit, and a significant minimization of the AA interference. An optimum biosensor architecture was made by polymerizing PPY for 40 minutes from a pyrrole solution containing 2,400 $U\,mL^{-1}$ of PPO and 10 mM of Fc, on top of which a thin Nafion film was formed. Using chronoamperometry as a detection technique, this biosensor yielded a DA detection limit

of 20 nM, which makes it suitable for monitoring DA levels in brain. Even a lower detection limit, 10 nM, and higher sensitivity were achieved by using electrochemical impedance spectroscopy (EIS) as a detection technique. Unfortunately, the developed biosensor lacked operational stability, predominately due to the leakage of PPO and Fc into the storage solution.

RÉSUMÉ

Le développement de capteurs et de biocapteurs électrochimiques pour la détection de la dopamine (DA) est décrit dans cette thèse. Le but est de développer des (bio)capteurs détectant continuellement et spécifiquement DA dans une large gamme de concentrations, avec une grande sensibilité et une basse limite de détection. Les matériaux d'électrodes utilisées sont le platine (Pt), le diamant dopé au bore (DDB), et la pâte de carbone (PC). Les deux premiers ont été employés pour élaborer des capteurs de DA, alors que le Pt s'est avéré supérieur pour la construction des biocapteurs. Les configurations des biocapteurs de DA sont basées sur l'utilisation d'une électrode de Pt comme surface de capteur, la polyphénol oxydase (PPO) comme enzyme, le ferrocène (Fc) en tant que médiateur, et le polypyrrole (PPY) et le Nafion comme membranes. Différentes techniques électrochimiques ont été utilisées dans cette recherche.

Il a été déterminé que l'oxydation de DA sur le Pt non-modifié est une réaction électrochimiquement réversible se produisant à de faibles surtensions et contrôlée par l'adsorption spontanée de DA sur la surface d'électrode. Le Pt et le DDB ont été efficacement employés comme capteurs pour déterminer la DA dans des solutions aqueuses. Afin de limiter l'interférence de l'acide ascorbique (AA), les surfaces de capteurs ont été modifiées par une mince couche de Nafion. Cette configuration s'est montrée efficace pour la détection sélective de la DA même lorsque l'AA était présent avec la DA à une concentration 1000 fois plus grande. La plus basse limite de détection de la DA a été obtenue à l'aide de capteurs non modifiés de DDB, 50 nM. Néanmoins, les capteurs de Pt et de DDB non modifiés et modifiés avec du Nafion permettent de détecter DA dans l'urine pour des concentration typiques.

Il a été démontré que la sensibilité et la limite de détection des biocapteurs de Pt développés dépendent de la quantité d'enzyme PPO et du médiateur d'électrons Fc incorporés dans la membrane de PPY, et également de leur ratio. La modification du biocapteur par une membrane de Nafion présente trois avantages: une augmentation de la sensibilité, une amélioration de la limite de détection, et une diminution significative de l'interférence de l'AA. Une architecture optimale du biocapteur a été obtenue en polymérisant du PPY pendant 40 minutes à partir d'une solution de pyrrole contenant

2,400 U mL^{-1} de PPO et 10 mM de Fc, sur lesquels une mince couche de Nafion a été déposée. En utilisant la chronoampérométrie comme technique de détection, ce biocapteur a permis de détecter DA avec une limite de détection de 20 nM, ce qui le rend approprié pour la surveillance des niveaux de DA dans le cerveau. Une limite de détection de 10 nM et une sensibilité plus élevée ont été obtenues en employant la spectroscopie d'impédance électrochimique (SIE) comme technique de détection. Malheureusement, le biocapteur développé possède une faible stabilité opérationnelle due en majorité à la perte de PPO et de Fc dans la solution de stockage.

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INTRODUCTION

Biosensors have been investigated extensively over the past three decades due to their low-cost and to their efficiency in detecting and monitoring specific species. The concept of an electrochemical biosensor was born with the pioneering work of Professor L C. Clark who invented, in 1956, the first oxygen sensor and later on in 1962 the first biosensor: an amperometric enzyme electrode for glucose [1]. Biosensors are defined as analytical tools combining a specific biological recognition component with a physical transducer [2]. It is the biological recognition element in the biosensor that distinguishes it from a chemical sensor. Two types of biosensors exist: enzyme-based biosensors and affinity biosensors. The biological recognition compound for the first type is an enzyme, a cell or tissues whereas for the second type it is a DNA sequence, antibodies, or membrane receptors. The purpose of the physical transducer is to convert the biological recognition event into an electrical signal that would be detected and related to the rate of the catalytic reaction or to the analyte concentration (Fig.1.1).

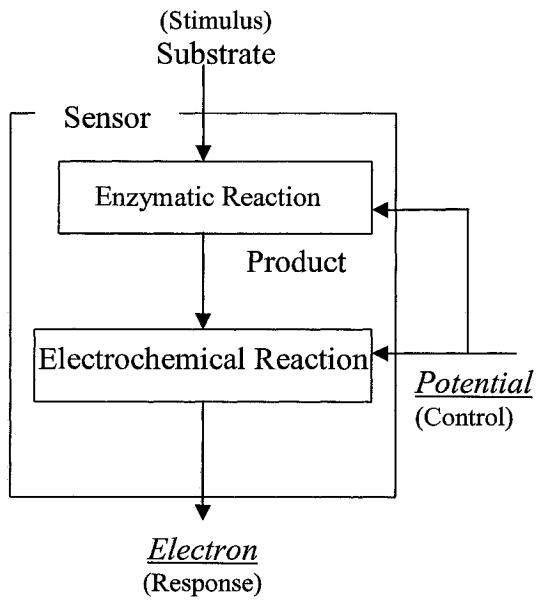


Figure 1.1 *Schematic of principle of work of an electrochemical biosensor* [3].

Both types of biosensors make use mostly of either optical, (electro)magnetic, radioactive, thermic, or electrochemical mode of transduction. Frequently used biosensors in hospitals, clinics, and especially in point-of-care applications are electrochemical ones, where a working electrode plays the role of the physical transducer. Electrochemistry has the advantage of being a cheap and simple method to use, providing high selectivity, good sensitivity and low detection limits. Therefore, the specificity, selectivity, speed and low cost of electrochemical biosensors explain their important emergence in the health care and pharmaceutical industries, environmental and military applications in recent years [2].

Neurology research has been the source of the most demanding application and of the greatest focus for biosensor development over the last few years. Neurotransmitters are molecules responsible for communication between cells in the brain. They play key roles in normal brain function and are linked to a number of neurological malfunctions such as Parkinson's and Alzheimer's disease, schizophrenia, depression, addiction, etc. In the brain, neurotransmitters are liberated by a presynaptic neuron into the synaptic cleft, as a result of the firing of an action potential, and are bound to specific receptors on the surface of the postsynaptic neuron (Fig.1.2). A change in the postsynaptic membrane potential occurs. This change can be either a direct depolarization (an excitatory postsynaptic potential) or hyperpolarization (an inhibitory postsynaptic potential), resulting either in the transmission or the elimination of the message [3,4].

Dopamine (DA) is an essential neurotransmitter in cerebral functions. Dysfunction in the dopaminergic transmission influences a variety of neurological and psychiatric disorders such as Parkinson's disease (decreased levels of dopamine) [5]. Dopamine plays an important role in motor control, in "reward", is related to addiction, and affects brain processes that control emotional response [6]. Thus, there is a great need to understand why dopamine deficiency causes the symptoms of Parkinson's disease, as well as what is exactly the normal role of dopamine. Monitoring dopamine's levels continuously *in vivo* could provide valuable information on the efficacy of various treatments aiming at healing or controlling Parkinson's disease. Therefore, an ideal DA (bio)sensor would have high sensitivity and selectivity in order to distinguish DA from

other electroactive species present in much higher concentrations. It should also have a fast response time and be able to detect low DA concentrations

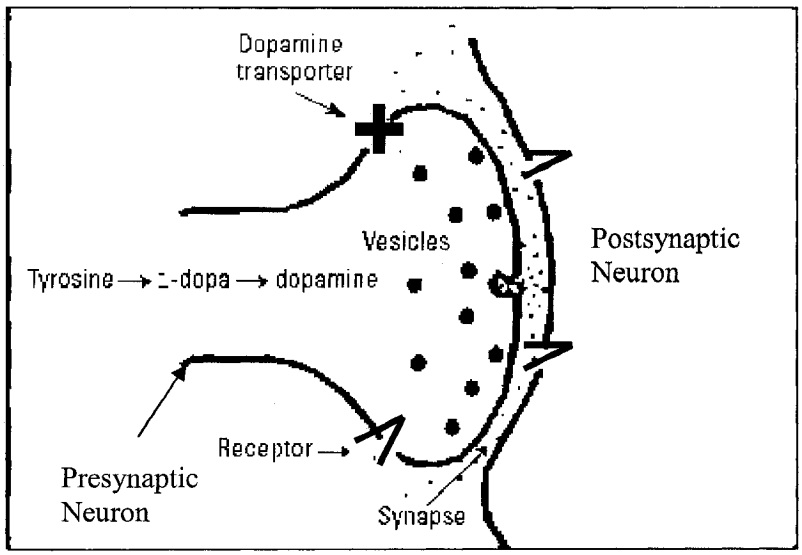


Figure 1.2 *Schematic of dopaminergic neuronal transmission: dopamine is formed in the presenaptic neuron, released from the vesicles into the synapse and bound to receptors of the postsynaptic neuron* [5].

This work reports results on the design and optimization of electrochemical (bio)sensors for the detection of a specific neurotransmitter, dopamine (DA). Two types of sensors were investigated: bare solid non-selective electrodes as sensors for the detection of DA, and specific enzyme-modified electrodes (biosensors) for the selective detection of DA. A range of electrochemical techniques were used in the development of DA (bio)sensors. More specifically, they were used for the detection of dopamine, for the study of the thermodynamics of dopamine adsorption on the electrode surface, for the construction of the (bio)sensors' calibration curves, as well as for the determination of (bio)sensors' sensitivity, selectivity, stability and detection limits.

OBJECTIVES

The main objective of this research is to design electrochemical (bio)sensors that would detect continuously and specifically dopamine over a wide concentration range with high sensitivity and low detection limits. The specific objectives of the thesis are:

(i) To investigate the redox kinetics of dopamine on a bare platinum surface.

(ii) To investigate the dopamine adsorption thermodynamics on a bare platinum surface.

(iii) To develop simple electrochemical dopamine sensors based on solid biocompatible electrodes (platinum and boron-doped-diamond), and investigate the effect of transducer surfaces and their modification on the selectivity and sensitivity in dopamine determination.

(iv) To develop and optimize a DA biosensor based on the use of enzyme polyphenol oxidase (PPO); to investigate the influence of various parameters (PPO loading, PPO/mediator ratio, surface Nafion film formation, etc.) on the sensitivity, selectivity and stability of the biosensor.

The thesis is divided into four major sections. The following section (Chapter 3) will give a thorough background relevant to the problem investigated, followed by a concise literature review on the work done on DA sensors and biosensors (Chapter 4). A detailed experimental procedure will be presented in Chapter 5, before presenting and discussing results in Chapter 6.

3 BACKGROUND

Electrochemical biosensors are devices that transform biochemical information such as analyte concentrations into an analytically useful signal: current or voltage. Biosensors usually contain two basic components connected in series: a biological recognition element (receptor) and a physical transducer (Fig.3.1). The purpose of the biological recognition element is to specifically detect the analyte of interest in a sample, while the physical transducer converts the biological recognition event into an electrical signal that would be detected and related to the analyte concentration. The biocomponent typically limits the biosensor's lifetime, stability and calibration requirements, unlike the physical transducer [7]. Most commonly used recognition elements in electrochemical biosensors are enzymes. Since active sites in enzymes are typically buried deeply inside the molecule, direct transfer of electrons between the active site and the transducer surface (electrode) is not possible (a maximum electron tunnelling distance is 1 nm). Hence, organic electron mediators (M) are commonly used for that purpose. The principle of work of an enzyme-based electrochemical biosensor is presented in Figure 3.1. The enzyme recognizes the substrate (S), and the enzyme active site oxidizes S (it could also reduce S, depending on the substrate, enzyme and reaction). The oxidized form of the mediator in turns regenerates (oxidizes) the enzyme active site, while the electrode (transducer) surface regenerates (oxidizes) M. Then, the current due to the oxidation of M is measured, and related to the amount of S in the solution.

33.0% of transducers materials are metals, with platinum being the most commonly used (23.6%) and 57.4% are carbon based [8]. Non-electrochemical transducers also used within biosensors are piezoelectric (shear and surface acoustic wave), calorimetric (thermistor), optical (fiber optic, surface plasmon resonance) and magnetic [9]. Electron mediators are usually electrochemical reversible redox organic molecules (viologens, ferrocenes, quinones, etc.) [10], but carbon-nano-tubes (CNT) have also attracted large attention as electron-conducting media due to their ability to penetrate inside enzymes to their active sites, and thus transfer electrons [11].

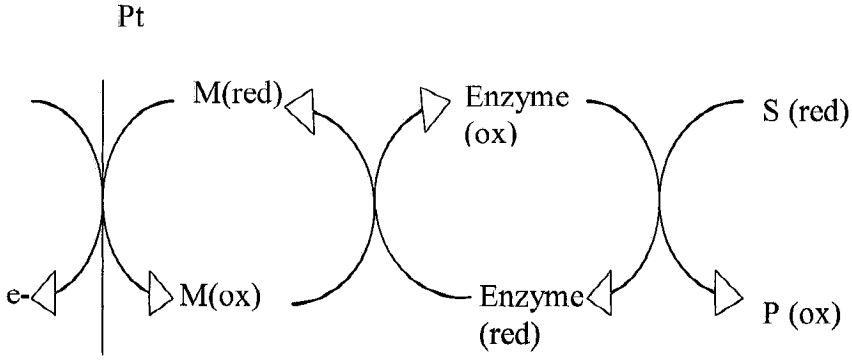


Figure 3.1 *Principle of work of an electrochemical biosensor S: substrate, P: product, M: mediator, ox: oxidized, red: reduced.*

3.1 Dopamine and its Function

Dopamine or 3, 4-dihydroxyphenylethylamine is a catecholamine. It consists of a benzene ring having two hydroxyl groups (catechol) with a monoamine attached to it (Fig.3.2). Dopamine is biosynthesized from tyrosine in a two step process taking place in dopaminergic neurons located in few discrete regions of the brain: the caudate putamen, nucleus acumbens and substantia nigra/ventral tegmental area. Like other neurotransmitters, it cannot cross the blood-brain barrier. Dopamine is involved in the modulation of arterial blood-flow, higher brain functions like cognition and learning and in anxiety-related behavior. Therefore, dopaminergic systems serve as a target for antipsychotic drugs. Dopaminergic hyperactivities lead to an accumulation of the neurotransmitter in the synaptic cleft and have been linked to some psychotic disorders, hallucinations and maniac states; whereas dopaminergic hypoactivities (deficit in the dopaminergic system) have been involved with motor dysfunction such as Parkinson's disease, deficits in motivation-dependent behavior, and imbalance of emotional perception [4]. Dopamine's basal brain concentration is around 100 nM. When the dopaminergic neurons are stimulated, dopamine is released according to two firing patterns: tonic and phasic [5]. The tonic firing occurs at frequencies of 2-5 Hz resulting in synaptic DA concentrations of ca. 10 nM, whereas the phasic firing occurs at frequencies of 15-100 Hz resulting in a transient increase of DA concentration up to 2 μM. After the release of dopamine due to the firing of action potential, about 80% is recaptured by a reuptake mechanism. Since the ultimate microbiosensor has to be implanted in the

interstitial space, it must have high temporal resolution, be sufficiently sensitive to detect DA concentrations between 5 nM and 2 μM, and be biocompatible, *i.e.* to produce minimal damage for the *in vivo* environment, and likewise the *in vivo* environment does not adversely affect the sensor performance [1]. Dopamine can also be detected by Positron Emission Tomography (PET) neuroimaging techniques [12], fluorescence probes, spectrophotometry or high performance liquid chromatography (HPLC).

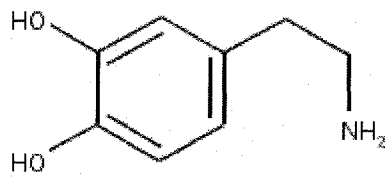
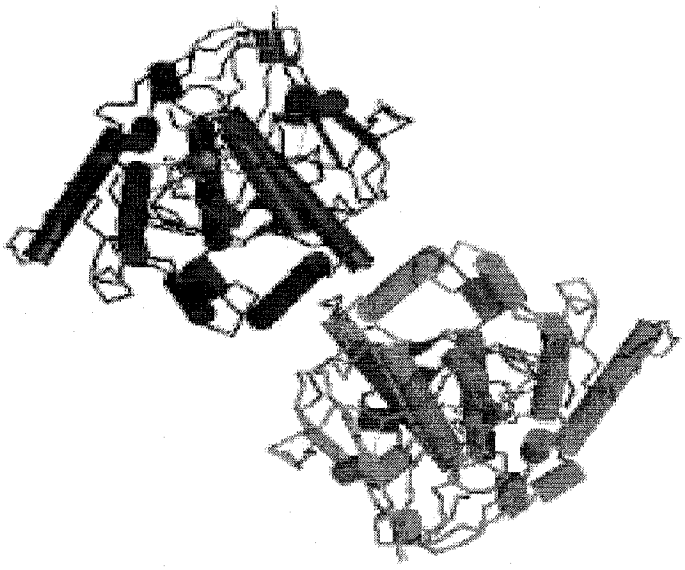


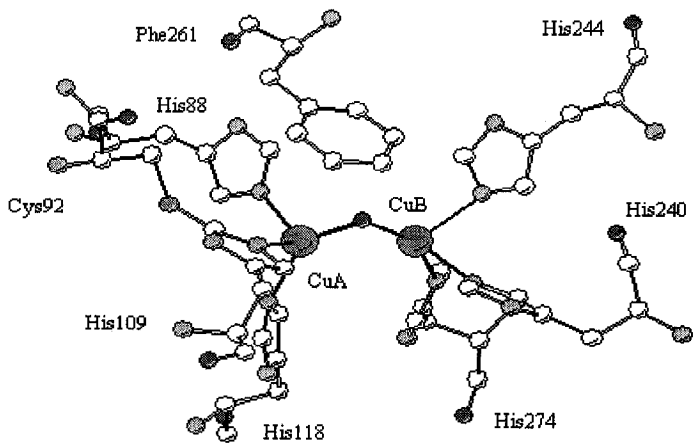
Figure 3.2 *Dopamine molecule.*

3.2 Biochemical Reaction between Dopamine and Polyphenol Oxidase – The Basis for DA Detection

As already mentioned, in order to specifically detect DA, a biorecognition element is needed, more specifically an enzyme that would recognize DA and catalyze its chemical transformation (Fig.3.1) In order to determine which enzyme to choose as a recognition element for dopamine, a thorough literature review has been done. It has been determined that polyphenol oxidase (PPO), also known as catechol oxidase or tyrosinase, is the enzyme specific to dopamine (Fig.3.3) [13-17]. The active site of PPO is a catalytic dicopper site that binds dioxygen resulting in its activation.



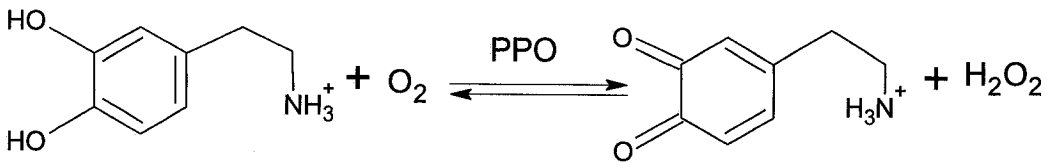
(a)



(b)

Figure 3.3 *(a) 3D structure of polyphenol oxidase, and (b) molecular structure of PPO around its active site: CuA and CuB are the dicopper active sites* [18].

PPO belongs to the family of oxidases, which catalyzes the oxidation of o-diphenols to o-quinones in the presence of molecular oxygen as the co-substrate:



(1)

The final product of this chain reaction is an insoluble polymer (melanin) that could be easily removed from the reaction mixture by centrifugation. In Equation (1) dissolved oxygen is converted to hydrogen peroxide through a two-electron transfer reduction by the reaction center of PPO at the binuclear copper site. Figure 3.4 [18] shows the catalytic reaction cycle of a catechol with the dicopper site of PPO.

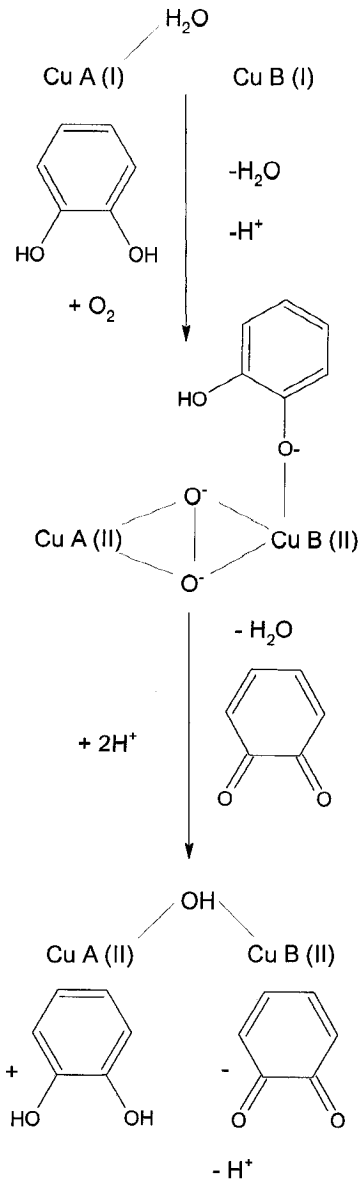


Figure 3.4 *Pathway of the reaction catalyzed by catechol oxzidase (PPO) in three steps: two molecules of catechol are oxidized, coupled with the reduction of molecular oxygen to water. CuA(I)-CuB(I): reduced dinuclear copper center. CuA(II)-CuB(II): oxidized dinuclear copper center* [18].

It has been shown that oxygen has to bind to the reduced copper center before the substrate catechol binds to the enzyme and gets oxidized to quinone. Vieira *et al.* [19] proposed a biochemical pathway of the PPO catalytic oxidation of dopamine to the final melanin product (Fig.3.5)

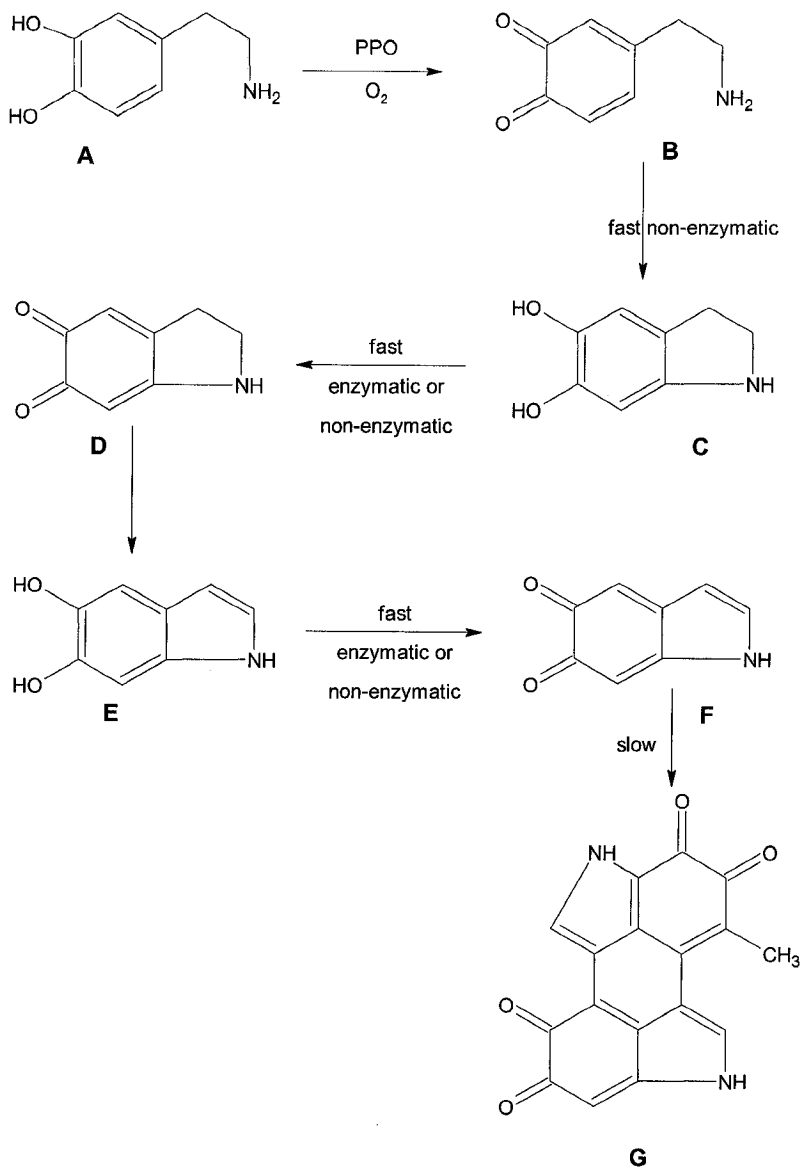


Figure 3.5 *Reaction steps of the catalytic oxidation of catecholamines by polyphenol oxidase. (A) dopamine, (B) dopaminequinone, (C) leucodopaminechrome, (D) aminochromes (methyldopachrome and dopaminechrome), (E) 5,6-dihydroxyindole, (F) indole-5,6-quinone and (G) melanin.*

In order to determine dopamine in pharmaceutical formulations these authors used an enzymatic spectrophotometric procedure. Crude enzyme extracts were added to known dopamine concentrations, and from the strong absorbance of dopaminechrome (molecule D) at 470nm, calibration curves were plotted. Therefore, unknown dopamine concentrations were determined in pharmaceutical formulations with close agreement to the labeled values and within an acceptable error margin (±5.4%). Figure 3.5 shows that quantification of dopamine is also possible using either the determination of oxygen consumption or monitoring dopaminequinone concentration (molecule B). However, dopaminequinone undergoes a fast non-enzymatic spontaneous auto-oxidation to leucodopaminechrome (molecule C) and hence is not a stable intermediate. One solution would be the reduction of dopaminequinone (molecule B) back to dopamine in a two-electron transfer reaction, and the subsequent use of dopamine as an enzyme substrate, thus giving rise to an enhanced detection signal [20]. Melanin (molecule G) detection might also be useful for dopamine detection since it is an electroactive molecule. The disadvantage would be the fouling of the electrode surface and the decrease in enzyme activity.

3.3 Biosensor's Architecture

Two major issues must be addressed when designing the dopamine biosensor:

1- *Oxygen dependence:* Polyphenol oxidase uses dioxygen as a cofactor that regenerates the enzyme to its active form (Fig.3.4). However, oxygen levels in blood or urine fluctuate and are difficult to control, thus causing limitations to the biosensor's efficiency. One solution could be the saturation of the electrolyte (blood or urine) with oxygen, or improving the relative surface availability of oxygen by the use of a membrane that would exclude the interference of other molecules and restrict the flux of the substrates [2,21]. Another possibility could be the use of fluorochemicals pasting liquids because they provide an internal supply of oxygen that would allow the biosensor to function [20]. However, the most common approach to eliminate oxygen dependence as well as interfering endogenous electroactive molecules such as ascorbic acid, uric acid or homovanillic acid, involves the use of *electron mediators.*

Mediators are molecules that can shuttle electrons between the redox center of the enzyme and the electrode surface, thus replacing O_2 and preferably lowering the possibility of the electrochemical interference of the various redox molecules mentioned above. In order to successfully mediate an enzyme reaction, a mediator must possess the following properties: low redox overpotential, reversible electrochemistry, fast electron transfer kinetics, and good stability [10]. Examples of diffusional electron mediators that have been used are ferrocyanide (7.5%), ferrocene and ferrocene derivatives (23.1%), conducting organic salts (2.8%) and quinones (10.4%) [2,3,22]. Ferrocene (Fig.3.6) is an organometallic complex that has a wide range of redox potentials independent of pH.

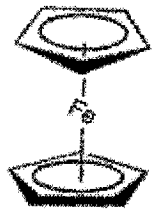


Figure 3.6 *Ferrocene molecule.*

2- *Immobilization of the enzyme (and electron mediator) layer, and the decrease of the electrochemical influence of interfering molecules:* a stable immobilization of the enzyme on the electrode surface with complete conservation of its activity is a crucial factor for the performance of an electrochemical biosensor. Several methods have been used for immobilizing the enzyme onto the electrode, including entrapment behind a dialysis membrane or within a polymeric film such as Nafion (10.6%) or polypyrrole (21.1%), covalent coupling through a cross-linking agent such as glutaraldehyde (2.3%), adsorption, and avidin-biotin (2.0%) binding [2,8]. The most efficient method is the electrochemical entrapment of the enzyme in organic conducting polymers during their electrogeneration on an electrode surface. The enzyme-containing surface polymer layer is formed from a solution containing the corresponding monomers and the enzyme by controlled potential electrolysis. A uniform and strongly adherent polymer film results,

deposited onto a small area with a high degree of geometrical conformity and controllable thickness [23]. The most commonly used conductive polymer is polypyrrole and its derivatives due to their versatile applicability and the wide variety of functional groups covalently linked to a pyrrole monomer [24]. In addition, polypyrrole is a biocompatible polymer, thus enabling the use of polypyrrole-modified biosensors *in vivo*. A Nafion layer (negatively charged sites) on top of the polypyrrole would be useful in repelling interfering anion species, such as ascorbic acid, whose concentration in the brain is up to 1000 times greater than that of dopamine. Nafion will also increase the sensitivity of the biosensor toward dopamine, however at the expense of a diminished temporal resolution. All commercial applications have employed polymer films to make the biosensor practical [7].

In this work, PPO was the biorecognition element used to oxidize DA. Ferrocene played the role of electron mediator. Platinum and carbon paste electrodes were used as physical transducers. Polypyrrole was the conducting polymer used to immobilize PPO and ferrocene onto the electrode surface, while Nafion was used in some experiments in order to eliminate the interference of ascorbic acid.

4 LITTERATURE REVIEW

The following section gives an overview of the literature related to the design of biosensors and sensors for the detection of dopamine (DA).

4.1 Enzyme-Based Dopamine Biosensors

The tailoring of a biosensor's architecture can be focused on several important parameters, such as enzyme immobilization, electron mediator immobilization, biosensor sensitivity, stability and detection limit, biosensor's fouling, etc.

As already mentioned previously, the recognition of a substrate molecule (e.g. dopamine) is done by using the corresponding enzyme, which is preferably immobilized on the electrode surface. In the case of DA, the immobilization of polyphenol oxidase (PPO) on the electrode surface has been done by the physical adsorption, covalent immobilization in a polymer film, entrapment under a dialysis membrane, or attachment to a self-assembled monolayer [2,22,24,25]. According to Sapelnikova *et al.* [26], the thickness of the enzymatic layer is an important parameter that should be carefully optimized since it governs the biosensor response time, sensitivity and stability. The optimum thickness must be at least three times the reaction length. Forzani *et al.* [27] physically adsorbed PPO on the electrode surface and entrapped it behind a dialysis membrane. The working electrode was a Teflon holder filled with carbon paste (CPE) containing 7,7,8,8-sodiumtetracyanoquinodimethane (NaTCNQ) as electron mediator. It was shown that the presence of NaTCNQ competes with the polymerization steps of dopaminequinone to melanin (Fig.3.5) and converts it back to DA, thus amplifying the signal detection. The lowest DA detection limit was 0.25 μM with a sensitivity of 48±5 $mA\,M^{-1}$. One disadvantage was the poor reproducibility of the analytical results caused by the inhomogeneity of the electrode surfaces. Vedrine *et al.* [28] used polythiophene to entrap PPO on a glassy carbon disk electrode. The immobilization of the enzyme was done by a one-step electrochemical polymerization of a solution of PPO and thiophene. Dissolved oxygen was used as an electron mediator and the experiment was carried out in an air-saturated phosphate buffer solution to keep a constant concentration of oxygen. This biosensor was based on the detection of the electrochemical reduction of o-quinone back to catechol. It showed high sensitivity to DA with a detection limit of 100 nM. The

enzyme electrode retained 30% of its initial activity after 12 days of operation. Tu *et al.* [17] immobilized PPO in a silk fibroin membrane on a ferrocene modified disposable working electrode. Silk fibroin is a favorable media to keep the bioactivity of the enzyme. The detection limit of DA was claimed to be 10 nM, however, the presented graph showed a detection limit of 20 nM only. The biosensor kept its activity for 500 times of use and it was successful in repelling ascorbic acid interference when the concentration was less than 500 times that of DA. Another paper by the same authors [16,29] dealt with a disposable screen-printed graphite electrode. Two electron mediators were used: ferrocene and tetramethylbenzidine (TMB). First, the electrode was modified with ferrocene, then, a mixture of β-cyclodextrin, glutaraldehyde and TMB constituted the inclusion complex for PPO and was deposited on top of the ferrocene layer. A more effective electron transfer and excellent analytical performances of the biosensor resulted: a very high selectivity to DA in the range from 1 nM to 1 μM with a detection limit as low as 0.5 nM. Also, the interference of ascorbic acid was reduced due to the performance of the membrane. The biosensor kept its activity for 15 days. DA was also amperometrically determined in pharmaceutical formulations by flow injection analysis (FIA) [13]. The biosensor was a carbon paste electrode modified with PPO and mediator 7,7,8,8 tetracyanoquinodimethane (TCNQ). It was used as flow-through detector. The stability of the biosensor was tested over a 60 days period and a loss of only 30% of the initial enzyme activity resulted. The biosensor showed good reproducibility and was able to detect DA linearly in the concentration range of 20 μM to 20 mM. A relative deviation less than 3.4% resulted when the biosensor was used to analyze DA concentrations in pharmaceutical formulations. Another paper [15] compared the properties of different carbon based electrodes modified with PPO: glassy carbon paste electrode (GCPE), graphite paste electrode (GPE) and glassy carbon electrode (GCE). GCPE was found to be the most electroactive surface due to the glassy carbon microparticles, to have the shortest potential window and highest background current. It was further analyzed for its effectiveness in detecting DA: the calibration curve of DA was linear up to a concentration of 11 μM. The sensitivity was 3.6 $mA\,M^{-1}$ and the detection limit 2.4 μM. The electrode was very stable, retaining 90% of its sensitivity after 4 months under dry conditions and it was also suitable for the determination of DA in pharmaceutical

formulations, with a relative error of less than 2%. Petit *et al.* [14] developed a solid-paraffin-graphite-particle electrode incorporating PPO within its matrix by direct mixing with the carbon paste. The biosensor was used in a flow injection analysis (FIA) system for the detection of DA. Various parameters were investigated to optimize the biosensor. The electrode response was fast and linear over a large DA concentration range from 0.1 μM to 1 mM. The biosensor was stable compared to a classical carbon paste electrode, and was able to provide a low DA detection limit of 50 nM. Deshpande *et al.* [30] used whole banana cells as the source of PPO. They were entrapped electrochemically in a polypyrrole film on a gold electrode. No mediator was used, thus DA was monitored by the oxidation current of hydrogen peroxide. A linear response was obtained for DA levels below 700 μM in plasma-based samples. The biosensor was stable but showed erratic response and irreproducibility for concentrations of DA above 1 mM. It also exhibited high biocatalytic activity, good response time, selectivity and reusability. Rubianes *et al.* [31] modified a carbon paste electrode by incorporating iridium microparticles and PPO. The resulting biosensor exhibited excellent electrocatalytic activity toward the reduction of quinone back to DA, thus enhancing the sensitivity and lowering the detection limit of DA down to 0.83 μM. Miscoria *et al.* [32] modified a carbon paste electrode with gold nanoparticles and PPO by mixing them with graphite particles and mineral oil. It is known that gold catalyzes the oxidation and reduction of hydrogen peroxide. Hence, lower potentials were needed for DA detection thus minimizing the interference of ascorbic acid. It was noticed that the presence of the nanoparticles and protein improved the electron transfer process. A very fast and sensitive response to DA was obtained with a sensitivity ranging from 1.5 $\mu A\,M^{-1}$ to 92 $\mu A\,M^{-1}$ and a detection limit of 0.2 μM. Only one paper by Zhou *et al.* [33] exists on the development of a PPO-based boron doped diamond (BDD) sensor for the detection of DA. The authors produced a covalently linked amine-terminated active BDD surface to immobilize PPO. The resulting biosensor responded rapidly within 10 seconds to the addition of DA aliquots and exhibited excellent selectivity to DA in the presence of AA. Moreover, linearity was observed within the range of 5 to 120 μM of DA with a sensitivity of 68.6 $mA\,M^{-1}cm^{-2}$ and a detection limit of 1.3 μM. The above enzyme electrode could maintain 90% of its original activity after intermittent use for 1 month when storing in a dry state at 4°C.

4.2 Enzyme-Free Dopamine Sensors

Many research groups have investigated the detection of DA using only bare electrodes, i.e., without the use of PPO as a recognition element. The major focus in the development of such enzyme-free electrodes (sensors) is on the modification of the sensor's surface in order to eliminate the interference of ascorbic acid, uric acid and/or 3,4-dihydroxyphenylacetic acid (DOPAC: metabolite of DA). The following section will give a review of various electrochemical sensors (electrodes) used to detect DA: platinum (Pt), gold (Au), boron-doped diamond (BDD) and carbon-based electrodes as well as *in vivo* DA sensors.

4.2.1 Platinum-based sensors

A Pt microelectrode was chemically modified by electrodepositing nickel hexacyanoferrate to catalyze the oxidation of DA and to eliminate electrode fouling caused by DA oxidation products on bare Pt [34]. Additionally, a Nafion film was formed on the electrode surface in order to eliminate the AA interference. The resulting DA oxidation currents increased linearly with DA concentration in a range from 0.1 mM to 15 mM, which are rather high concentrations for physiological monitoring of DA. Pt electrodes were also modified with conducting polymers such as poly[4,4-bis(butylsulfanyl)-2,2-bithiphene [35] or poly(3,4-ethylenedioxythiophene)/Prussian blue films [36]. The resulting modifications led to increased oxidative currents showing a linear response in a DA concentration range from 0.1 mM to 2 mM, and to higher sensitivity to DA. However, this concentration range is also too high for monitoring physiological effects of DA. In another paper [37], DA was pre-concentrated at a Pt electrode prior to electrochemical analysis, since DA is known to adsorb rapidly onto Pt surfaces. DA detection limits as low as 50 nM were obtained.

4.2.2 Gold-based sensors

In electrochemical applications, gold electrode surface is usually modified with organic self-assembled monolayers (SAMs). Liu *et al.* [38] synthesized a new reagent 2-amino-5-mercapto-(1,3,4) triazole to fabricate SAMs on a gold electrode for the first time. The electrochemical behavior of DA and uric acid (UA) was studied at the resulting

sensor. A peak separation of the two components was achieved, thus allowing their simultaneous determination. The calibration curve of DA was linear in a concentration range from 2.5 μM to 500 μM, with a detection limit of 0.8 μM. This sensor was also used to detect UA in real urine and serum samples with satisfactory results. Shervedani *et al.* [39] produced a gold cysteamine SAMs modified electrode that was successful in detecting DA at a concentration of 2.31 μM in the presence of 1.0 mM AA. The voltammetric waves of the two species were well separated in an acidic medium and the calibration curve for DA presented two linear regions. Zhang *et al.* [25] developed an *in-situ* functionalized SAM of 4'-mercapto-N-phenyl-quinone diimine (MNPD) on gold which exhibited catalytical effects toward DA and AA oxidation. The peak current due to the oxidation of DA obtained from differential pulse voltammetry was linear in a concentration range between 5 μM and 125 μM, with a detection limit of 1.2 μM. The modified electrode showed a good sensitivity and selectivity to DA in the presence of AA. The initial sensitivity decreased by 14% after one month. Further, a polycrystalline gold electrode was modified with gold nanoparticles immobilized on an amine-terminated SAM for the detection of DA in the presence of excess AA [40]. Gold nanoparticles exhibited enhanced catalytic activity toward the detection of DA, clearly separating the peak potentials of DA and AA. The detection limit of DA was 0.13 μM. The developed sensor was stable, reproducible, sensitive and selective. Gold electrodes were also modified with polymeric films and composites such as ultrathin polypyrrole-tetradecyl sulfate (PPy-TDS) film [41] poly(aniline boronic acid)/carbon nanotube composite (PAB/CNT) [42] and tannic acid-doped polypyrrole (PPy/TA) film [43]. The first modified gold sensor (Au/PPy-TDS) was successfully used for the simultaneous determination of DA, UA and AA. It showed excellent selectivity due to the catalytic function of the PPy-TDS film, high stability and good anti-fouling properties. A detection limit of 0.4 μM was achieved for DA. The second modified sensor (Au/PAB/CNT) was able to detect DA concentrations as low as 1 nM with very high sensitivity, making it a potential device for the diagnosis of Parkinson's disease. The third sensor (Au/PPy/TA) was also efficient in repelling AA up to a concentration of 5 mM, above which the oxidation current increased. The detection limit of DA was 2.0 μM in the absence of AA

and 0.3 μM in the presence of AA. This unexpected behavior was due to the AA-catalyzed reduction of dopaminoquinone back to dopamine.

4.2.3 Boron-doped-diamond (BDD)-based sensors

The attractive features of BDD were explored to develop selective, sensitive and stable dopamine sensors. Fujishima *et al.* [44] examined an anodically treated diamond electrode that was able to detect dopamine at a concentration of 50 nM while separating the AA and DA peaks. The electrode showed a good stability over a period of 3 months. Olivia *et al.* [45] developed BDD microfiber electrodes covered with overoxidized polypyrrole in order to eliminate AA and DOPAC interferences. The limit of detection for DA was 0.1 nM and the calibration curve was linear from 0.5 nM to 100 μM. The sensor was highly reproducible with a relative standard deviation of 5.4%. The sensor was stable during 7 days with efficient rejection of AA and DOPAC. Another BDD sensor was electrochemically modified with a cationic polymer film of *N,N*-dimethylaniline (PDMA) [46] to produce a selective, stable and sensitive sensor for DA in the presence of AA in their range of physiological concentrations, i.e. AA 100 to 1000 times higher than DA. The detection limit of DA was 60 nM. Sopchak *et al.* [47] used a rotating and hydrodynamically modulated rotating disk electrodes (RDE) of BDD in order to improve the analytical sensitivity by reducing the background current and to extend the linear current response for dopamine analysis to 200 nM. Unfortunately, this RDE sensor was unsuccessful in resolving DA and AA peaks.

4.2.4 Carbon-based sensors

These are the most commonly used transducers in research on sensors due to their ease of preparation, low background current, reproducible surface, low cost and chemical and mechanical stability. Examples of carbon-based electrodes are carbon paste electrodes, carbon-fiber microelectrodes, multi-wall carbon nanotubes, glassy carbon electrodes and graphite electrodes. These different electrodes were modified chemically and/or physically in order to selectively detect low DA concentrations in the presence of AA and UA. Ramesh *et al.* [48] have used unmodified, exfoliated graphite electrodes and were successful in detecting a DA concentration of 50 nM in the presence of 100 μM

AA. A glassy carbon electrode was modified with a suspension of multi-wall carbon nanotubes and covered with Nafion [49], thus exhibiting excellent selectivity towards DA in the presence of 500-fold excess of AA and 150-fold excess of UA. A detection limit of 2.5 nM was achieved after accumulation of DA at open-circuit potential for two minutes. Carbon-polyvinylchloride (C-PVC) composite electrode was constructed by Aguilar and co-workers [50]. It was selective and stable for the detection of DA in a mixture containing excess AA and UA in acidic medium. The limit of detection of DA was 0.2 μM. Polymer films were commonly used with carbon-based electrodes to reject anions and prevent fouling of the electrode surface. An overoxidized polypyrrole (dodecyl sulphate) film-coated glassy carbon electrode [51] successfully detected 40 nM of DA while repelling AA. Another paper [23] reported a carbon paste electrode modified with polypyrrole/ferrocyanide film that was able to detect DA and AA separately with a detection limit of 15.1 μM of DA. Different carbon electrodes were modified with a melanin-type polymer film for the quantification of DA. Depending on the nature of the carbon material used, the analytical performance of the sensor varied greatly. Rubianes *et al.* [52] showed that glassy carbon electrode offered excellent selectivity (3% interference from DOPAC and 7% interference from AA), stability, and low detection limit (5 nM) in detecting DA. Glassy carbon electrode was also modified with TiO_2 nanoparticles covered with Nafion film [53]. The prepared electrode showed excellent electrocatalytic behavior for DA oxidation while preventing the AA interference. A detection limit of 9.5 nM was obtained with high sensitivity. Salimi *et al.* [54] have reported the modification of glassy carbon electrode with catechin hydrate as a natural antioxidant covered with Nafion. The resulting sensor was sensitive with a low detection limit of 11 nM and selective to DA even in the presence of a 200-fold concentration excess of AA. An unmodified, overoxidized carbon-fiber microelectrode was used *in vitro* and *in vivo* for the detection of DA [55]. *In vitro* measurements showed improved adsorption of DA leading to an increased sensitivity at the expense of temporal response and a detection limit lower than 5 nM. In a paper by Lin *et al.* [56], glutamic acid was used to modify a graphite electrode. This allowed a peak separation of AA and DA when present in the same solution and a detection limit of 1.8 μM of DA. However, the interference of UA could not be resolved. Carbon nanotube paste modified electrodes [11] have been

prepared and proved to be promising tools for the detection of DA with good sensitivity and detection limit around 0.5 μM. Also, these electrodes are biocompatible with biological tissues and their nanometer size makes them suitable for *in vivo* measurements. A new potential sweep voltammetric technique developed by Yoo *et al.* [57] made use of a carbon ultramicroelectrode of 10 μm diameter to detect DA. This method showed excellent dynamic linearity for DA with a detection limit of 127 pM, the lowest limit reported in the literature, and with a high sensitivity. Different papers reported the properties of modified carbon paste electrodes with zeolites [58], nafion-coated clinoptilolite [59], nanosized cobalt phthalocyanine particles [60] and iron(II)tetrasulfophthalocyanine [61]. The resulting sensors had good electrocatalytic activitiy to DA, good stability and reproducibility, effective peak separation of DA and AA and low detection limits ranging from 10 nM to 1 μM.

4.2.5 *In vivo* sensors

In vivo sensors are intended for cerebral applications in order to study the relationship between release of DA and human behavior. Most of *in vivo* sensors are made of carbon fiber microelectrodes, even though some studies made use of carbon paste and Pt electrodes. Nafion coating procedures have been investigated in order to design *in vivo* sensors that would eliminate signals from electroactive interferents such as AA. Crespi *et al.* [62] reported the use of Nafion on top of carbon fiber microelectrodes. The sensor showed good selectivity for detecting positively charged dopamine *in vitro*, whereas a mixture of Nafion and a crown ether compound allowed the detection of basal extracellular levels of dopamine *in vivo*. However, one disadvantage of such biosensor was the inability to detect consistently dopamine in dopaminergic brain areas without electrical or pharmacological stimulation of dopamine release. Another paper [63] reported the use of Nafion-coated carbon paste electrodes *in vitro* and *in vivo* for AA and DA concentrations. Even though the presence of Nafion eliminated the AA interference, the sensor sensitivity to DA remained unchanged *in vitro*. Furthermore, it was found that carbon paste voltammetric electrodes were suitable for monitoring AA release rather than endogenous DA release. Yokoyama *et al.* [64] used Pt-disk microelectrodes for simultaneous monitoring of temporal changes in hydrogen peroxide (H_2O_2) and

dopamine (DA) levels. The electrode was capable of detecting each target substance without any interference. For an *in vivo* application, the electrodes were implanted into the striatum of rats and used successfully to monitor intrastriatal changes in H_2O_2 and DA simultaneously. Brown *et al.* [65] also reported the development of a Pt sensor covered with temperature treated Nafion intended for *in vivo* applications. Elimination of interference from AA and UA was achieved; however there was a complete blocking of DA signal. It was concluded that this type of sensor was more suitable for detection of gaseous neurochemicals such as nitric oxide.

5 MATERIALS AND METHODS

5.1 Reagents and Solutions

The electrolyte used in all experiments was 0.05 M phosphate buffer solution (PBS) pH 7.0 prepared by dissolving 0.68 g of anhydrous, monobasic potassium phosphate (KH_2PO_4, Sigma Chemical Society 99.7%) in 29.1 mL of 0.1 M sodium hydroxide (NaOH, Sigma-Aldrich Laborchemikalien) and further diluted by conductivity water (Nanopure, resistivity = 18.2 MΩ cm) to a volume of 100 mL. Solutions of dopamine (DA, Sigma-Aldrich) and ascorbic acid (AA, Sigma-Aldrich) were prepared by dissolving appropriate amounts of DA and AA powders in 0.05 M phosphate buffer (pH 7.0). The necessary amounts of the enzyme polyphenol oxidase (PPO, Sigma-Aldrich E.C.1.14.18.1) and of the mediator ferrocene (Fc, Sigma-Aldrich 98%) were dissolved in 0.1M of sodium sulfate (Na_2SO_4, Fisher Scientific, anhydrous) in which 5 mL of ethanol (90%v/v) and water (10%v/v) was added. Pyrrole solution (Aldrich Chemical Co. 98%) was added to 5 mL of this mixture in order to form a polypyrrole film, an electronically conducting polymer that incorporated PPO and Fc. Other reagents include Nafion perfluorinated ion solution (dissolved in 5% lower aliphatic alcohols, Aldrich Chemical Co.) used to coat the working electrode, paraffin oil (Fluka) and graphite powder (Sigma-Aldrich) for the preparation of the carbon paste electrode. For all washing, rinsing and preparation of solutions, conductivity water was used. All experiments were conducted at ambient temperature.

5.2 Electrochemical Cell and Electrodes

A standard three-electrode, single compartment electrochemical cell was used in all measurements (Fig.5.1).

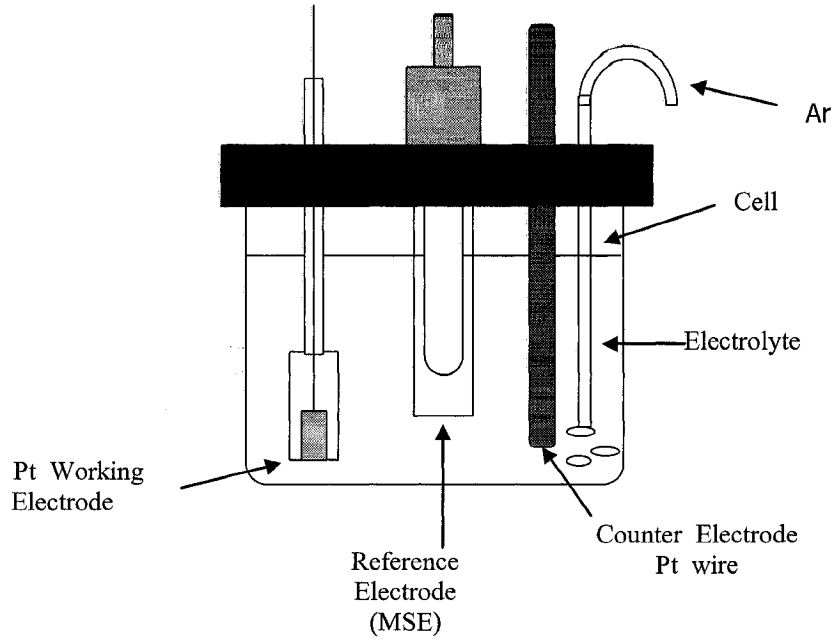


Figure 5.1 *Electrochemical cell used in experiments.*

The counter electrode (CE) was made of high purity platinum (99.99%, Johnson-Matthey) and stored in 98% sulfuric acid when not in use. The reference electrode (RE) was a commercial radiometer mercury/mercurous sulfate reference electrode (MSE, +650 V vs. standard hydrogen electrode, SHE). For the polymerization experiment, the electrochemical cell was a plastic 60mL syringe filled with 5 mL of the polymerization solution (PPO and Fc dissolved in EtOH/H_2O/Na_2SO_4+0.1 M pyrrole). Saturated calomel electrode (SCE, +0.244 V vs. SHE) was used as a reference electrode in this case. In experiments in which a PPY film was formed on the electrode surface, the working electrode (WE) was a platinum disk (diameter = 0.3 cm) of high purity (99.99%, Johnson-Matthey) embedded in a Teflon holder giving a two-dimensional area exposed to the electrolyte. Before experiments, the platinum working electrode was electrochemically cleaned in 0.5 M sulfuric acid solution by potentiodynamic cycling between the hydrogen evolution reaction and the oxygen evolution reaction regions [66]. When a reproducible cyclic voltammogram (CV) was obtained the Pt electrode was considered to be clean and ready to use. A carbon paste electrode and a boron-doped diamond electrode were also used as WEs. Boron-doped diamond electrode was cleaned

in a solution of ethanol and sodium hydroxide and was placed in an ultrasonic bath for 10 minutes.

To remove any dissolved oxygen and to provide a well-mixed bulk solution in the electrochemical cell, the solution was purged by argon. The arrangement of the electrodes in the cell shown in Figure 5.2 ensured that the purge stream was placed as far as possible from the WE to avoid disturbances from the gas bubbles, and that the RE was close to the WE to minimize ohmic losses, since they are a function of the cell geometry.

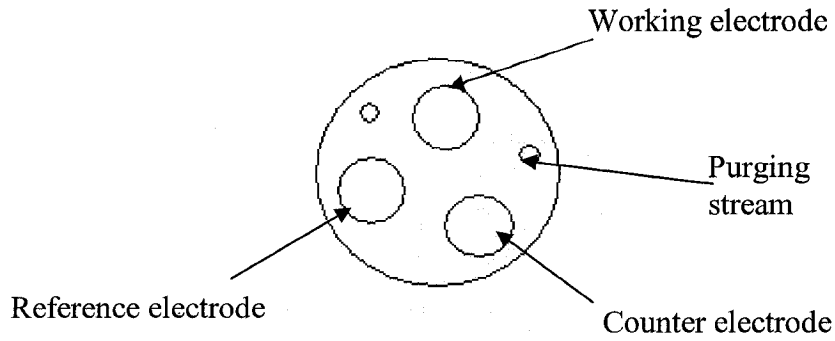


***Figure 5.2** Arrangement of electrodes in the electrochemical cell cover.*

5.3 Working Electrodes – (Bio) Sensors

Platinum (Pt) and boron-doped-diamond (BDD) electrodes were first used "bare" as sensors, i.e. without the enzyme and the electron mediator, in order to investigate the adsorption behaviour and kinetics of DA as well to determine DA concentrations in unknown (urine) samples. Nafion was also used to modify the electrodes and study its efficiency in repelling interfering species.

5.3.1 Platinum Electrode

Platinum is a noble metal widely used in electrochemistry due to its chemical inertness and excellent catalytic properties. 23.6% of the electrodes used in biosensors applications are made of platinum [8]. The following procedure was used to construct a Pt-based biosensor: the biosensor architecture was based on the immobilization of PPO enzyme and Fc mediator in the polypyrrole polymer film on the electrode surface. A solution of 6,120 U (1,200 U/mL) PPO, 10 mM Fc and 0.1 M pyrrole monomer [67] in $EtOH/Na_2SO_4/H_2O$ solution was used for the biosensor construction. The solution was purged by argon for 15 minutes prior, and then during the experiment to have all the

components well mixed/distributed. The polymerization proceeded for either 20 or 40 minutes. A black uniform polypyrrole (PPY) layer, incorporating PPO and Fc, was formed on the Pt surface. The biosensor was rinsed with nanopure water, placed in the electrochemical cell, and measurements were taken. When Nafion was used to form a membrane on top of the PPY layer, a drop (~9μL) of the Nafion solution was placed on top of the polymer layer and left to dry/polymerize for 30 minutes before using the biosensor in the electrochemical cell.

5.3.2 Carbon Paste Electrode

Carbon paste electrodes have received special attention in electroanalysis due to their simplicity and rapid preparation. They also offer reproducible surfaces and low residual current [68]. The CPE constructed in this work had the following weight composition [14,15,52]: 60% graphite powder, 39% paraffin oil, 0.3% PPO and 0.7% Fc. For the preparation of 1 g of carbon paste, 3 mg of PPO (11,700 U) and 7 mg of Fc were mixed with 0.39 g of paraffin oil in a mortar with a pestle for 3 minutes, then 0.6 g of graphite powder was added and mixed thoroughly for 15 minutes to form a homogeneous and compact paste. The mixture was then packed firmly into a plastic syringe (internal diameter 4 mm) and the electric contact was made through a copper wire inserted into the paste. When not in use, the CPE was stored in a freezer at -20°C and in a fridge at 4°C one day before conducting the experiments.

5.3.3 Boron-Doped-Diamond Electrode

Boron-doped diamond electrode (2D diameter = 0.3 cm) was used as a sensor surface because of its wide electrochemical inert window, corrosion resistance, chemical inertness and very low capacitance [44,46,69]. Before each set of experiments, the electrode was cleaned in a solution of EtOH and NaOH then sonicated in an ultrasonic bath for 10 minutes. Before the first utilisation, BDD was activated by an anodic treatment in 2M H_2SO_4 solution at a potential of +2.0 V vs. MSE for 30 minutes. It was then rinsed thoroughly with nanopure water and placed in the electrochemical cell.

5.4 Electrochemical Equipment

All electrochemical experiments were done using an Autolab potentiostat/frequency-analyzer PGSTAT30/FRA2 equipped with GPES 4.9 and FRA softwares. Cyclic voltammetry (CV), ac-voltammetry (ACV) and chronoamperometry (CA) experiments were run and data were analyzed using the GPES software, while electrochemical impedance spectroscopy (EIS) experiments were run and data were analyzed using the FRA software. Calculations were made using Microsoft Excel software.

5.5 Experimental Techniques

Several electrochemical techniques were used in this research:

5.5.1 Cyclic Voltammetry (CV)

CV is a useful technique to determine redox potentials, the extent of reaction reversibility, and the presence of coupled homogeneous reactions and of adsorption. The working electrode is polarized linearly between two potentials at a specified scan rate in the forward and reverse direction [46,70]. A potential-dependant current response, commonly known as a cyclic voltammogram (CV), results from such experiments (e.g. see Fig.6.1.1). CVs are chemical signatures that distinguish different chemicals, for instance dopamine and ascorbic acid. From the peaks generated, they indicate if ascorbic acid or any other electroactive metabolite such as uric acid or 3,4-dihydroxyphenylacetic acid (DOPAC) were interfering with dopamine, whether the reaction of dopamine was reversible, electron-transfer or mass-transport controlled, etc. CV was the technique of choice to determine the oxidation potential of dopamine, ascorbic acid as well as of the mediator ferrocene and of the monomer pyrrole.

5.5.2 Chronoamperometry (CA)

Chronoamperometry is the most commonly used electrochemical technique for substrate detection. It is also the method of choice to follow the chemical changes that occur during very rapid important neurochemical events such as the release of dopamine in the synaptic cleft and its subsequent oxidation to dopaminoquinone [18,46]. In this

technique, potential is controlled (kept constant) and current is measured as a function of time (and substrate addition), as shown in Figure 5.3.

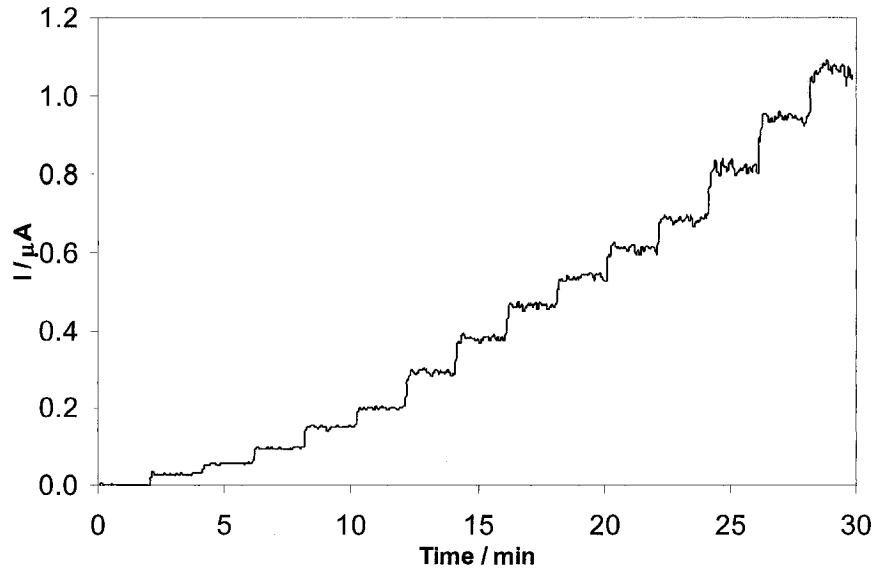


Figure 5.3 *Chronoamperometric response of a platinum sensor to increasing addition of dopamine. E=-0.1V (vs. MSE).*

Chronoamperometry provides the best temporal resolution allowing the direct measurement of kinetic events instead of their extrapolation from other techniques. However, chronoamperometry has little chemical selectivity, for example, when ascorbic acid is present with dopamine (two electroactive molecules), it is impossible to distinguish the current for each component. In this work, chronoamperometry was used to determine the detection limit of dopamine, the response times of the biosensor and to construct calibration curves of the biosensor in use. The oxidation potential of dopamine at which the calibration curve was carried out was determined from CV measurements, and it was varied depending on the transducer in use and on the surface modifications.

5.5.3 Alternating Current Voltammetry (ACV)

ACV is a technique that is sensitive to the presence of adsorbed species on the electrode surface. An *ac* potential of a fixed frequency and amplitude is applied in a selected potential range, and a capacitive behavior of the system is monitored (e.g. see Fig.6.1.6). The frequency is fixed so it probes the response of an electrochemical double

layer (usually around 25 Hz). The technique was used to study the variation of the capacitance of the system with dopamine concentration and to characterize the adsorption of dopamine on the Pt electrode surface. Increasing dopamine concentrations were added to the PBS and capacitance versus voltage curves were obtained for each concentration.

5.5.4 Electrochemical Impedance Spectroscopy (EIS)

EIS is a non-destructive technique. It provides information on processes occurring at the solid-liquid interface (adsorption, charge and mass transfer, charging of the double layer, etc.). The working electrode is held at a specific *dc* potential and an *ac* sinusoidal signal of small amplitude (±5 - 10 mV) and a certain frequency is applied over it (the excitation signal). The measured signal is *ac* current and phase angle [70]. Impedance, which is a measure of the resistance of a circuit to a flow of electric current, can be determined for different processes due to their different time constants. Hence, the system is scanned over a wide frequency range in order to get a response of different time constants. Then, an electrical equivalent circuit (EEC), comprising of resistors and capacitors (see Fig.6.3.11), is used to model the electrode/electrolyte interface and corresponding processes, thus obtaining information on the kinetic, mass transport and electric/dielectric properties. Hence, the behavior of an electrochemical system is quite similar to the behavior of an analogous electrical circuit made of real electronic elements. A possibility of using EIS as a detection technique was investigated on a Pt-based biosensor.

6 RESULTS AND DISCUSSION

This section of the thesis is dedicated to the analysis and discussion of the experimental results obtained. It is divided into three parts. The first part will discuss the results concerning the dopamine (DA) oxidation kinetics and adsorption thermodynamics on a bare (unmodified) platinum electrode surface. The remaining two sections will discuss the results related to the detection of DA using sensor and biosensor electrodes. Namely, in the second section the results on the detection of DA using platinum and boron-doped diamond electrodes will be discussed, while the detection of DA using platinum-based and carbon paste-based *bio*sensor electrodes will be presented in the third section of the chapter.

6.1 Kinetics of DA oxidation on a Pt electrode

Platinum was used as both a sensor electrode and one of the biosensor transducers. Therefore, it was of an importance to investigate some fundamental aspects of the electrochemical interaction of DA with the bare Pt surface (oxidation potential and DA adsorption).

In order to investigate the interaction of DA with a Pt surface under potentiodynamic conditions, a series of cyclic voltammetry measurements was carried out in phosphate buffer pH 7.0 containing various concentration of DA (0 to 300 μM). The major aim of these experiments was to determine a potential region of DA oxidation, which would serve as a basis for further experiments. Figure 6.1.1 shows an example of the cyclic voltammograms of the Pt electrode recorded in a phosphate buffer solution (PBS) and in DA-containing solutions. The response of the electrode in the DA-free solution is rather featureless. A slight increase in anodic current (a right-going polarization direction) is due to the adsorption of oxygen-containing species on the Pt surface, i.e. Pt-OH formation [71], while the small cathodic shoulder, S, obtained during the cathodic bias (a left-going polarization direction) represents the reduction of the surface Pt-OH layer to pure metallic Pt. However, in the presence of DA in the bulk solution a well-defined anodic, A, and cathodic, C, peaks are recorded. The anodic peak at -0.2 V is related to the oxidation of DA to dopaminequinone (DOQ) [15,27,29] according to Equation (2):

$$DA \leftrightarrow DOQ + 2e^- + 2H^+ \quad (2)$$

While the cathodic peak at -0.3 V is related to the reduction of DOQ back to DA. However, the CV curves show that DA starts oxidizing already at ca. -0.35 V. Hence, it is safe to conclude that DA can be oxidized on Pt electrodes at potentials positive of -0.35 V. This experimental DA oxidation potential is quite close to the thermodynamic value reported in the literature, -0.30 V vs. MSE [72].

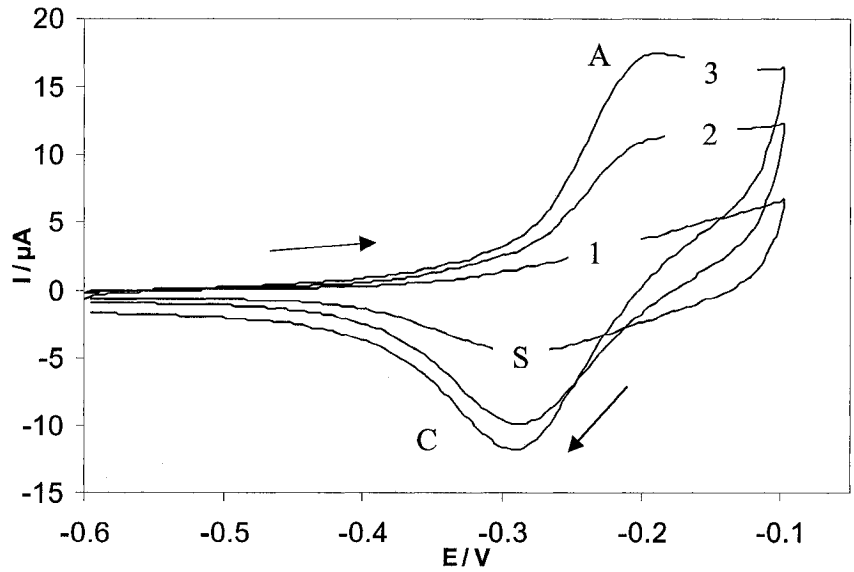


Figure 6.1.1 *Cyclic voltammograms of the bare Pt electrode in 0.05 M phosphate buffer pH 7.0 recorded in a (1) DA free solution, (2) 0.13 mM DA solution and (3) 0.25 mM DA solution. A=anodic peak, C=cathodic peak, S=cathodic shoulder. Scan rate=100mVs^{-1}.*

CV measurements were also done at various scan rates (polarization rates). The aim of these experiments was to determine whether the DA oxidation reaction is, under the experimental conditions applied, diffusionally or surface/adsorption controlled [70]. Figure 6.1.2 shows that with an increase in scan rate the DA oxidation and DOQ reduction peak current increases. This increase for the DA oxidation peak was found to be linear with scan rate (Fig.6.1.3), which indicates that the adsorption of DA is the reaction rate determining , not its mass-transport from the bulk solution to the electrode surface [70,73].

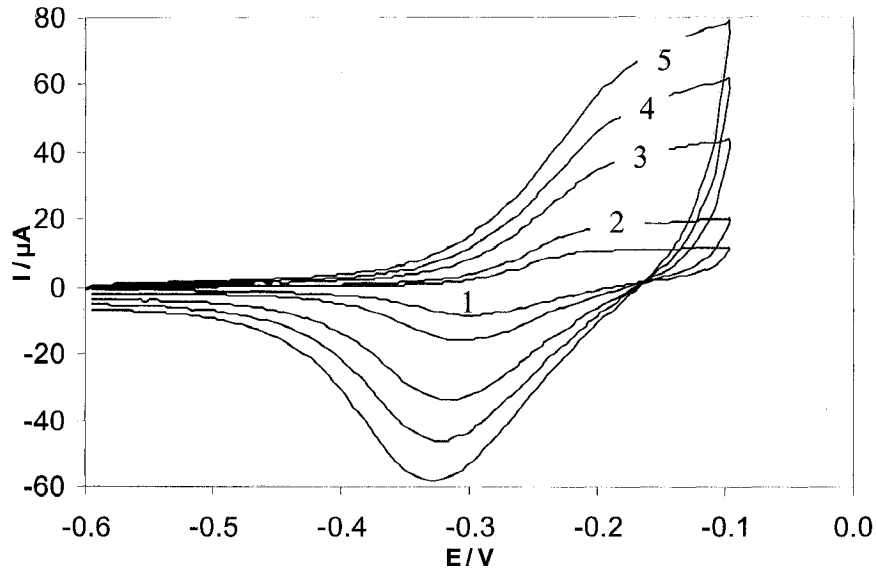


Figure 6.1.2 *Cyclic voltammograms of the bare Pt electrode in a 0.15 mM DA solution recorded at various scan rates: (1) 0.1, (2) 0.2, (3) 0.5, (4) 0.75, and (5) 1* Vs^{-1}.

Wightman *et al.* [74] also found that DA oxidation is not diffusion-controlled, but rather dominated by its adsorption on an electrode surface (carbon-fiber microelectrodes). Ranganathan *et al.* [75] have also reported that DA adsorbs on a glassy carbon electrode, and that the observed increase in electron transfer rate correlated with a DA adsorption strength. Bath *et al.* [73] showed that the stronger adsorption of DA was found to be correlated with an increased sensitivity of the carbon fiber electrodes, while DuVall *et al.* [76] concluded that the adsorption is essential for the rapid electron transfer to the catechol/orthoquinone redox center for DA and several other catechols.

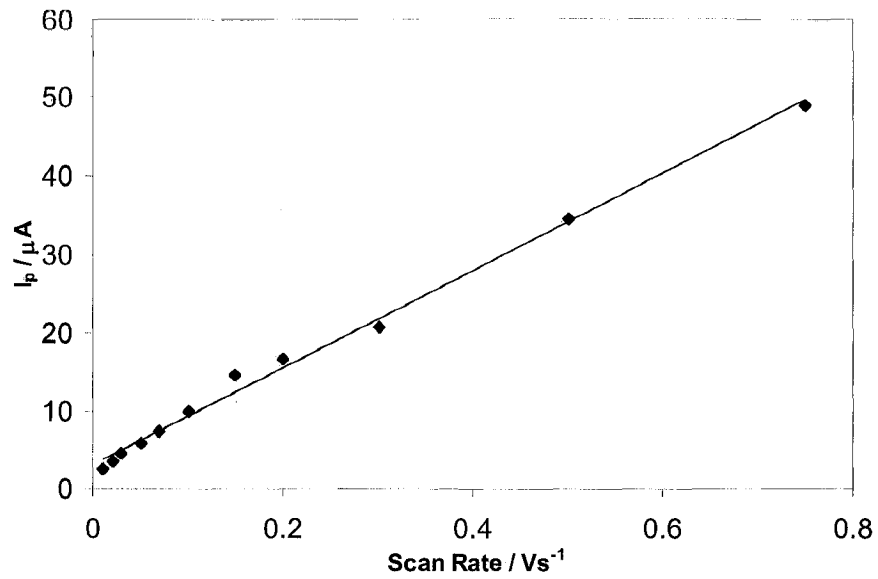


Figure 6.1.3 *Dependence of the oxidation currents on scan rate obtained at the potential of -0.142 V (vs. MSE) from cyclic voltammograms of the bare Pt electrode in 0.15 mM DA solution.*

6.1.1 Adsorption of DA

The DA oxidation reaction on a Pt electrode is a heterogeneous reaction. It was already shown that DA adsorption represents a rate determining step in the overall DA oxidation mechanism. In CV experiments in Figure 6.1.1 and 6.1.2, DA was not allowed to equilibrate on the Pt surface before the measurements. However, if DA is allowed to reach an equilibrium surface coverage (i.e. surface concentration), then the DA peak oxidation current would be directly proportional to the amount of DA adsorbed on the surface (i.e. surface concentration, Γ/mol cm^{-2}), $I_p \propto \Gamma$. Hence, CV equilibrium adsorption measurements were done at various DA concentrations in the bulk solution, and the DA oxidation peak was then further analyzed. Figure 6.1.4 represents the dependence of the DA oxidation peak current on the DA bulk solution concentration. Indeed, the data display a shape quite characteristic for an adsorption process. The graph shows a classical Langmurian-type adsorption trend, in which the surface concentration initially increases with an increase in concentration of DA in the bulk solution and then levels off to a plateau at a bulk DA concentrations above ca. 30 µM.

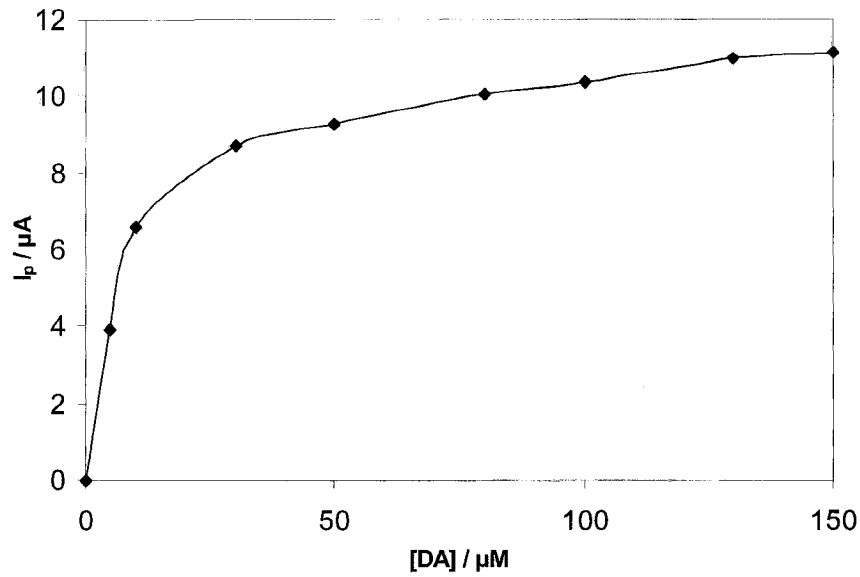


Figure 6.1.4 *Isotherm for the adsorption of DA on a Pt surface: dependence of the oxidation peak current on the bulk solution DA concentration obtained from equilibrium CV experiments.*

In order to obtain thermodynamic data related to the adsorption of DA on the Pt surface, the experimental data was modeled using the Langmuir isotherm [71,77-79]:

$$I_p = \frac{B_{ADS} I_{p,\max} c}{1 + B_{ADS} c} \tag{3}$$

In which c (mol cm^{-3}) is the equilibrium concentration of the adsorbate (DA) in the bulk solution, I_p (A) is the DA oxidation peak current (which is directly proportional to the DA surface concentration), $I_{p,max}$ (A) is the maximum value of I_p, and the parameter B_{ADS} (cm^3 mol^{-1}) reflects the affinity of DA molecules towards surface adsorption sites. Further rearrangement of Equation (3) into a linear form gives:

$$\frac{c}{I_p} = \frac{1}{B_{ADS} I_{p,\max}} + \frac{c}{I_{p,\max}} \tag{4}$$

If the Langmuir isotherm is valid for the observed system, a plot of c/I_p versus concentration c should yield a straight line with parameters $I_{p,max}$ and B_{ADS} derived from the slope and intercept, respectively. Figure 6.1.5 shows the linearized form of the adsorption experimental data from Figure 6.1.4, and indeed the c/I_p *vs.* c dependence is quite linear. This demonstrates the applicability of the proposed adsorption model when describing the adsorption of DA on Pt.

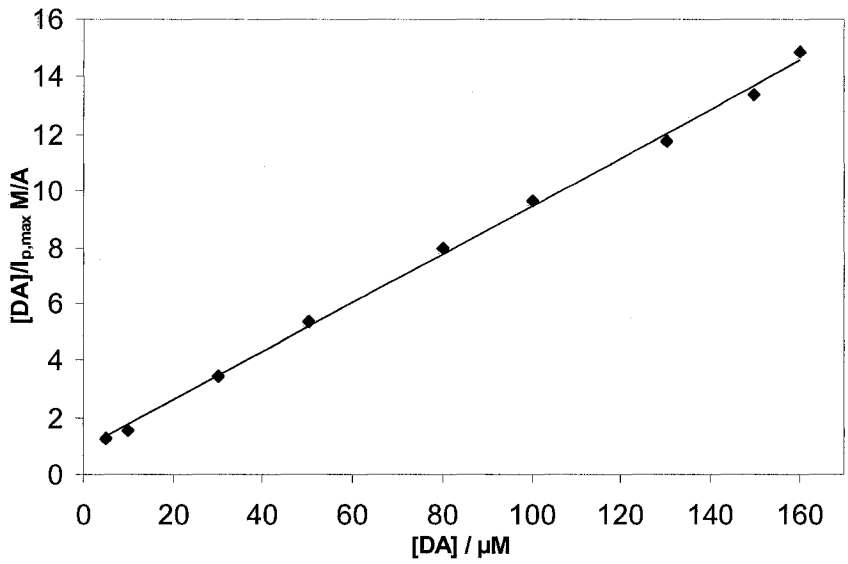


Figure 6.1.5 *Linearized form of the Langmuir isotherm for the adsorption of DA on platinum. The symbols represent the experimental data, while the line is the modeled Langmuir isotherm.*

The parameter B_{ADS}, which reflects the affinity of the adsorbate molecules towards adsorption sites at a constant temperature, can be presented as:

$$B_{ADS} = \frac{1}{55.5}\exp\left(\frac{-\Delta G_{ADS}}{RT}\right) \tag{5}$$

Where R (J mol^{-1} K^{-1}) is the gas constant, T (K) is the temperature, ΔG_{ADS} (J mol^{-1}) is the Gibbs energy of adsorption, and 55.5 is the molar concentration of water (mol dm^{-3}), which is used as a solvent. Using this equation, the Gibbs energy of adsorption of DA onto the Pt surface in phosphate buffer solution was calculated. The Gibbs energy adsorption value obtained was -38 kJ mol^{-1}. The high negative Gibbs energy of adsorption indicates that the equilibrium for the adsorption process lies well in favor of adsorption of DA onto the Pt surface, *i.e.* the DA adsorption process is highly spontaneous. Vasquez *et al.* [80] reported a Gibbs adsorption energy of -34 kJ.mol^{-1} suggesting that the degree of adsorption is intermediate between what is considered as weak adsorption and strong adsorption. As for Soriaga *et al.* [81], their results indicated that the adsorption process is energetically favorable being limited primarily by the space available at the surface.

Adsorption of DA on the Pt surface causes alterations in the double-layer structure, namely the blockage of Pt surface (A), a decrease of a relative dielectric constant (ε_{DA}=2 - 3, while ε_{water}=80) [77], and an increase in the thickness of the double-layer region (d). Since the electrode/electrolyte interface can be represented by a double-plate-capacitor model [73,82]:

$$C_{DL} = \varepsilon_r \varepsilon_0 \frac{A}{d} \tag{6}$$

Where ε_0=8.85×10^{-14} F cm^{-1} is the permittivity of vacuum, the adsorption of DA on the Pt surface should result in a decrease in the double layer capacitance, C_{DL} (F). Figure 6.1.6 shows a set of ACV curves recorded at various concentrations of DA in the bulk solution. As can be seen, with an increase in DA bulk concentration the recorded capacitance decreases, indicating that DA adsorbs on the Pt surface.

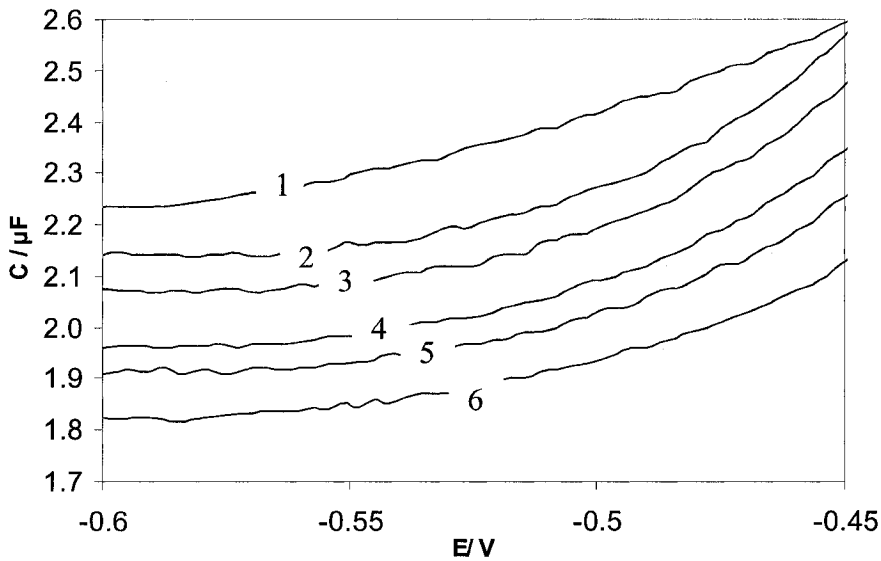


Figure 6.1.6 *Dependence of the double layer capacitance on DA concentration in a non-faradaic potential region of the bare Pt electrode in 0.05M phosphate buffer pH 7.0. (1) DA free solution, (2) 0.03 mM, (3) 0.05 mM, (4) 0.08 mM, (5) 0.1 mM, and (6) 0.2 mM of DA in the solution. Frequency 25 Hz, phase angle -90°.*

The surface coverage by the adsorbed DA layer, θ_{DA}, can be determined using the following equation [83]:

$$\theta_{DA} = \frac{C_O - C^{DA}}{C_O - C_{\min}} \tag{7}$$

Where C_O (F) is the capacitance in the absence of DA, C^{DA} is the capacitance in the presence of DA, and $C_{\min}$ is the capacitance in the presence of DA obtained at the maximum (saturated) DA surface concentration. The DA surface coverage, θ_{DA}, can be defined as the ratio between the DA surface concentration at a particular bulk DA concentration Γ, and the maximum (saturated) surface concentration, Γ_{max}, $\theta_{DA}=\Gamma/\Gamma_{max}=I_p/I_{p,max}$ Taking this definition into account, the original Langmuir isotherm Equation (3) can be further modified to give:

$$\frac{c}{\theta_{DA}} = \frac{1}{B_{ADS}} + c \tag{8}$$

The plot c/θ_{DA} versus c should yield a straight line if the proposed isotherm is valid for the description of the experimental data. Then, from the intercept of the line the adsorption affinity constant, B_{ADS}, can be calculated and subsequently the value of the Gibbs energy of adsorption. Taking the ACV capacitance data presented in Figure 6.1.6 at potential -0.6 V, and using Equations (7) and (8), the surface coverage for the adsorption of DA onto a Pt surface was calculated, and presented in a linearized form (Equation (8)) in Figure 6.1.7. The agreement between the experimental and simulated data was very good ($R^2 = 0.996$).

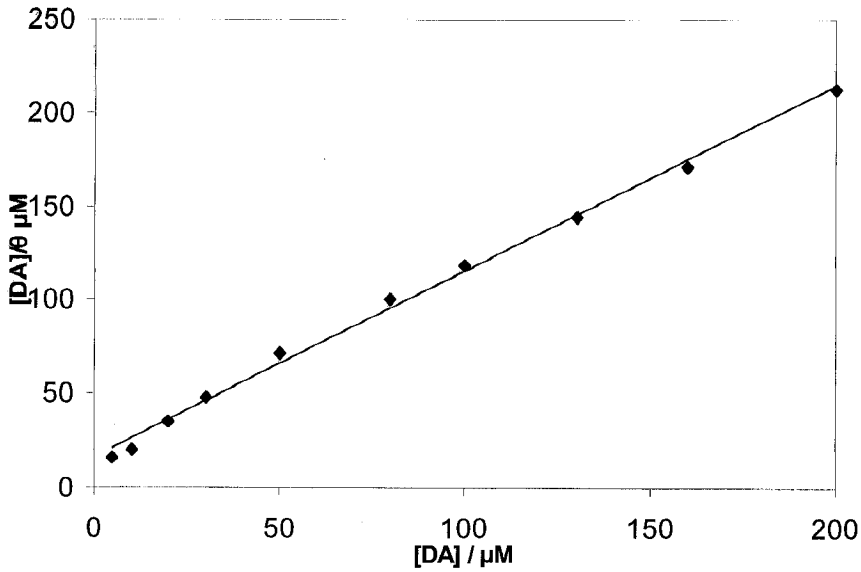


Figure 6.1.7 *Linearized form of the Langmuir isotherm for the adsorption of DA on the Pt electrode. The symbols represent the experimental data, while the line is the modeled Langmuir isotherm. The data were obtained from ACV measurements.*

The value of Gibbs energy of adsorption calculated from the plot is -37.0 kJ mol^{-1}, which is very close to the value calculated on the basis of the DA oxidation peak current values from CV measurements (-38 kJ mol^{-1}). Hence, the data shows that there is a very good agreement between the two experimental methods used for the investigation of the adsorption of DA on a bare Pt surface.

6.2 Dopamine Sensors

In order to detect DA in tissue slices or mammalian brains, unmodified microelectrodes (microsensors), i.e. without the use of polyphenol oxidase (PPO) enzyme as a recognition element, are needed. In this work, platinum (Pt) and boron-doped diamond (BDD)-based sensors were constructed and tested for their efficiency in detecting DA *in vitro*. Due to a high inertness and biocompatibility of these two materials, they are good candidates for the use in *in vivo* measurements. The major parameters of interest in the development of such sensors are their sensitivity, selectivity and detection limit. The required *sensitivity* for DA is determined by the concentration levels found in the measurement environment of interest; it is defined as a slope of the (quasi)linear response (calibration) curve of the sensor. *Selectivity* is expressed as the ratio of the signal output measured with DA alone to the one measured in the presence of interfering substance at the same concentration as DA. The *detection limit* pertains to the DA concentration that generates a response (signal) typically two to three times the noise level [7,84].

6.2.1 Pt-based Sensor

A two-dimensional (2D) platinum electrode (diameter = 0.3 cm) was first investigated as a sensor for the detection of DA. Chronoamperometry (CA) was the major electrochemical technique used to investigate the response of the sensor to DA and to construct a response calibration curve. With CA, the Pt-sensor is held at a constant potential corresponding to the oxidation of DA. Before the injection of aliquots of DA into the supporting electrolyte solution, the sensor is left to stabilize until a quasi-steady-state (background) current is reached. This is followed by the injection of DA aliquots, and the resulting DA oxidation current is recorded as a function of time. An optimum value of DA oxidation potential was determined from CV experiments discussed in Section 6.1, and is denoted in figure captions. It is worth noting that CV measurements (Fig.6.1.1) demonstrated that DA could be oxidized at any potential positive of -0.35 V. The DA oxidation rate, and thus the response signal, increases with an increase in oxidation potential, and hence, it would be more beneficial to detect DA at higher anodic potentials. However, this is true only when DA is the only redox species present in the

solution. When interfering species (e.g. ascorbic acid, AA) are also present in the sample, their oxidation rate also increases with an increase in detection potential, and since the DA oxidation potential is lower than the AA oxidation potential [85], it is desirable to detect DA at low oxidation potentials. An optimum detection potential value is chosen in such a way to balance between the low DA oxidation rate (poor signal) and the interference of other redox species in the sample. This potential is pH dependant, and is thus different on (bio)sensors modified with a Nafion membrane.

Figure 6.2.1 shows the typical staircase response of the Pt sensor. Each staircase represents a response recorded after an aliquot of DA is added into the electrolyte. Thus, with an increase in DA concentration in the bulk solution, the response current also increases. Upon addition of DA in the electrolyte, a sudden increase in current is recorded, which corresponds to the oxidation of DA to DOQ (Equation (2) Section 6.1). A steady-state DA oxidation current is reached ca. 22 seconds after the DA addition. This response time scale is controlled mostly by mass-transport of DA from the injection point to the electrode surface, rather than the kinetics of DA oxidation. Nevertheless, a value of steady-state DA oxidation current is directly proportional to the concentration of DA in the bulk solution, thus allowing the construction of a DA calibration curve.

The inset to Figure 6.2.1 represents the DA calibration curve of the Pt-based sensor, where the steady-state oxidation currents were normalized with respect to the background current before being plotted against the corresponding bulk DA concentrations. It can be noticed that the curve yields two linear regions, one in the low (0 to 20 μM) and the other in the high (30 to 120 μM) DA concentration region. The calibration curve shows that the sensor offers twice the sensitivity for DA in the low concentration region (15.5 $mA\,M^{-1}$) compared to the higher concentration region (8 $mA\,M^{-1}$). A larger sensitivity, 116 $mA\,M^{-1}$, was reported by Hiratsuka *et al.* [86] on bare Pt for DA concentrations ranging from 0 to 600 μM. However, their Pt electrode had a surface area 1.5 times larger than the one we used in this experiment.

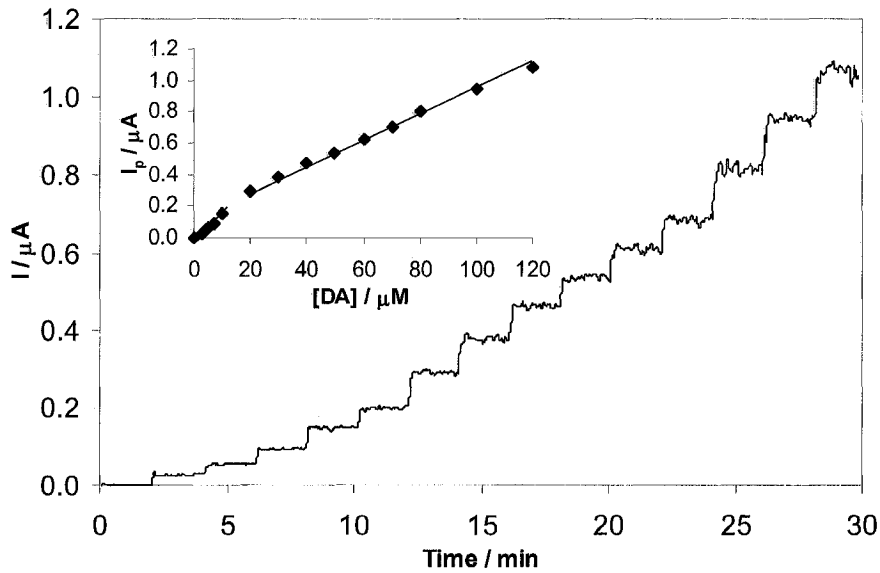


Figure 6.2.1 *Current-time response of a Pt-based sensor for increasing DA concentrations (from 0 to 120 μM). Each current step represents a response of the sensor after addition of an aliquot of DA. E=-0.2V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0). Inset: Calibration curve obtained from steady-state current values of the graph on the main plot.*

CA provides the best temporal resolution but has little chemical selectivity, i.e. it cannot resolve the chemical identity of different electroactive species present in the same solution. This is a problem when ascorbic acid (AA) is present with dopamine, because it has a comparatively high concentration in the brain, ca. 100 to 1000 times higher than DA concentration [1]. Therefore, the Pt surface has to be modified in order to not only differentiate between the two electroactive species, but also to provide better sensitivity to DA. This could be done by blocking (or at least minimizing) the contact of AA with the sensor surface. Since at neutral (physiological) pH DA is a positively charged molecule, while AA is negatively charged, by modifying a Pt-sensor to yield a negative surface charge, AA could be efficiently repelled, while DA would be attracted to the surface by coulombic (electrostatic) interactions. In this work, we have chosen to modify a Pt surface with a Nafion membrane (film), which is a cation-exchange polymer that is highly permeable to cations, while being almost impermeable to anions. Nafion was also chosen as a surface modifier due to the ease of a surface film formation (see the experimental part of the thesis, Chapter 5).

The Nafion-covered Pt-sensor was first tested for its sensitivity to DA detection. Figure 6.2.2 shows a CA curve where aliquots of DA were added into the electrolyte and the DA oxidation current was recorded with time. As with the unmodified Pt sensor, a similar staircase increase in current was observed. However, a higher noise level was recorded due to the presence of the Nafion film on the electrode surface. Although the Nafion film allows DA to pass through to the electrode surface and gets oxidized, it also offers resistance to mass-transport, which is on the CA curve manifested as noise. Similarly to the bare Pt-sensor (Fig.6.2.1), the Pt-sensor/Nafion calibration curve (inset to Fig.6.2.2) shows two linear regions.

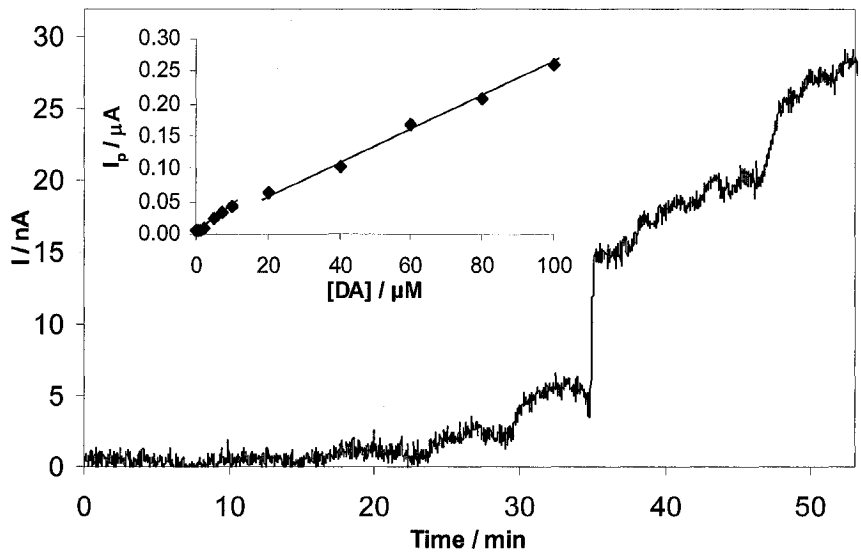


Figure 6.2.2 *Current-time response of a Pt-based sensor modified with Nafion for increasing DA concentrations (from 0 to 100 µM). E=-0.1V (vs. MSE,) Argon saturated 0.05 M phosphate buffer (pH 7.0). Inset: Calibration curve obtained from steady-state values of the graph on the main plot.*

This sensor also showed better sensitivity at lower DA concentrations, with a calibration curve slope of 4.0 mA M^{-1} in the lower concentration region, and 2.5 mA M^{-1} in the higher concentration region. Hence, the presence of Nafion on the Pt-sensor surface does not seem to influence the concentration-sensitivity ratio (ca. 2:1), but it does decrease the absolute sensitivity of the sensor by almost a factor of four. This is due to the blockage of the sensor's surface by a portion of the Nafion film that is directly attached to it, thus resulting in a decrease in the electrode area available for DA

oxidation. Nevertheless, a detection limit of the sensor is 200 nM, which makes it suitable for DA determination in urine DA samples, but is too high for the monitoring of DA changes in brain.

The next step was to verify whether the modified Pt-based sensor would repel AA. Figure 6.2.3 shows a Pt/Nafion-sensor CA curve recorded for increasing AA concentrations. Upon addition of AA, the current instantly decreases, due to the initial blockage of the Nafion layer by AA, which, in turn, blocks the access of water to the Pt surface and thus its oxidation ($Pt + H_2O \rightarrow PtO + 2H^+ + 2e^-$). However, due to the unfavorable electrostatic interactions between Nafion and AA, the membrane gets unblocked with time, and the Pt surface oxidation reaction (PtO formation) recovers, finally reaching a steady state. If the Nafion film were not selective to AA, with each increase in AA concentration in the bulk solution, the steady-state current would increase due to the AA oxidation. However, the result in Figure 6.2.3 demonstrates that the steady-state oxidation current remains the same in the whole AA concentration region investigated (5 to 100 μM), thus confirming that a surface Nafion film is efficient in preventing AA oxidation, i.e. interference with DA. Hence, the use of Nafion with the Pt-based sensor was useful in repelling AA. Additionally, it provided a greater selectivity to DA, however, at the expense of a decrease in the sensor's sensitivity [7,84].

Zhou *et al.* [34] have successfully electrocatalyzed the oxidation of DA by modifying a Pt microelectrode with nickel hexacyanoferrate. Moreover, they used Nafion to eliminate AA interference at a 50-fold concentration of DA. Another paper by Lupu *et al.* [36] reported the modification of Pt electrode with poly(3,4-ethylenedioxythiophene)/Prussian blue film. The resulting sensor showed increased oxidative DA currents that were linear in the DA concentration range from 0.1 mM to 2 mM, however, the sensor was not successful in eliminating AA oxidation and thus, its interference with DA. Hiratsuka *et al.* [86] generated a thin hexadiene plasma-polymerized film (PPF) on a Pt electrode that showed a DA sensitivity one and a half times larger than that of a bare Pt electrode, as well as a high selectivity between DA and AA compared with a bare Pt electrode (selectivity ratio of 12 compared to 4.6).

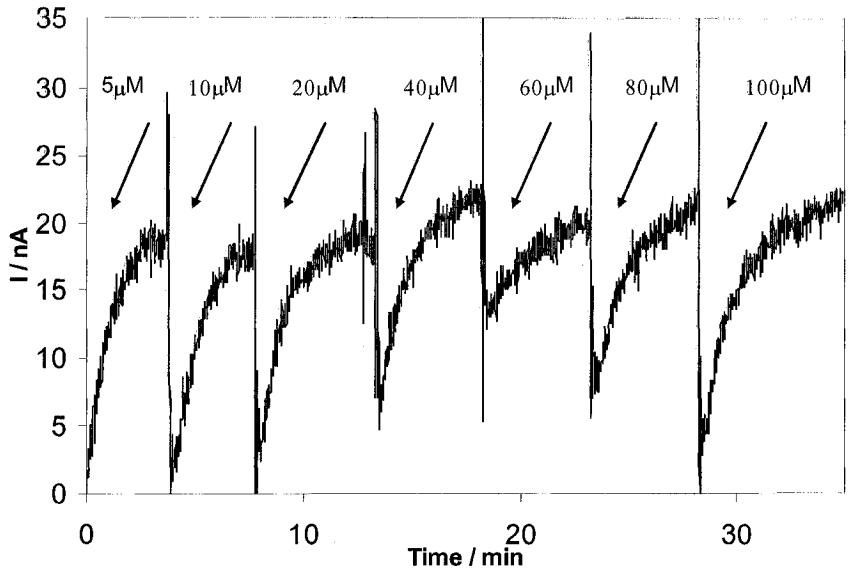


Figure 6.2.3 *Current-time response of a Pt-based sensor modified with Nafion for increasing AA concentrations. E=-0.4V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

6.2.2 BDD-based Sensor

BDD is a new material used in a wide range of applications. It is very interesting as an electrode material since it offers a wide inert potential window for redox reactions, extremely high corrosion resistance, chemical inertness, and very low capacitance and background current [44,46,47,69]. Hence, it could be a very interesting material in electroanalysis, i.e. for electrochemical sensors. A BDD electrode (diameter = 0.3 cm) was also used as a possible sensor surface for DA detection in this thesis. A similar approach used to characterize the Pt-sensor was adopted for the BDD-sensor. First, a bare BDD-sensor was used to detect DA, and then the surface was modified with a Nafion film to provide selectivity toward DA while repelling AA.

Figure 6.2.4 represents a CA response of the BDD-sensor with increasing DA concentrations in the bulk solution. A typical staircase current response can be observed. The background current and noise levels were low, as expected for a BDD electrode, but it took longer time to reach steady-state DA oxidation current values (ca. 60 seconds compared to 22 seconds for the Pt-based sensor). Since the configuration of the electrochemical cell and the experimental conditions were the same in both cases, the

slower response of the BDD sensor cannot thus be related to the influence of mass-transport, but to the difference in kinetics of the DA oxidation reaction on the two surfaces at the potentials applied.

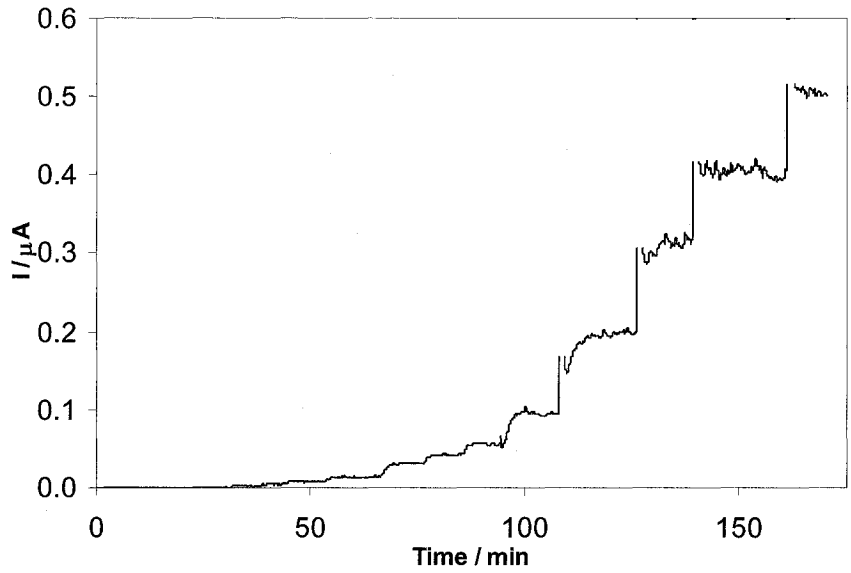


Figure 6.2.4 *Current-time response of a BDD-based sensor for increasing DA concentrations (from 0 to 100 μM). E=0 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

The response of a Nafion-modified BDD sensor (Fig.6.2.5) was also very similar to the unmodified sensor (Fig.6.2.4), but with higher DA oxidation currents that took longer time to reach steady-state. Figure 6.2.6 shows the calibration curves of both types of BDD-sensors. One can notice that high linearity of both calibration curves was obtained in the whole DA concentration range, from 0 to 100 μM. Moreover, when the BDD-sensor was covered with Nafion, a 27% increase in sensitivity to DA was obtained. This could be due to a local increase in DA concentration inside the membrane. However, a drawback was a decrease in the detection limit of the BDD/Nafion-sensor (200 nM), compared to the BDD-sensor (50 nM), because the noise level was higher at low DA concentrations. Thus, a higher sensitivity does not automatically infer a low detection limit.

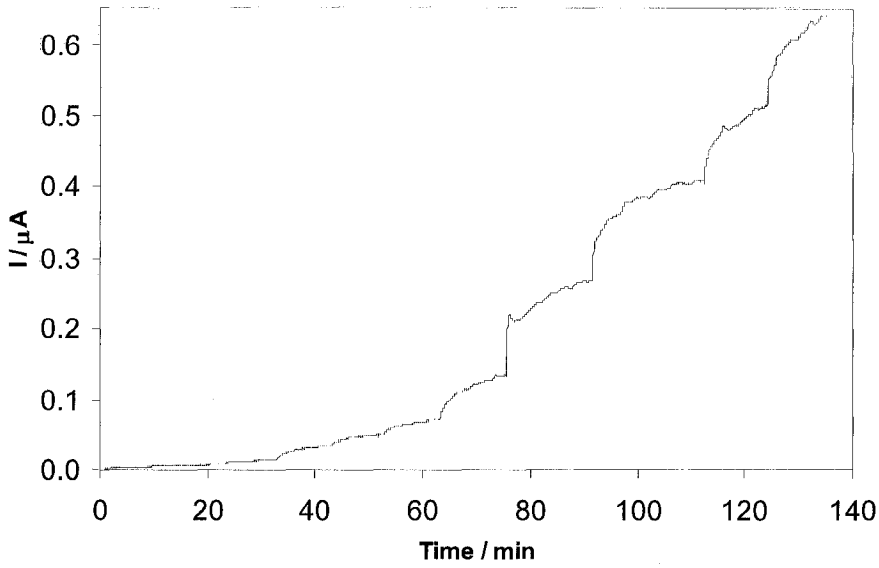


Figure 6.2.5 *Current-time response of a BDD-based sensor modified with Nafion for increasing DA concentrations (from 0 to 100 µM). E=+0.4V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

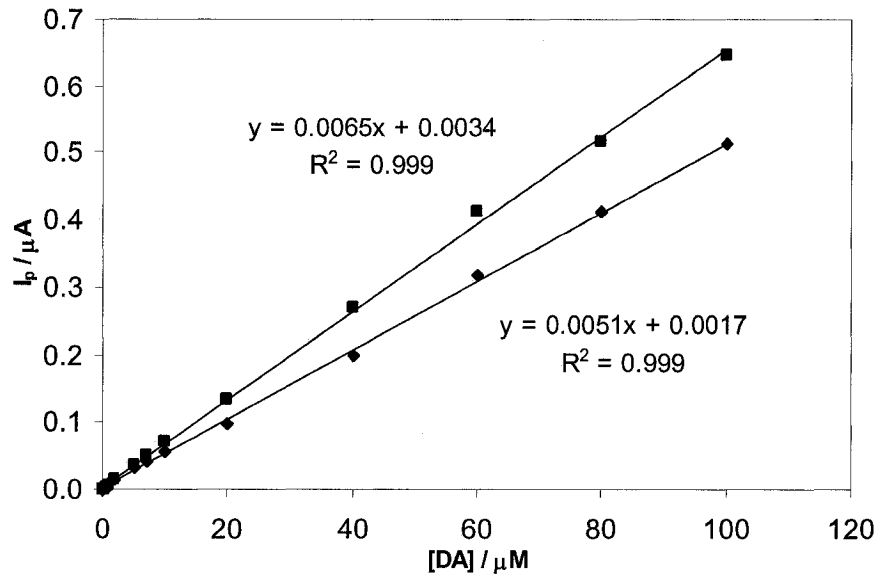


Figure 6.2.6 *Calibration curves of a (■) BDD/Nafion, and (◆) BDD sensor. Steady-state currents were obtained from CA curves in Figure 6.2.4 and 6.2.5.*

A comparison between the modified Pt and BDD-based sensors shows that the main difference lies in their sensitivities: BDD being 2.6 fold more sensitive to DA than

Pt. While the noise level was three times higher for the Pt-based sensor, both sensors had a detection limit of 200 nM.

The performance of a Nafion-coated BDD sensor was also tested in the presence of AA in the test solution. Figure 6.2.7 shows the CA curve of a BDD/Nafion-sensor with successive additions of AA (dashed arrows at shorter times) and then DA (solid arrows at longer times). It can be noticed that the background current and noise levels are very low compared to the Pt/Nafion-sensor (Fig.6.2.2). With an increasing concentration of AA in the bulk solution, the response current (signal) also increased in a stepwise manner. A similar behaviour is recorded with increasing concentrations of DA. However, it should be noted that the sensitivity of the sensor to DA is excellent, since the sensor was able to detect DA in the presence of a 1000-fold excess of AA. Additionally, the sensor had higher selectivity to DA than to AA. Indeed, for a concentration difference of 100 μM of AA and 60 μM of DA, the sensor was 100 times more selective to DA than to AA. Furthermore, in the presence of an excess AA concentration (83 fold), the current response to DA was still 8 times larger. Hence, the DA-to-AA response signal of the BDD/Nafion-sensor at the same DA and AA concentration is 664:1.

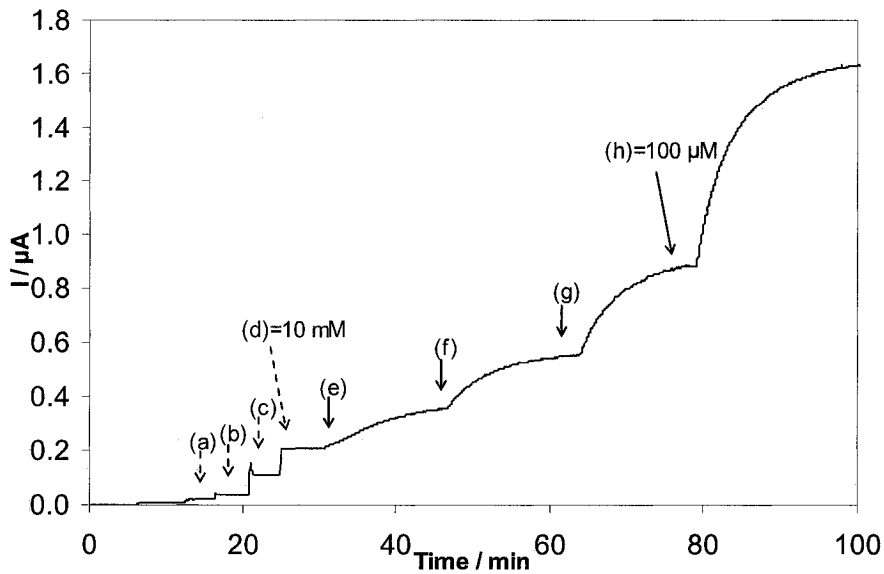


Figure 6.2.7 *Current-time response of the BDD-based sensor modified with Nafion for increasing AA concentrations (a) 0.5 mM, (b) 1 mM, (c) 5 mM, and (d) 10 mM, followed by increasing DA concentrations (e) 10 μM, (f) 20 μM, (g) 40 μM, and (h) 100 μM. E=+0.4 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

Published results on BDD have shown that detection limits of DA as low as 0.1 nM were attainable with an overoxidized polypyrrole modified BDD microfiber electrode [45]. The corresponding calibration curve was linear from 0.5 nM to 100 μM and the sensor successfully repelled AA and DOPAC. Other BDD sensors detected 50 nM [44] and 60 nM DA [46] while providing good selectivity to DA in the presence of AA in their range of physiological concentrations [46]. BDD sensors were anodically treated [44] and electrochemically modified with a cationic polymer film [46], respectively. In a paper by Sopchak *et al.* [47] the authors used a rotating disk electrode (RDE) of BDD in order to improve the analytical sensitivity by reducing the background current. Unfortunately, this BDD sensor was unsuccessful in resolving DA and AA peaks.

Other DA sensors include gold and carbon-based electrodes. In electrochemical applications, gold electrode surface is usually modified with self-assembled monolayers (SAMs) or with polymeric films. Many authors reported results on DA detection with gold electrodes modified with SAMs [38-40,87]; the resulting sensors showed catalytic activity toward DA and AA while separating their oxidation peaks, thus allowing their simultaneous determination, unlike the developed Pt sensor that prevented completely the oxidation of AA. Furthermore, DA detection limits varied from 0.13 μM [40] to 2.31 μM [39] which are not low enough to determine DA concentrations *in vivo*, and the oxidation currents were linear in the range from 2.5 μM to 500 μM [38] and 5 μM to 125 μM [87]. As for gold surfaces modified with polymeric films and composites [41-43], they were also efficient in either separating DA, AA and UA peaks [41] or repelling AA interference up to a concentration of 5 mM [43]. The lowest DA detection limit of 1 nM was obtained with a gold electrode modified with poly(aniline boronic acid)/carbon nanotube composite [42] with very high sensitivity, making it a potential device for the diagnosis of Parkinson's disease. Numerous papers reported chemically and/or physically modified carbon-based sensors to selectively detect low DA concentrations in the presence of AA and UA. Polymer films were commonly used with carbon-based electrodes to reject anions and prevent fouling of the electrode surface. An overoxidized polypyrrole(dodecyl sulphate) film-coated glassy carbon electrode successfully detected 40 nM of DA while repelling AA [51]. Another paper [23] reported a carbon paste

electrode modified with polypyrrole/ferrocyanide film that was able to detect DA and AA separately with a detection limit of 15.1 μM of DA. Different carbon electrodes were modified with a melanin-type polymer film for the quantification of DA. Depending on the nature of the carbon material used, the analytical performance of the sensor varied greatly. Glassy carbon electrode was the best in terms of selectivity (3% interference from DOPAC and 7% interference from AA), stability, and low detection limit of DA of 5 nM [52]. Glassy carbon electrode was also modified with TiO_2 nanoparticles covered with Nafion film [88]. The prepared electrode showed excellent electrocatalytic behavior for DA oxidation while preventing the AA interference. A detection limit of 9.5 nM was obtained with high sensitivity. Salimi *et al.* [54] have reported the modification of glassy carbon electrode with catechin hydrate as a natural antioxidant covered with Nafion. The resulting sensor was sensitive with a low detection limit of 11 nM and selective to DA in the presence of a 200-fold concentration excess of AA. A glassy carbon electrode was modified with a suspension of multi-wall carbon nanotubes and covered with Nafion, thus exhibiting excellent selectivity towards DA in the presence of 500-fold excess of AA and 150-fold excess of UA. A detection limit of 2.5 nM was achieved after 2min. of open-circuit potential accumulation [49].

6.2.3 Determination of DA using the *standard addition method*

The previous sections described the development of Pt- and BDD-based sensor for the analysis of DA employing a direct detection method based on a calibration curve. Another procedure for quantitative analysis of DA concentrations in unknown liquid samples, such as urine, is the *standard addition method.* In this method, the sample solution of unknown concentration is spiked several times with a standard DA solution in such a way so as to give equally spaced increasing concentrations of the DA to be determined. A plot of the current response versus final bulk solution concentration of DA is constructed. Then, the unknown concentration can be found from the intercept of the plot on the concentration axis (abscissa). The applicability of this method was tested in both a low (0 to 4 μM) and high (0 to 40 μM) DA concentration region using a Pt/Nafion-based sensor. Figure 6.2.8 represents the dependence of steady-state oxidation current values (signal) obtained from CA curves on the *known* DA concentration in the bulk

solution. The first signal point (zero concentration) corresponds to the *unknown* DA concentration (which was, in this case, 1 μM). From the intercept of the line with the concentration axis, a DA concentration of 1.2 μM was obtained. Hence, the determination error was 20% (or the recovery was 80%). For the higher DA concentration range (20 μM, figure not shown), the error was 15%. Since DA levels in urine are ca. 1-2 μM, these experiments demonstrate that the *standard addition method* can be successfully used to determine DA in unknown samples.

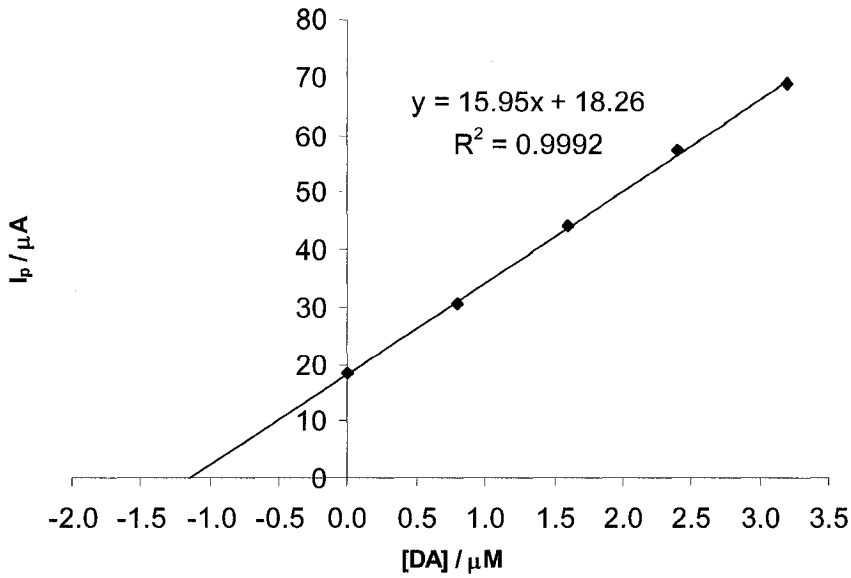


Figure 6.2.8 *Application of the standard addition method in the determination of unknown DA concentration using a Nafion-coated Pt-based sensor. Injected 'unknown' DA concentration: 1μM. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

The standard addition method was also used to determine DA employing high-performance liquid chromatography (HPLC) and fluorimetry techniques. A large number of publications have appeared on the determination of catecholamines in the brain and body fluids using HPLC [89]. For recovery determination of DA by HPLC, Hollenbach *et al.* [90] employed three different concentrations (0.4, 0.6, 1.2 pM per 50 pL injection volume). The recoveries for DA were slightly lower than other catecholamines in plasma 94.6 % and in urine 94.3%, respectively. There were no differences in plasma and urine with regard to recoveries. Fluorimetry was also an efficient method used to determine DA in urine samples [91]. A linear relationship was obtained between the relative

fluorescence intensity and concentration of dopamine in the range from 0.53–18.45 μM. The detection limit of this method was 0.43 μM and recovery was from 104.2 to 106.6%. The results obtained by this method agreed with those by HPLC.

6.3 Dopamine Biosensors

Electrochemical dopamine (DA) biosensors have attracted large attention in the past two decades due to the important role DA plays in neurotransmission. In addition, the analytical determination of DA in urine has been reported to provide a valuable diagnosis of cardiovascular and renal diseases in patients [90]. In order to provide adequate performance, DA biosensors must be able to distinguish DA among interfering species, such as ascorbic acid (AA) in the brain or AA and uric acid (UA) in urine so as to lead to a better understanding of biological functions. Thus, an ideal DA biosensor would combine selectivity, stability, and an appropriate temporal resolution within acceptable sensitivity and detection limits [1]. The following section discusses results on the development of DA biosensors. Platinum (Pt) and carbon paste electrode (CPE) were the physical transducers used to build the DA biosensors. First, the Pt-based biosensor was developed and optimized for DA detection and AA repulsion by investigating various parameters, including polymerization time, Nafion coating and enzyme loading. Then the optimized Pt biosensor was tested for short-term stability. By implementing the previously determined optimized conditions, a CPE-based biosensor was built and compared to the optimized Pt biosensor.

6.3.1 Development and Optimization of the Pt-based Biosensor

A two-dimensional (2D) platinum electrode (diameter = 0.3 cm) was first investigated as a biosensor transducer for the detection of DA. The working principle of a Pt-based biosensor is illustrated in Figure 6.3.1. DA is first oxidized to dopaminoquinone (DOQ) by the active copper site (-Cu-O-O-Cu-) of the enzyme polyphenol oxidase (PPO). Then, ferrocenium, which is the oxidized state of the electron mediator ferrocene (Fc), converts the reduced enzyme (-Cu-O-Cu-) back to its active oxidized state. Finally, the reduced Fc shuttles the electron to the Pt electrode and gets oxidized to ferrocenium,

thus generating a current signal proportional to the bulk DA concentration. The Pt-based biosensor therefore combines the enzyme's specificity for recognizing DA with the transduction of the biocatalytic reaction rate into a current signal through the electron mediator Fc.

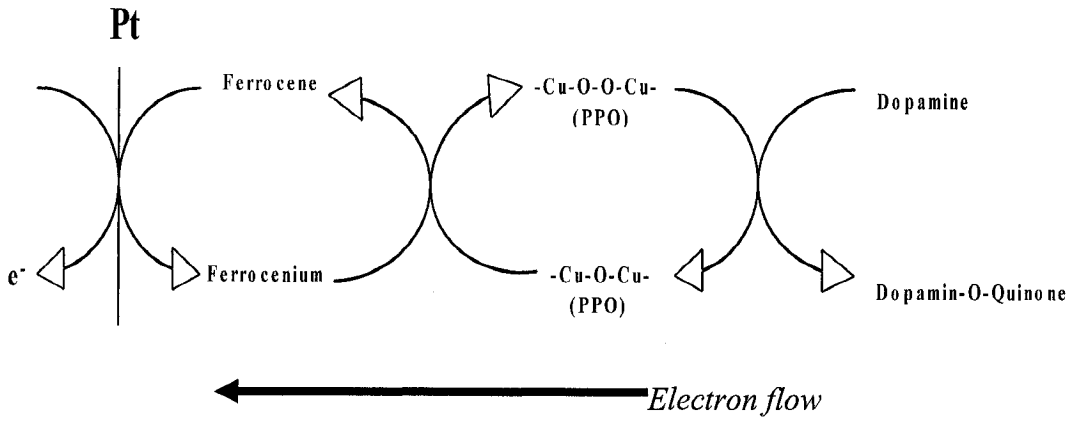


Figure 6.3.1 *The principle of work of a DA electrochemical biosensor: -Cu-O-O-Cu-= oxidized copper site, -Cu-O-Cu-= reduced copper site.*

The reaction mechanism corresponding to Figure 6.3.1 is the following:

$$DA + PPO_{ox} \rightarrow DOQ + PPO_{red} \quad (9)$$

$$PPO_{red} + Fc_{ox} \rightarrow PPO_{ox} + Fc_{red} \quad (10)$$

$$Fc_{red} \rightarrow Fc_{ox} + 2e^{-} \text{ (measured at the electrode)} \quad (11)$$

Where (ox) and (red) correspond to the oxidized and reduced states of PPO and Fc.

The biological recognition element, in this case PPO, typically limits the biosensor's lifetime, stability and calibration requirements because of its activity loss [7]. In order to extend the enzyme activity, to ensure its high local concentration, and to decrease the reaction diffusion path, the enzyme should be immobilized on the transducer surface. Since the mediator serves as an electron shuttle, it should also be immobilized on the surface, together with the enzyme. Various immobilization methods have been investigated and were listed in Section 3.3. In this work, in order to immobilize PPO and entrap Fc onto the Pt surface, a polymer film, polypyrrole (PPY), was electrochemically formed on the transducer (Pt) surface by controlled potential electrolysis of an aqueous solution containing the monomer pyrrole, the enzyme PPO and the electron mediator Fc.

Therefore, PPO and Fc species present in the polymerization solution were incorporated in the growing polymer film. One of the main advantages of this electrochemical polymerization technique is that films can be easily prepared in a rapid one-step procedure.

6.3.1.1 Polymerization time

To optimize the biosensor, the first parameter investigated was the polymerization time, which directly determines the amount of the enzyme and mediator entrapped in the polypyrrole film. The resulting biosensor architecture is Pt/(PPY+Fc+PPO). Polymerization times of 20 and 40 minutes were applied. The current-time (chronoamperometric) response of the biosensor constructed by applying a 40-minute polymerization time is shown in Figure 6.3.2.

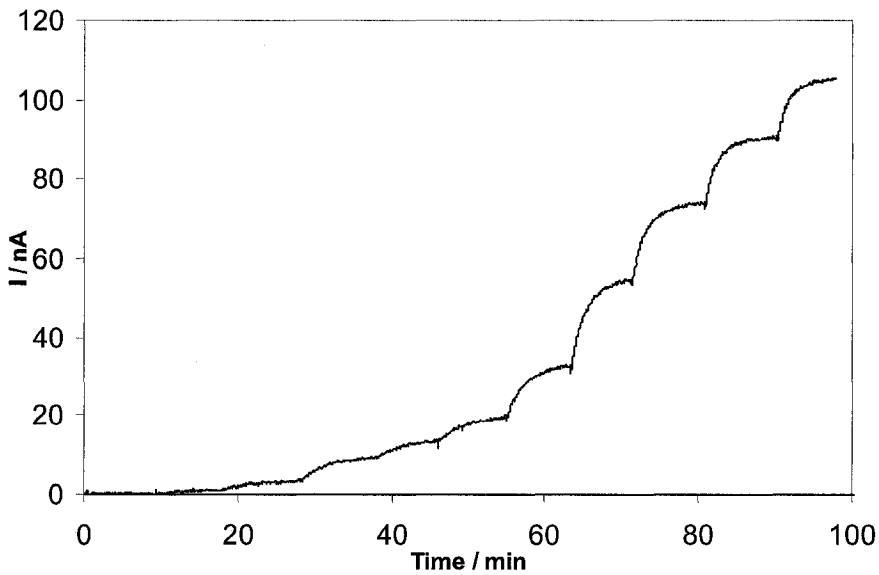


Figure 6.3.2 *Current-time response of a DA Pt/(PPY+Fc+PPO) biosensor constructed by applying a 40-minute polymerization time. Each current step represents a response of the biosensor after addition of an aliquot of DA. E=-0.1V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

This figure shows a staircase response very similar to the one obtained with the Pt sensor described in Section 6.2.1. Upon addition of DA to the electrolyte, an instant increase in current is recorded, corresponding to the mediated oxidation of DA to DOQ

by the active copper PPO site (Fig.6.3.1 and Equations (9-11)). The steady-state DA oxidation current is reached approximately 5 minutes after each DA addition, compared to ca. 22 seconds with the Pt sensor (Fig.6.2.1). This difference is due to an increased mass-transport resistance through the polypyrrole layer deposited on the Pt surface. Nevertheless, from the steady-state DA oxidation current values, which are proportional to the bulk DA concentrations, calibration curves of the Pt/(PPY+Fc+PPO) biosensors at 20 and 40 minutes polymerization times were constructed and shown in Figure 6.3.3.

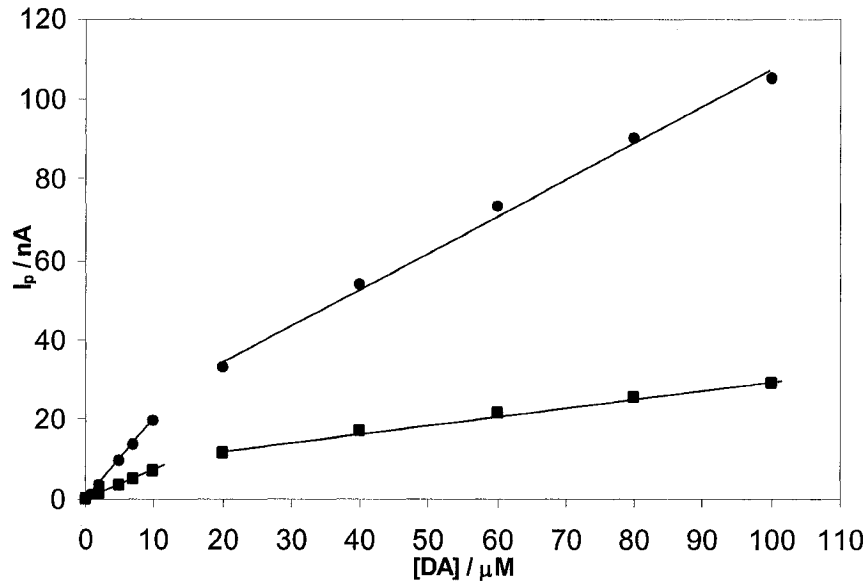


Figure 6.3.3 *Calibration curves of Pt/(PPY+Fc+PPO) biosensors constructed by applying a polymerization time of (■) 20 and (●) 40 minutes. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

The steady-state oxidation currents were normalized with respect to the background current before being plotted against the corresponding bulk DA concentrations. It can be noticed that both curves have two linear regions, the low region from 0 to 10 μM, and the high region from 20 to 100 μM of DA. When polypyrrole polymerization was carried out for 40 minutes, the biosensor current response (signal) increased in the whole DA concentration region. A longer polymerization time allowed for more enzyme and mediator to be entrapped within the polymer layer being formed, thereby increasing the number of active sites available for the DA oxidation and electron shuttling. As a result, the biosensor's sensitivity towards DA detection, which is

determined from the slope of the calibration curve, increased. At low (Fig.6.3.4) and high (Fig.6.3.3) DA concentration ranges, the corresponding slopes showed that doubling the polymerization time increases the biosensor's sensitivity by a factor of three (1.92 mA M^{-1} vs. 0.68 mA M^{-1}) and a factor of four (0.9 mAM^{-1} vs. 0.22 mAM^{-1}) respectively. Additionally, a 40-minute polymerisation time yielded a biosensor with 1 μM detection limit making it suitable for DA determination in urine samples, as opposed to the 2μM detection limit resulting from a 20-minute polymerization time.

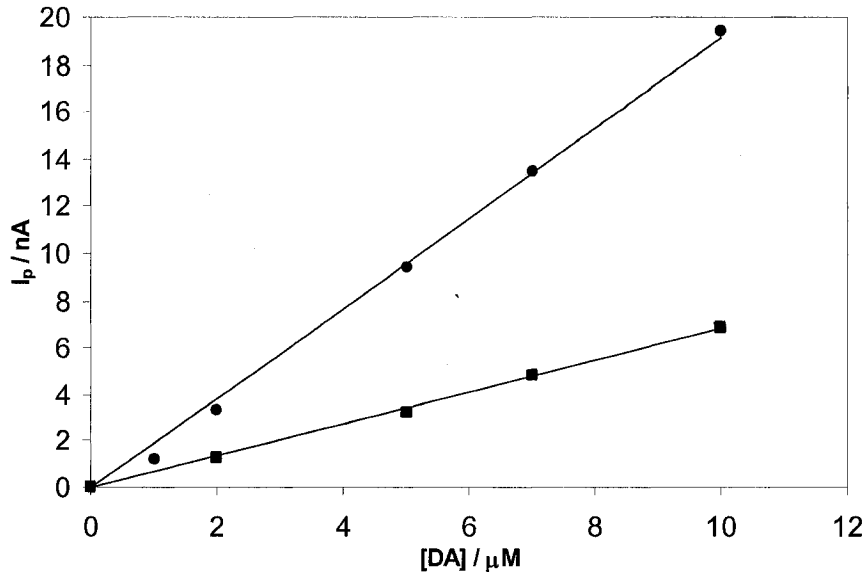


Figure 6.3.4 *Calibration curves of Pt/(PPY+Fc+PPO) biosensors with a polymerization time of (■) 20 and (●) 40 minutes, for the low DA concentration range, 0 to 10 μM. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

Cosnier [24] also constructed a DA biosensor using a glassy carbon transducer surface (diameter = 0.5 cm) and by polymerizing pyrrole for 20 minutes and immobilizing PPO without the use of a mediator. Oxygen dissolved in the electrolyte served as an electron mediator. The reported sensitivity and detection limit were 11 mA M^{-1} and 50 nM respectively for a surface area of 0.196 cm^2 and an enzyme loading of 38,700 U mL^{-1}. If the sensitivity value obtained by Cosnier were normalized with respect to the electrode surface area and enzyme loading, it would fall to 0.135 mA M^{-1} and would be five times lower than ours. On the other hand, Deshpande *et al.* [30]

incorporated whole PPO-containing cells within a polypyrrole film for a 2-hour polymerization time. The sensitivity of the resulting biosensor was 1.5 $mA\,M^{-1}$ for DA levels below 0.7 mM, and the detection limit was 0.4 mM, both values being worse than the sensitivity of 1.92 $mA\,M^{-1}$ and detection limit of 1 μM obtained in our case for a 40-minute polymerization time. Further optimization of our biosensor resulted in a significant improvement of the detection limit, giving a value (10 nM) significantly better than in the report of Cosnier (50 nM) [24]. This will be discussed in detail later in the thesis.

Our experiments showed that the longer polymerization time (40 minutes) results in a better sensitivity over the whole DA concentration range, and also a lower detection limit. Hence, this time was used in subsequent experiments for the constriction of Pt-based biosensors. The notation used is Pt/(PPY_40+Fc+PPO).

6.3.1.2 Nafion-modified biosensor

The second optimizing parameter investigated was the addition of a thin Nafion film on top of the Pt/(PPY_40+Fc+PPO) biosensor and the study of its effect on the biosensor sensitivity, detection limit and later on its selectivity to DA (*i.e.* resistivity to ascorbic acid).

Figure 6.3.5 shows the calibration curves of the Nafion-free biosensor, Pt/(PPY_40+Fc+PPO), and the Nafion-modified Pt/(PPY_40+Fc+PPO+Nafion) biosensor. One can notice that the presence of a Nafion film on top of the biosensor's surface produces a large difference in the response in the whole DA concentration range. This is opposite to the result obtained on the Pt sensor (Fig.6.2.2) where the presence of a Nafion film decreased the sensitivity of the sensor. The precise reason for such behavior is not currently known, but could be partially related to the synergetic action of the two polymers, Nafion and PPY, involving an interplay of electrostatic and amphiphilic forces. Also, when Nafion is deposited directly on top of the Pt sensor surface (Fig.6.2.2), it partially blocks the area of the sensor (Pt/Nafion contact points), thus reducing the useful Pt area available for the DA detection, lowering the sensor's sensitivity. On the other hand, when Nafion is deposited on top of the PPY layer (Fig.6.3.5), it does not block the

electrode surface area at all, and only its positive coulombic effect is thus seen, resulting in an increase in detection sensitivity. Figure 6.3.5 demonstrates that the Nafion-based biosensor offers a three fold increase in DA sensitivity (2.44 $mA\,M^{-1}$ vs. 0.73 mAM^{-1}) and a five-time lower detection limit (100 nM vs. 500 nM). To the best of the author's knowledge, no DA biosensors coated with Nafion have been reported in literature. Nafion has been used only in DA sensors applications.

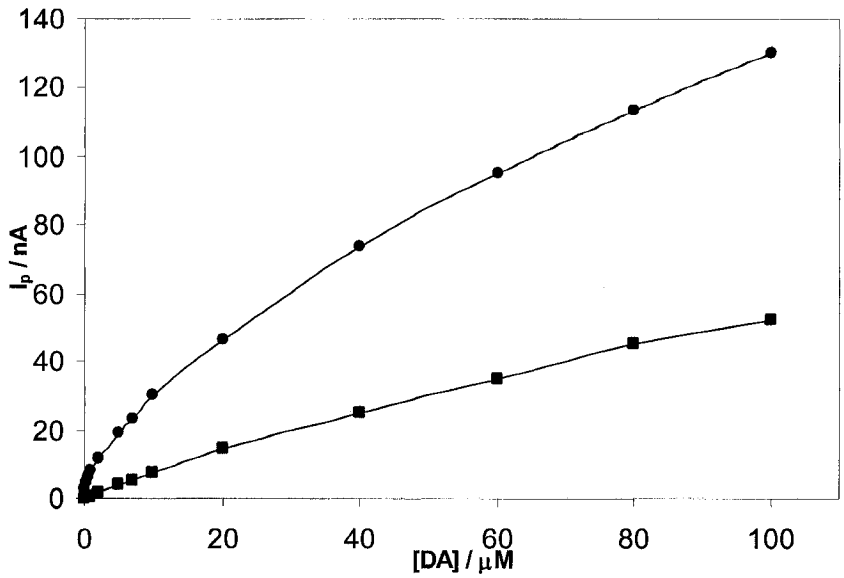


Figure 6.3.5 *Calibration curves of a (■) Pt/(PPY_40+Fc+PPO), and (●) Pt/(PPY_40+Fc+PPO+Nafion) biosensor. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

6.3.1.3 Enzyme loading

In order to further increase the sensitivity of the Pt/(PPY_40+Fc+PPO+Nafion) biosensor over a wide DA concentration range and decrease its detection limit, the effect of enzyme loading in the polypyrrole film was investigated.

The difference between these experiments and those discussed in Section 6.3.1.2 is that now the PPY film thickness, mediator loading and Nafion coating were kept the same, while only the PPO loading was changed. The initial concentration of PPO in the modification solution was ~1,200 $U\,mL^{-1}$, while the concentration of Fc was kept at 10 mM. The notation for this sensor is Pt/(PPY_40+Fc+PPO_1X+Nafion). A double amount of PPO in the modification solution is marked as "2X", and so on. The actual loading of

PPO in the polymer layer was not determined, and thus, the values discussed are referred to the amount of enzyme dissolved in the modification solution. However, we assume that the amount of PPO entrapped in the PPY layer was proportional to the amount of PPO in the modification solution. The response of the 1X Pt/(PPY_40+Fc+PPO+Nafion) biosensor is shown in Figure 6.3.6, where increased aliquots of DA added to the electrolyte generated oxidation current responses that reached steady-state within ca. 8 minutes at low DA concentrations (0 to 10 μM) and ca. 5 minutes at high DA concentrations (20 to 100 μM). The higher time needed to reach steady-state at low DA concentrations is due to a lower flux at lower DA concentrations. The 1X biosensor gave a DA detection limit of 100 nM. Figure 6.3.7 shows the calibration curves for different PPO loadings.

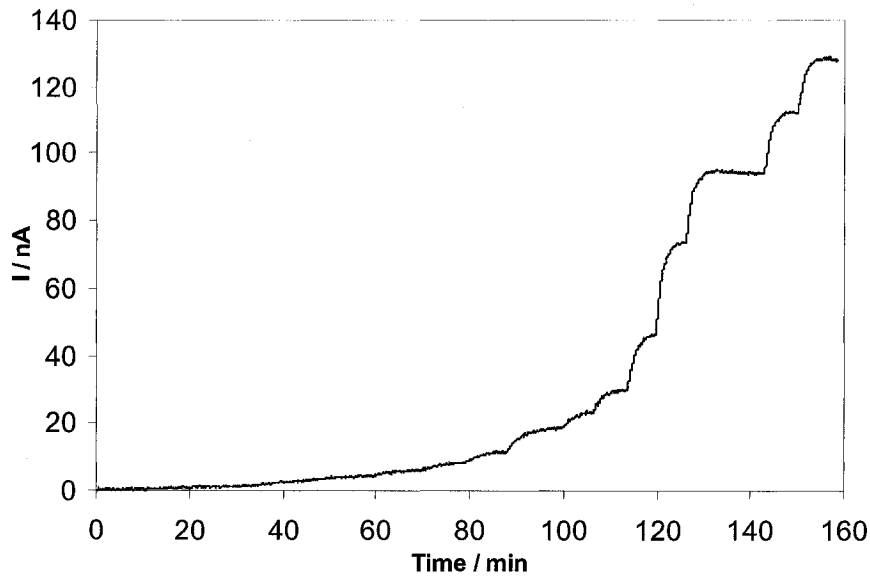


Figure 6.3.6 *Current-time response of a Pt/(PPY_40+Fc+PPO_1X+Nafion) biosensor. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

Next, when the enzyme loading was doubled to 2,400 $U mL^{-1}$, Pt/(PPY_40+Fc+PPO_2X+Nafion), (Fig.6.3.7, circles) the response of the biosensor improved significantly, giving a higher sensitivity over the whole DA concentration range. This is particularly apparent in the 0.1 to 10 μM concentration range, where the current response was twice that of the 1X biosensor (triangles). Indeed, increased PPO

concentrations in the polymerization solution presumes a proportional increase within the polymer film, hence a higher concentration of active copper sites are available for the oxidation of DA. In addition, the high sensitivity of 20 mA M^{-1} (over 0 to 1 μM DA) and low DA detection limit of 20 nM indicates that sufficient Fc molecules were entrapped within the polypyrrole film to transport the electrons to the working electrode, thus generating the large current signal reaching steady-state in ca. 3 minutes.

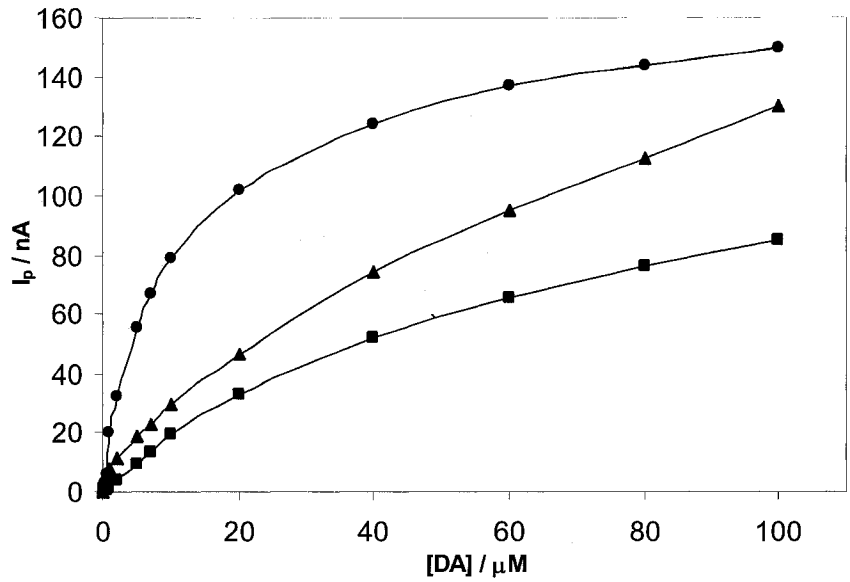


Figure 6.3.7 *Calibration curves of a Pt/(PPY_40+Fc+PPO+Nafion) biosensors for enzyme loadings of (▲) 1X PPO, (●) 2X PPO and (■) 4X PPO. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

To verify whether even a more sensitive biosensor could be obtained, the enzyme loading was further increased to 4,800 UmL^{-1} (4X) in order to yield a 4:1 PPO to Fc ratio in the polymerization solution (Fig.6.3.7, squares). However, the graph shows that the steady-state current response to increased DA concentrations was poorer than in the cases of 1X and 2X biosensors with lower PPO loadings. One of the possible explanations could be that the higher PPO to Fc ratio in the solution favored the entrapment of PPO in the polypyrrole film at the expense of Fc. As a result, even if a high number of active enzyme centers were available in the PPY layer, there was not enough of Fc entrapped in the polymer layer to shuttle the electrons to the electrode surface (Fig.6.3.1). Therefore, lower current responses were observed and a detection limit of 500 nM was obtained. It

was thus concluded that the optimum PPO to Fc concentration ratio was 2:1, since it resulted in a low DA detection limit of 20 nM and significantly better sensitivity in the whole DA concentration range.

Various authors studied the effect of enzyme loading on the biosensor performance. Vedrine *et al.* [28] reported a gradual increase in the biosensor response as a PPO loading in a surface polythiophene film was increased. The biosensor, a glassy carbon electrode with a disk-shaped active surface of 0.07 cm^2, showed a sensitivity of 27 $mA\,M^{-1}$ (normalized with respect to PPO concentration) to DA concentrations from 0 to 200 μM, but a poorer detection limit (100 nM) than ours. However, with a further increase in the PPO loading in the polymer layer, a gradual decrease in the biosensor response was observed, quite similar to our observations (Fig.6.3.7). On the other hand, Petit *et al.* [14] found that the carbon paste biosensor response increased non-linearly by raising the enzyme loading in the paste and opted for an intermediate value of 5% (w/w) PPO because it gave a better background current stability than an 8% (w/w) loading. Similarly, Rubianes *et al.* [31] varied the enzyme content in the iridium-modified carbon paste biosensor from 5 to 20% (w/w) and noticed a linear increase in sensitivities for DA. However, a drawback was an increase in noise due to an increase in the resistance of the bioelectrode. Miscoria *et al.* [32] reported an increase in sensitivity of a gold-nanoparticles-modified carbon paste DA biosensor with an increase PPO loading from 0.5 to 2.0% (w/w). Cosnier *et al.* [92] also observed that a glassy carbon-based DA biosensor exhibited a sensitivity which increased almost linearly with the enzyme loading, and concluded that the enzyme amount in the polymer layer and its turnover capacity were controlling the electrode kinetics rather than internal diffusion limitations.

When testing for DA concentrations in unknown samples such as urine, it is important for the biosensor to selectively detect DA and minimize the interference of AA and UA since these analytes are present in much higher concentrations than DA and they also get oxidized close to DA oxidation potentials. In this work, the Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor was tested for its ability to repel AA. Figure 6.3.8 represents the response of the optimized Pt biosensor to increasing DA concentrations (*a*) to (*e*), followed by increasing AA concentrations (*f*) to (*j*).

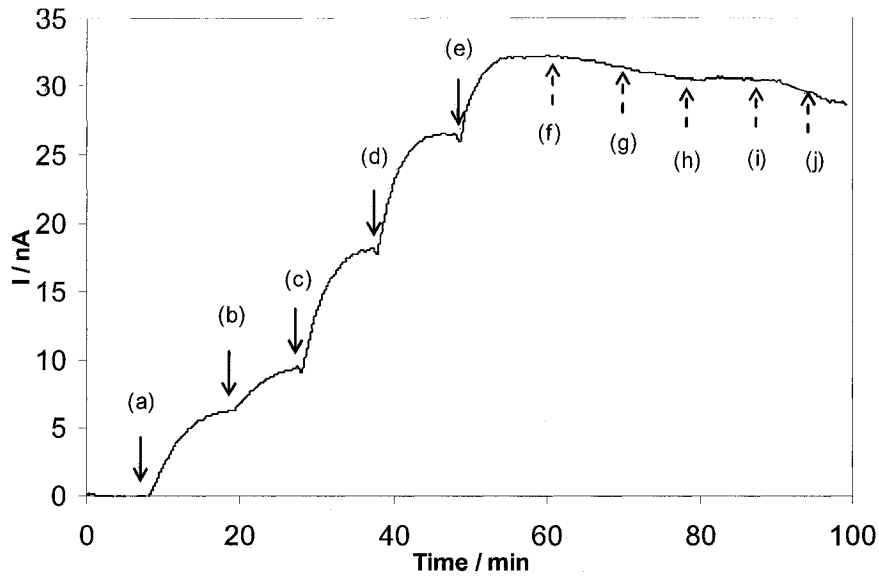


Figure 6.3.8 *Current-time response of the Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor after addition of increasing DA concentrations: (a) 15 μM, (b) 20 μM, (c) 40 μM, (d) 80 μM and (e) 100 μM, followed by the addition of increasing AA concentrations: (f) 10 μM, (g) 20 μM, (h) 40 μM. (i) 100 μM and (j) 1000 μM. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

It can be noticed that as the DA concentration in the solution increases from 15 to 100 μM, a typical staircase increase in the current corresponding to the oxidation of DA is observed. However, as AA was added gradually up to a concentration of 1000 μM, no AA oxidation current was detected. Even when the AA concentration was 46 fold higher than the DA concentration, the sensor did not show any response to AA. If the Nafion film were not repelling AA, with each addition of AA aliquot to the bulk solution, an increase in steady-state current due to the AA oxidation would be recorded. Thus, a Nafion film biosensor surface was proved selective to DA detection in the presence of excess of AA in the bulk solution.

To the author's knowledge, two papers on the effectiveness of DA biosensors to repel AA have been reported. Miscoria *et al.* [32] used carbon paste electrode modified with PPO and gold nanoparticles to construct an AA tolerant DA biosensor. They observed that the addition of AA to the electrolyte already containing DA, in a concentration up to 180 times that of DA, shows no interference, therefore concluding that the incorporation of PPO into the composite containing carbon paste and gold nanoparticles forms a highly sensitive and selective DA biosensor. Tu *et al.* [16,29] were

successful in building a DA biosensor that minimized the AA interference when its concentration was less than 500 times that of DA. Furthermore, when the AA-to-DA ratio was 2500, the AA current was only twice that of DA, thus confirming the selectivity of the biosensor to DA. The minimization of the AA interference was based on modifying the biosensor with Fc and PPO mixed in a silk fibroin solution.

The results in Figure 3.6.7 lead to the conclusion that the Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor was successfully optimized to be selective in detecting DA with good sensitivity and low detection limits. The best response obtained from the optimized Pt biosensor using CA as a detection technique is shown in Figure 6.3.9 and 6.3.10, and had a detection limit of 20 nM at a signal to noise ratio of 2.

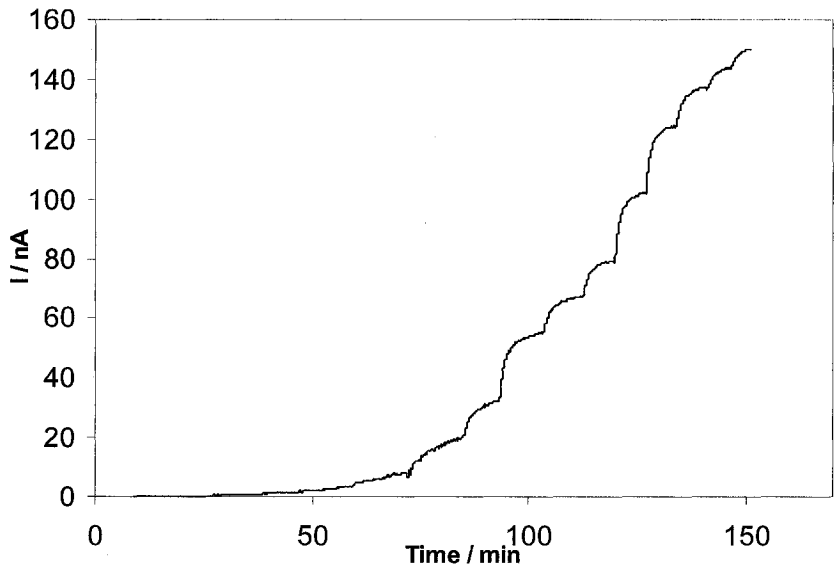


Figure 6.3.9 *The current-time response of the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor to increased additions of DA concentrations. E=-0.1V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

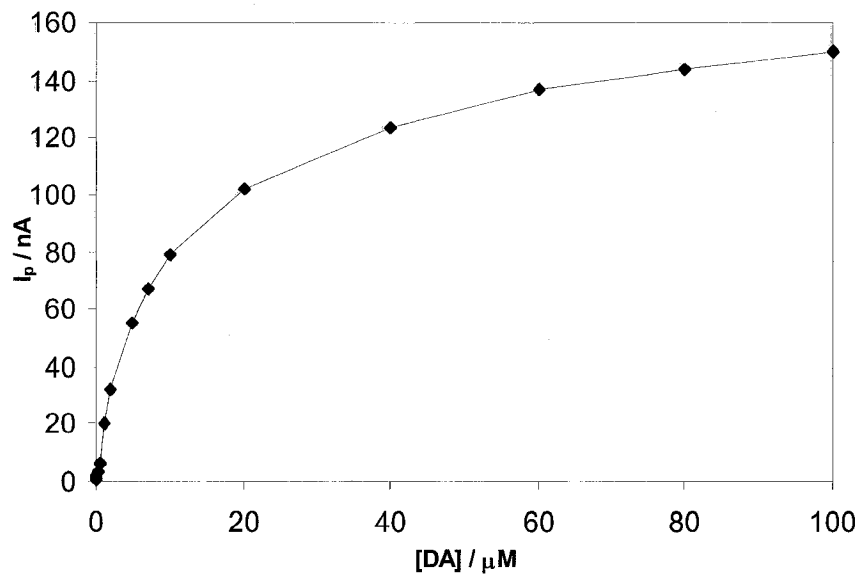


Figure 6.3.10 *The calibration curve of the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor obtained from the data in Figure 6.3.9.*

The previously reported results were obtained using chronoamperometry as a DA detection technique. Although this is a simple and most commonly used technique in biosensors development, it does not always offer a satisfactory performance. On the other hand, electrochemical impedance spectroscopy (EIS) has been shown to yield a much higher sensitivity to faradaic processes. Therefore, EIS was used in order to investigate the response of the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor, with the aim to decrease the detection limit of the biosensor.

Figure 6.3.11 shows an EIS response of the biosensor to increasing DA concentrations. The data is presented in a form of a Nyquist plot (imaginary impedance part versus real impedance part). It is quite obvious that even the addition of DA to give a 10 nM concentration in the bulk solution results in a rather large change in the EIS response. However, in order to quantify the response, it is necessary to model the spectra using an electrical equivalent circuit (EEC). The presentation of the EIS data in the form of a Nyquist plot reveals the presence of two semicircles. This, in turns, indicates that the response of the sensor should be described by a two-time-constant EEC, which is shown as an inset to Figure 6.3.11. The parameters of the EEC have the following meaning: R_{el} is the resistance of the electrolyte between the reference electrode and the biosensor's

surface (Ω), R_1 is the charge transfer resistance, *i.e.* the resistance related to the kinetics of the electron-transfer reaction, CPE_1 is the constant phase element ($\Omega^{-1}s^n$) representing the capacitance of the electrochemical double layer, R_2 is the resistance of the PPY/Nafion layer to mass-transport, and CPE_2 is the corresponding Warburg mass-transport impedance. Hence, the kinetic response of the biosensor can be quantified by the sum of R_1 and R_2 (total resistance), which is inversely proportional to a steady-state current response in chronoamperometry through Ohm's law. Further, R_1 and R_2 can be, in turn, visualized as diameters of the two semicircles in Figure 6.3.11. Hence, as DA was added to the solution, the diameter of both semicircles decreased, indicating a drop in the total resistance, *i.e.* an increase in DA oxidation current.

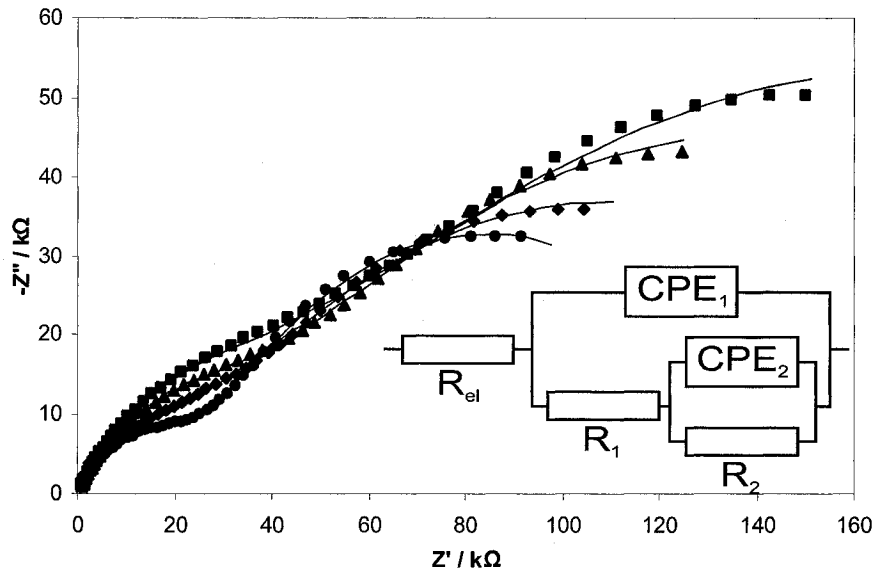


Figure 6.3.11 *Nyquist plot of the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor in (■) background electrolyte, and with the addition of (▲) 10 nM DA, (◆) 60 nM DA and (●) 100 μM DA. Symbols are experimental data, and lines represent modeled data obtained using the EEC model. Inset: Electrical equivalent circuit (EEC) used to model the impedance response of the biosensor. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

With a further increase in DA concentration, more ions and electrons are transferred through the electrolyte and within the Nafion and polypyrrole layers, hence a larger oxidation current results and therefore, the total charge transfer resistance (R_1+R_2) decreases.

Figure 6.3.12 represents the dependence of the inverse of total resistance on the DA concentration in the bulk solution, obtained by modeling the EIS spectra. With an increase in DA concentration, the signal rapidly increases. When 10 nM of DA was added in the electrolyte, the EIS recorded a 22% drop in the total resistance. This demonstrates a high sensitivity of the EIS method in determining DA using the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor. The inset to Figure 6.3.12 shows a semi-log EIS calibration plot of the biosensor. Two linear regions could be seen, with a lowest attainable DA detection limit of 10 nM. Although lower DA concentrations were not investigated, based on the 22% response change at 10 nM, the author's assumption is that even lower detection limit could be obtained by using the EIS and optimized biosensor.

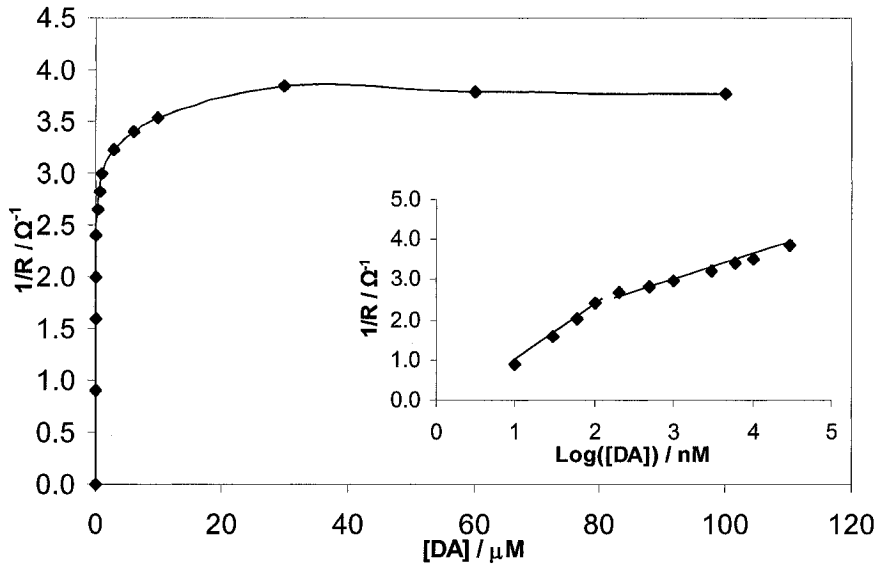


Figure 6.3.12 *The calibration curve of the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor obtained from the data in Figure 6.3.11. Inset: linearized semi-log calibration curve for the low DA concentration range.*

To the best of the author's knowledge, EIS has never been used before as a DA detection technique. In our case EIS was used to confirm that the optimized Pt biosensor was effectively detecting low DA concentrations. However, although the technique is significantly more sensitive than chronoamperometry, it is not suitable for commercial applications due to a rather long measurement time and a need to model the experimental data.

6.3.1.4 DA biosensor stability

One of the important requirements for application of biosensors is its stability. The stability ensures a repetitive usage of a biosensor over a relatively long period of time with no or little loss in the response signal resulting from the detection of the analyte of interest. Operational stability may vary considerably depending on the response rate-limiting factor, on the biosensor geometry, method of preparation, as well as on operational conditions: analyte concentration, pH, temperature and buffer composition [84]. For commercial applications, operational (use) and storage (shelf or long-term) stability of biosensors is crucial and should be determined (known).

The operational stability of the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor was tested over a period of 8 days at room temperature and at pH 7.0. The biosensor was stored in PBS 4°C when not in use [17,28,31,93]. Figure 6.2.13 represents the steady-state current responses of the optimized biosensor in a wide DA concentration range on days 1, 3, 4 and 8. The figure demonstrates that there is a significant decrease in the sensitivity of the sensor from day 1 to day 3. A reason for the observed sensitivity decrease could be the loss of the enzyme's activity, and also the leakage of both the enzyme and mediator into the storage solution. Kranz *et al.* [94] reported that enzyme inactivation and not electrode fouling was responsible of the poor long term stability of DA biosensors. In literature, most of operational stability analyses were monitored by measuring the biosensor response to one DA concentration everyday. Vedrine *et al.* [28] developed a DA biosensor that retained about 30% of its initial activity after 12 days. A biosensor developed by Tu *et al.* [16,29] maintained the same response value for 15 days when used every day for several measurements. Bezerra *et al.* [13] studied the operational stability of the modified carbon paste electrode over a period of 60 days. It was possible to carry out 500 determinations with a loss of 30% in the initial enzyme activity. Petit *et al.* [14] developed a biosensor that maintained 100% and 90% of its initial flow injection analysis response after 12 hours and 24 hours of continuous use, respectively.

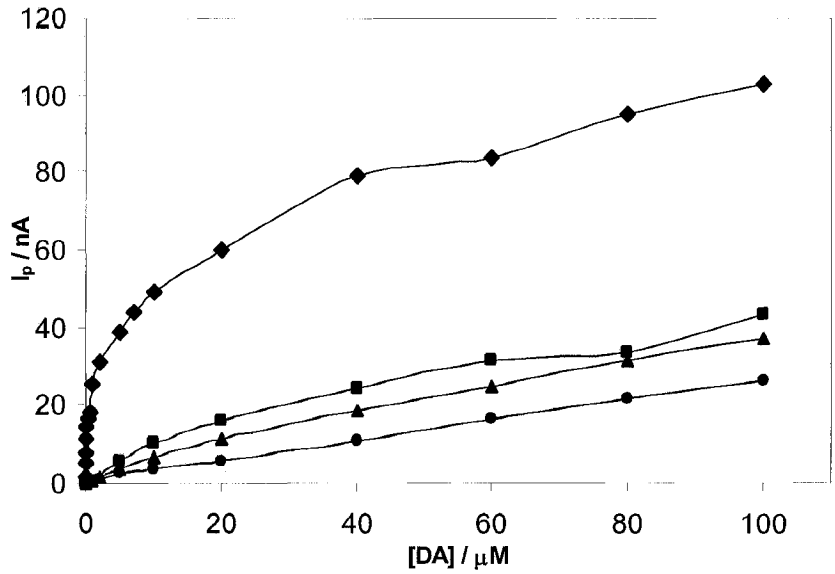


Figure 6.3.13 *Stability of the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor over a period of 8 days. Calibration curves for (◆) day 1, (■) day 3, (▲) day 4 and (●) day 8. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

Although the operational stability of the optimized biosensor is low, its reproducibility is quite satisfactory. An error in determining an unknown DA concentration in the mid (10 μM) and high (40 μM) concentration range was rather low, 8 %.

6.3.2 Carbon paste-based Biosensor

As it was shown in the previous section, the main disadvantage of the developed biosensor is its short-term stability, thus making it quite irreproducible when used for a prolonged time. On the other hand, carbon paste electrodes (CPE) offer an attractive solution due to a possibility of easy and multiple renewals of its surface. Hence, a CPE DA biosensor was constructed in order to compare its efficiency to the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor.

Carbon Paste Electrodes (CPE) have the advantages of being easy to produce by mixing specific amounts of PPO and Fc with graphite and paraffin oil, to form a homogeneous compact paste (Section 5.3.2). When not in use, the CPE biosensor was dry-stored in a freezer at -20°C, so that the enzyme would retain most of its activity for a

long period. Figure 6.3.14 shows the calibration curve obtained using the CPE DA biosensor, together with a curve obtained using the optimized Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensor. Note that the figure has two y-axes, the left axis is in μA for the CPE biosensor response, and the right axis is in nA for the Pt biosensor response. It can be noticed that the CPE biosensor gave a much higher current response (sensitivity), but at the expense of a high non-linearity. In addition, the CPE biosensor showed a ten time poorer detection limit (200 nM). Nevertheless, the aim of this part of the work was not to develop and optimize the CPE DA biosensor, but rather to compare a complex Pt-based biosensor to a simple, renewable and stable CPE biosensor.

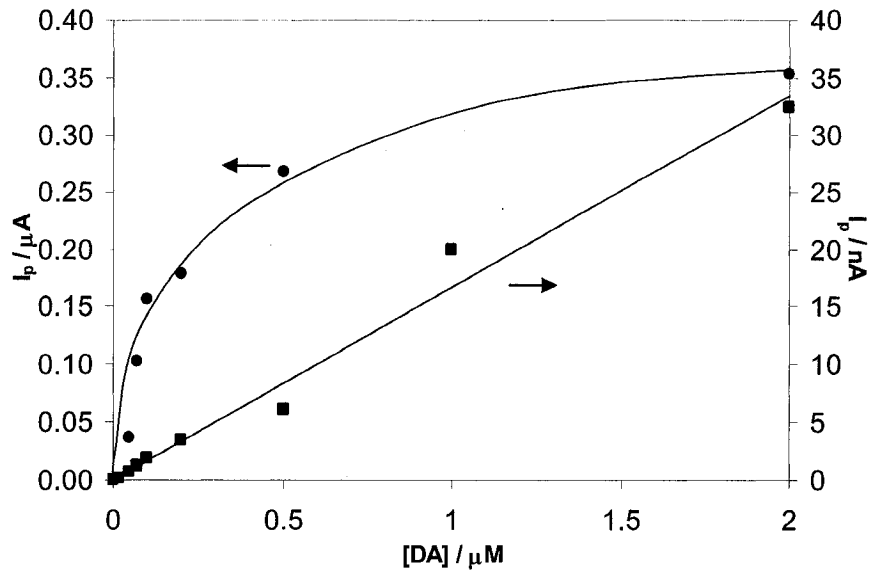


Figure 6.3.14 *Calibration curves of a (●) CPE and (■) Pt/(PPY_40+Fc+PPO_2X+Nafion) biosensors. E=-0.1 V (vs. MSE), Argon saturated 0.05 M phosphate buffer (pH 7.0).*

When comparing to literature work that made use of carbon paste electrodes as DA biosensors, a broad range of behaviors have been reported: the electrode response was linear over a large DA concentration range, from 0.1 μM to 1 mM [14] or from 20 μM to 20 mM [13], with sensitivities ranging from 0.0058 $mA\,M^{-1}$ [32] to 11 $mA\,M^{-1}$ [14], and detection limits as low as 50 nM [14], and high as 2.4 μM [15].

7 CONCLUSIONS

The main objective of this research was to design electrochemical (bio)sensors that would continuously and specifically detect dopamine (DA) over a wide concentration range with high sensitivity and low detection limits. A range of electrochemical techniques were used for this purpose.

It was shown that the oxidation of DA on bare platinum (Pt) surface is a surface-controlled reaction, occurring at low overpotentials, i.e. close to the reversible potential. The reaction is electrochemically reversible.

The equilibrium experiments showed that DA adsorbs on a Pt surface. The adsorption can be described by the Langmuir isotherm. The Gibbs energy of adsorption value (-38 kJ mol^{-1}) showed that the process is highly spontaneous.

Two types of DA sensors were investigated/developed: solid electrodes for the direct determination of DA (*sensors*), and enzyme-modified electrodes for the mediated determination of DA (*biosensors*).

Platinum (Pt) and boron-doped-diamond (BDD) surfaces were used as electrodes for the development of DA *sensors*. It was shown that both surfaces could be efficiently used to determine DA in aqueous solutions. The interference of ascorbic acid (AA) was successfully eliminated by forming a thin Nafion film on the Pt and BDD electrode surfaces. A detection limit for both the Pt/Nafion and BDD/Nafion sensors was 200 nM, while the BDD sensor without the surface Nafion film (unmodified BBD sensor) was able to detect 50 nM of DA. The sensitivity of the Pt- and BDD-based sensors varied between 2.5 mA M^{-1} and 15.5 mA M^{-1}, depending on the sensor and DA concentration range investigated. Hence, both types of sensors would be suitable for monitoring DA levels in urine.

The standard addition method was also investigated as a possible method for DA determination in unknown samples. Chronoamperometry used with a Pt electrode gave good results even at low DA levels, 1 μM, typical for urine samples, with 80% recovery.

In order to make the chronoamperometric determination of DA highly selective and to further lower the detection limit, a *biosensor* based on the use of a Pt electrode as a

transducer surface, polyphenol oxidase (PPO) as an enzyme, ferrocene (Fc) as a mediator, and polypyrrole and Nafion as membranes, was constructed and optimized. It was shown that the sensitivity and detection limit of the biosensor depends on the amount of PPO and Fc incorporated into the PPY membrane, and also on their ratio.

The modification of the biosensor by a Nafion membrane offered three benefits: an increase in sensitivity, an improvement in detection limit, and a significant minimization of the AA interference.

The optimum biosensor architecture was made by polymerizing PPY for 40 minutes from a pyrrole solution containing 2,400 $U\,mL^{-1}$ of PPO and 10 mM of Fc, on top of which a thin Nafion film was formed. Using chronoamperometry as a detection technique, this biosensor yielded a DA detection limit of 20 nM, which makes it suitable for monitoring DA levels in brain. However, the biosensor lacked operational stability, predominately due to the leakage of PPO and Fc into the storage solution.

A possibility of applying electrochemical impedance spectroscopy to detect DA using the optimized biosensor architecture was also investigated. It was shown that this experimental technique is significantly more sensitive than chronoamperometry, providing a DA detection limit as low as 10 nM. However, the technique requires more complex and expensive equipment, and mathematical modeling of the raw data is also needed.

A simple, cheap, very stable and renewable carbon-paste (CP) DA biosensor was also constructed. Although this biosensor offered several advantages over the Pt-based biosensor (higher sensitivity, simplicity, and much longer shelf-life), its detection limit was poorer, 200 nM, thus making it unsuitable for the monitoring of DA in brain, but quite suitable for the DA monitoring in urine samples.

In short, this research resulted in the development of Pt- and BDD-based sensors and Pt- and CP-based biosensors possibly suitable for the monitoring of DA levels in urine and brain. The lowest detection limit of the two Nafion-modified, and thus AA-resistant sensors, was 200 nM, while the unmodified BDD sensor offered a detection limit of 50 nM. The optimized Pt biosensor, in combination with electrochemical

impedance spectroscopy, offered a very high sensitivity and low DA detection limit, 10 nM, making it potential candidate for monitoring DA levels in brain.

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