

Point-of-Care Testing – Biosensor for Norepinephrine Determination

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Abstract — An useful electrochemical sensing approach was developed for norepinephrine (NE) detection based on semiconducting polymer (9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole) and laccase modified platinum electrode (Pt). The miniature Pt biosensor was designed and constructed via the immobilization of laccase in an electroactive layer of the electrode coated with thin polymeric film. This sensing arrangement utilized the catalytic oxidation of NE to norepinephrine quinone. The detection process was based on the oxidation of catecholamine in the presence of enzyme – laccase. With the optimized conditions, the analytical performance demonstrated selectivity in a wide linear range (0.1–200x10⁻⁶ M) with a detection limit of 240 nM and a quantification limit of 365 nM. Moreover, the method was successfully applied for selective NE determination in the presence of interfering substances.

Keywords—biosensor, cyclic voltammetry, laccase, norepinephrine, point-of-care

I. INTRODUCTION

ONE of the primary goals of world-wide scientific endeavors is to improve the *quality of life*. Achieving this assumption is directly related to the rapid analysis of common disorders, quality control in the food industry and environment monitoring. Constant, fast and sensitive *in situ* monitoring is a priority in diagnostic control, most of all, in medical diagnostic. The devices that meet these requirements are biosensors. The dynamic development of sensor technology in recent years and mutual cooperation of several scientific disciplines - biology, chemistry, electronics, and material engineering, enables the development and upgrading of research tools and measurement for quick and sensitive detection. The purpose of these devices is to control and evaluate, e.g. health status of patients, monitoring of the quality of food products and medicines, and study of changes in the environment.

Recently, a pronounced increase of interest of neurotransmitters determination is observed. Neurotransmitters, such as epinephrine (EP), norepinephrine (NE), dopamine (DA) and serotonin (5-HT) belong to the group of catecholamines and play an essential role in the human organism as chemical messengers. These species are responsible for processes like “fight or flight” response, transmitting signals across a chemical synapse and modulating blood flow throughout the body. Neurotransmitters are present in human serum at nM level, nevertheless, any dysfunction of these catecholamines may lead

to numerous, serious health problems, like: Alzheimer disease, Parkinson disease, schizophrenia, depression, Huntington disease, severe head trauma, various neuroblastoma, adrenocortical carcinoma, pituitary adenoma, pheochromocytoma, and cancerous tumors or cardiovascular problems [1-4]. Due to this fact, rapid and sensitive catecholamines detection is extremely important in modern medicine, however, there is still any available device, which will show the concentration of these compounds in patient’s body.

Many classical techniques allow to investigate catecholamines concentration in the sample, like high-performance liquid chromatography (HPLC), gas chromatography (GC), spectrophotometry, chemiluminescence, electrochemistry, fluorescence-based measurements, capillary electrophoresis (CE) [5-10]. Still, most of these procedures are expensive, do not allow continuous monitoring, are active only for a short period of time or have low sensitivity and selectivity.

From the literature are known biosensors for norepinephrine detection. Samdani et al. presented an electrochemical sensor for NE determination based on glassy carbon electrode (GCE) modified with FeMoO₄ (FM) nanorods. Such a system has been able to catalyze the NE oxidation due to the formation of Fe (II)-dioxygen complexes. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques have been employed for the characterization of the sensor. The amperometric response of NE on the FM/GCE showed a linear increase in the current between 5.0x10⁻⁸ M and 2.0x10⁻⁴ M with a detection limit of 3.7x10⁻⁹M [11]. Another electrochemical detection system for NE and serotonin determination has been present by Wang et.al. The sensor has been fabricated by modification of screen-printed electrode (SPE) with multi-walled carbon nanotubes (MWNTs) and zinc oxide/chitosan (MWNTs-ZnO/chitosan SPE) complex. The electrochemical behavior of NE was investigated using CV and square wave voltammetry (SWV) techniques. The peak currents of NE was linearly dependent on its concentrations in the range of 0.5–30 μM, with the limit of detection of 0.2 μM. The modified electrode had been stored stably for at least 3 months at 4°C in a refrigerator [12]. Lu et al. describes the preparation and characterization of an electrodeposited DNA membrane doped with gold nanoparticles. The gold nanoparticles-doped DNA composite electrode was successfully used for the selective determination of norepinephrine in the presence of ascorbic acid (AA). A detection limit of 5 nM NE was obtained by using a DPV technique in static solutions [13].

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The beginning of the use of diagnostic systems for point-of-care (POC) research is considered to be the 60's and 70's of the 20th century, the date of development and implementation of a glucometer, an all-in-one apparatus enabling independent measurement of glucose in the blood. Towards the end of the 1970s, one-time tests, such as pregnancy tests, have revolutionized the testing of disposable household products. Currently, we can find fertility tests, menopausal tests, tests detecting the presence of psychoactive substances, or the presence of infection. Designing devices allowing POC analysis in the clinical sense (enabling continuous monitoring of given substances) has been developing rapidly for two decades, enabling quick and sensitive "bedside" diagnostics [14]. POC testing is generally defined as tests that are performed away from the medical laboratory. There is still not any universal definition of such testing, however, any tests that can be done quickly, simply and without the need for sophisticated laboratory equipment is defined as POC. According to Peeling et al. the fundamental requirements for POC are accuracy, sensitivity, specificity, user-friendliness, rapid and robust, equipment-free and delivered (ASSURED) at settings away from traditional laboratory [15]. In comparison with conventional analytical testing POC possess many advantages, they are more efficient, the turnaround time for results is decreased, which is directly connected with faster patient diagnosis, allow for rapid response and continuous monitoring of critical parameters, they are portable and more convenient, an indirect costs by avoiding travel can be reduced [16].

The semiconducting polymers are known to be excellent materials for the immobilization of biomolecules and rapid electron transfer for the fabrication of efficient biosensors. Conducting polymers (CPs) are an interesting alternative to organic polymers, which are mostly insulators. A characteristic feature of CPs is the presence of conjugated π electrons (a system having conjugated bonds C=C), thanks to which they exhibit unique electronic properties, such as low optical transition energy, low ionization potential and high electron affinities [17]. Due to the presence of aromatic units in the polymer backbone, the immobilization was performed with the help of π - π stacking interactions of the polymer and enzymatic protein. These strong interactions stabilize the tertiary structure of proteins effectively [18]. What is more, CPs are cheap, easy to synthesize and universal, because their properties can be easily modulated by physical changes (such as pH, temperature) even after their synthesis [19,20].

In this study, we present a unique polymer-based electrochemical biosensor for the determination of norepinephrine level, based on an enzyme-dependent redox reaction. The novel procedure reported here may offer a few advantages: high sensitivity, application of stable materials, stable matrix for a protein anchoring, which also ensures stability during measurements and also simple and quick measurements. However, the main advantage over the other systems known from literature is the low detection limit and excellent selectivity. The fabricated platinum electrode modified with semiconducting polymer poly[9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole] and laccase or exhibit exquisite electrochemical behavior. The presented study provides a simple, selective and sensitive method, making described biosensors a good tool for catecholamines detection.

II. EXPERIMENTAL

1) Reagents and materials

Laccase (from *Cerrena unicolor*, EC 1.10.3.2, ≥ 10 U/mg), as well as norepinephrine hydrochloride (NE), tetrabutylammonium-tetrafluoroborate (TBA-TFB), dichloromethane, uric acid (UA), ascorbic acid (AA), L-cysteine (CYS) were purchased from Sigma-Aldrich Co. Citric acid (CA), NaOH, NaH_2PO_4 , Na_2HPO_4 , KH_2PO_4 , Tris, HCl, CH_3COONa , CH_3COOH , NaCl, KCl, glutaraldehyde (GA) were purchased from POCH (Part of Avantor, Performance Materials, Poland). All chemicals were of analytical grade and were not further purified before use. All buffers needed to wash unbound protein or to immobilization (phosphate buffer, acetate buffer and Tris-HCl) were prepared according to generally known, obligatory standards.

2) Modification of electrode

The platinum electrode (Pt electrode, diameter 3 mm, produced by BASi, MF-2013) was polished before experiment with 3 μm fine diamond polish and rinsed thoroughly with double distilled water. Such prepared electrode for NE determination was modified with a thin layer of poly[9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole] and laccase. The synthesis of the monomer is described in the literature [21]. The electropolymerization of monomer was performed by a potentiostat/galvanostat AUTOLAB PGSTAT128N with GPES software. A three-electrode electrochemical cell (10 ml) equipped with a working electrodes (Pt), a silver-silver chloride reference electrode (Ag/AgCl), and a coiled platinum wire as the counter electrode, was used for all electrochemical experiments. All the electrochemical measurements were performed at room temperature. Due to synthesis of the polymeric layer onto a surface of the clean Pt electrode, monomer (1mM) was dissolved in a dichloromethane solution containing an electrolyte - 0.1 M tetrabutylammonium-tetrafluoroborate (TBA-TFB). The electrodes were dipped into 8 mL of the monomer solution. The polymer layers deposition was carried out through cyclic voltammetry (CV). The Pt electrode was scanned in a potential range 0.0 – 1.4 V vs Ag/AgCl for 10 cycles, at a scan rate of 50 mV/s. Then, the modified Pt electrode with poly[9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole] was washed with dichloromethane.

Knowledge about a biological element or recognition receptors, has a significant meaning due to biosensors designing, because the receptor plays a key role in the successful determination of the desired compound. Biomolecular recognition is related to non-covalent specific binding, and weak interactions (such as hydrophobic forces, Van der Waals force or hydrogen bonding) between molecules – in case of biosensors one is a biologically active material and the other is an analyte. Recently, there has been much progress in understanding the forces that drive the formation of such complexes, and how these forces are related to the physical properties of the interacting molecules and their environment allows rational design of molecules and materials that interact in specific and desired ways. Due to this, an assortment of the appropriate receptor and adequate platform for this bio-receptor is the most essential part in the design of biosensors. Immobilized biomolecules have to maintain their structure and their function, to retain their biological activity after

immobilization and to remain tightly bound to the surface. An ideal biosensor has to be stable for long-term applications. The type of immobilization method significantly affects the activity and stability of enzymatic biosensors. Physical immobilization is a simple and inexpensive method but there is a risk that the biocatalyst will be desorbed during the use of the device. Whereas chemical immobilization provides a strong and stable covalent bonding between the matrix and the enzyme, but it is an expensive and more complicated method and most importantly often results in protein conformational changes. The sensitivity of the sensor decreases if immobilization causes enzyme denaturation or if the enzyme has been modified, especially on its active site [22,23]. Each immobilization method presents advantages and drawbacks. The choice of the most appropriate technique depends on the enzyme nature, the transducer and the associated detection mode [24].

In this case, the immobilization processes were provided by physical adsorption of laccase in a phosphate-citric buffer (pH 5.2) at room temperature onto the surface of polymer-modified Pt electrode. This physical adsorption last for 2 h and then the setup was crosslinked with glutaraldehyde (GA) (10 minutes). Additional use of the GA for cross-linking allows a relatively stable and active immobilization of the proteins onto the electrode surface, because of covalent bonds creation.

The excess of unbounded proteins was washed with phosphate (pH 7.0), acetate (pH 5.2) and Tris-HCl (pH 7.2) buffers, respectively.

Enzyme immobilized by physical adsorption with cross-linker does not require any other activation. According to this procedure modified Pt-E/9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole/Laccase electrode was obtained and was stored at 4°C when not in use. The described modified electrode has been catalytically active for c.a. 90 reaction cycles.

3) Electrochemical measurements

Norepinephrine detection was performed using cyclic voltammetry (CV) with a potentiostat/galvanostat AUTOLAB PGSTAT128N with GPES software. The measurements were carried out with a typical three-electrode system in the 8-ml cell. The Pt electrode for NE detection modified with thin polymer film of poly[9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole] and laccase was used as working electrode, together with a coiled platinum wire as the auxiliary electrode and a silver-silver chloride reference electrode (Ag/AgCl). Cyclic voltammetry (CV) measurements, showing the whole redox cycle were carried out by repeated potential scanning in range -0.5 – 1 V. All the electrochemical measurements were performed at a scan rate of 50 mV/s, in room temperature with air-opened conditions.

4) Influence of interfering substances

Interfering substances (ascorbic acid (AA), uric acid (UA), L-cysteine (CYS) and mix of all interfering substances) in a concentration of 50 µM were added to NE standard solutions at concentration 1, 50 and 100 µM to check the interferences in deficiency, balance, and excess. Mentioned species were mixed each time with epinephrine solutions in volume ratio 1:1.

III. RESULTS AND DISCUSSION

1) Characterization of polymers

A crucial problem in the design of enzymatic electrodes is to enhance the speed and reversibility of charge transfer between

the enzyme and the electrode. In the electrochemical devices, where preservation of the enzyme activity at the nanocomposite/enzyme interface is vital for designing efficient electrodes, charge transfer between the enzyme and electrode should be fast and reversible. It has already been published that the use of conductive materials in the construction of biosensors (e.g. polymers [25]) improves the electron transport between the active site of the enzyme and the electrode surface, hence the sensor has short response time and high sensitivity. Biosensor efficiency depends mainly on the surface architecture, interaction among the enzyme and electrode surface and protection of the 3D structure of biocatalyst.

Conductive polymer's charge transfer capacity acts as an excellent matrix for biomolecules providing enzyme mimetic environment [18,26]. Due to the presence of aromatic units in the polymer backbone, the immobilization was performed with the help of π - π stacking interactions of the polymer and enzymatic protein. These strong interactions stabilize the tertiary structure of proteins effectively. In addition, one of the classes of compounds intended for electrode modification is bis(selenophene)carbazole derivatives. There are research results that prove that the presence of small amounts of selenium in the medium has a positive effect on the catalytic activity of laccase [27].

In this paper polymer built of 9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole (Fig. 1) has been electrochemically synthesized in the presence of 0.1 M TBA-TFB. The polymer occurs in the conducting oxidized state.

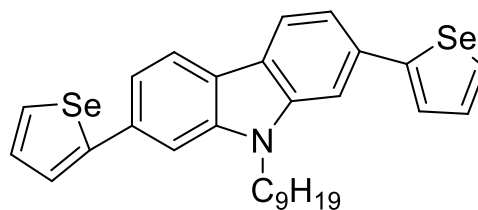


Fig. 1. Structure of 9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole

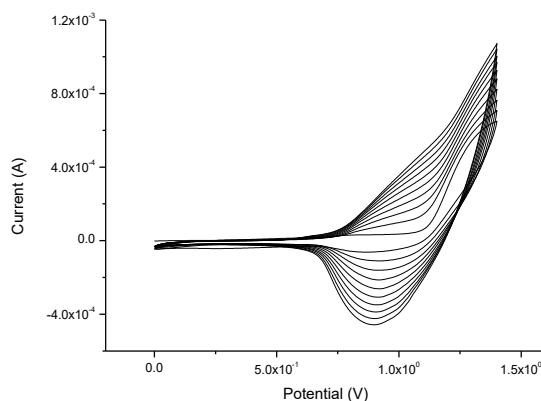


Fig. 2. Cyclic voltammograms of investigated monomer - electropolymerization of 9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole (monomer concentration - 1mM) in 0.1 M TBA-TFB. Measurement conditions: scan rate 50 mV/s, Ag/AgCl – reference electrode, 10 cycles.

In order to obtain the conducting polymer on the Pt electrode, the cyclic voltammetry was used. The optimal potential range for polymer deposition was found to be 0.0 – 1.4 V and the polymerization lasted for 10 cycles. Figure 3 exhibit

the results of the electrochemical polymerization of the carbazole. The voltammograms demonstrate the formation of the electroactive polymer layers of 9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole on the Pt electrode. The graph exhibits the increase of peak currents in subsequent scans, which suggests the embedding of the conducting polymer.

2) Principle of electrochemical measurements and detection assays for neurotransmitters

A bio-system for norepinephrine determination is the system using the Pt electrode modified with poly[9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole] and laccase (Fig. 3) in the presence of a wide range of concentration of the NE (0.1 – 200 μM).

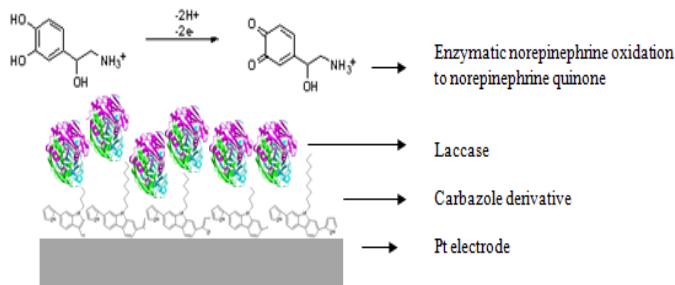


Fig. 3. Scheme of measuring platform for enzymatic norepinephrine determination

In Figure 4A are presented results obtained for biosensor responses to the given NE concentration - as can be observed signals precisely respond to concrete NE concentration. The increased electrical signal is due to the charge (electron) released in the oxidation/reduction process, occurring between an enzyme - laccase and an analyte - norepinephrine. An oxidation peak of NE is c.a. 0.22 V. Biosensor response is in that case linear and proportional, as the concentration increases, at the same time, current increases. The electrochemical nature of NE was examined in a wide range of concentrations (0.1 - 200 μM) employing the CV method (applied potential -0.5 – 1 V, scan rate = 50 mV/s) in oxygen-saturated conditions for 20 scans. Figure 4B presents the results of the linearity of the biosensor based on Pt-E/9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole/Laccase with good linear response to NE in the investigated concentration range, a very good linear coefficient ($R^2=0.991$) is also observed. The slope of the calibration curve is associated with the biosensor's sensitivity which is related to the limit of detection (LOD).

One of the most important parameter in the construction of biosensor is the limit of detection. LOD is the lowest concentration of analyte which can be checked with statistical certainty using the presented technique. The LOD was calculated with using the following equation:

$$LOD = 3.29 \cdot \frac{\sigma_B}{b} \quad (1)$$

where σ_B is the standard deviation of the population of blank responses and b is the slope of the regression line [28].

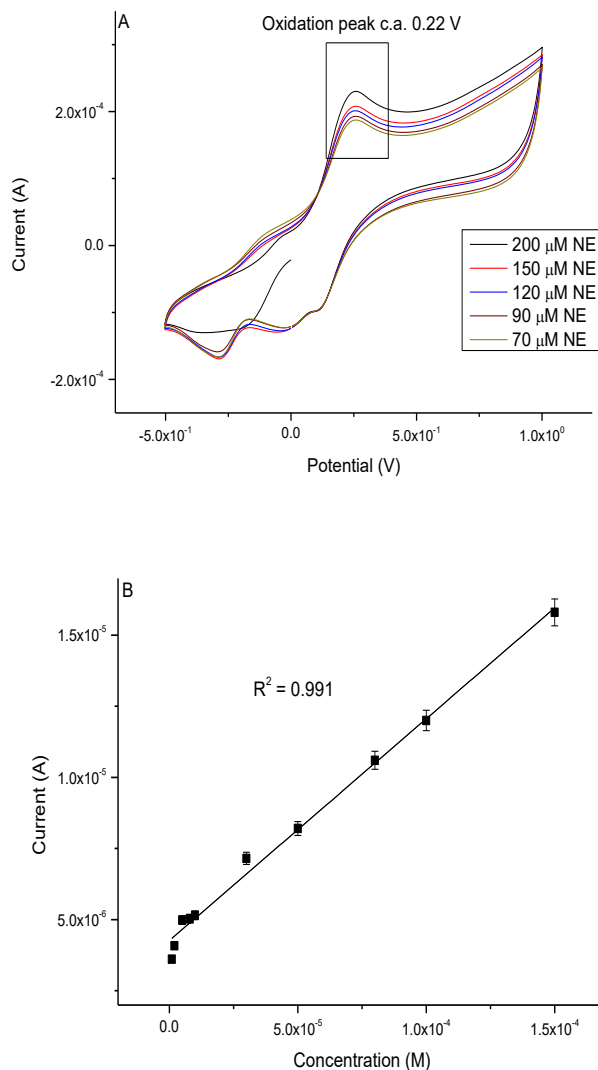


Fig. 4A,B. A: Representative CV-scans for increasing concentration (NE concentration 70, 90, 120, 150, 200 μM); B: relationship between 5-HT concentration and current (biosensor response)

The limit of detection for system is equal to 240 nM. The low detection limit demonstrates the ability of the constructed platform to detect low NE concentrations. This value can be compared with other biosensor systems for neurotransmitters determination known from the literature (Table I).

Additionally, the limit of quantification (LOQ) was also determined (calculated with using equation 2) and it equals 365 nM.

$$LOQ = 5 \cdot \frac{\sigma_B}{b} \quad (2)$$

where σ_B is the standard deviation of the population of blank responses and b is the slope of the regression line [28]. Parameters demonstrating an analytical validation are shown in Table II.

TABLE I
COMPARISON OF BIOSENSORS AND SENSORS FOR NEUROTRANSMITTERS
DETECTION

Analyte	Detection complex	Measurement	LOD	Ref.
Serotonin	Catechin/Carbon Paste Electrode	Electrochemical	3 nM	[29]
Dopamine	Functionalized-CuInS ₂ QDs	Optical	200 nM	[30]
Dopamine	GQDs/pDAcomplex	Optical	8 nM	[31]
Norepinephrine	Single-Walled Carbon Nanotube/Glassy Carbon Electrode/Tyrosinase	Electrochemical	0.1 μM	[32]
Epinephrine	Laccase-carbon Paste Electrode	Electrochemical	1.84 μM	[33]

TABLE II
ANALYTICAL PARAMETERS OF THE PRESENTED BIOSENSOR

Linear range	LOD	LOQ	R ²
0.1 – 200 μM	240 nM	365 nM	0.991

The results presented for the biosensor are characterized by the wide linear range and low detection limit, which proves, that this constructed system may pretend to be a very good platform for clinical applications to accurately determine the level of norepinephrine.

3) Selectivity

Presented here enzyme-based biosensor is designed for quantitative NE detection in human body fluids' samples. In that case, created bio-tool should be selective only for described neurotransmitter determination, not for any interfering species present in the sample. Human plasma or urine contains a number of interfering substances, like the most common ascorbic acid (AA) and uric acid (UA) have high concentrations in the human body and possess similar oxidation potentials to NE. Interfering compounds may have an impact on oxidation peak potentials and current values during NE examination in human samples, which in consequence may lead to an incorrect result of a measurement. Figure 5 presents this compounds which can disturb signals obtained during measurements, including: ascorbic acid (AA), uric acid (UA), cysteine (CYS) and mix of all examined substances, which were added to investigated NE samples at concentration 1, 50, 100 μM.

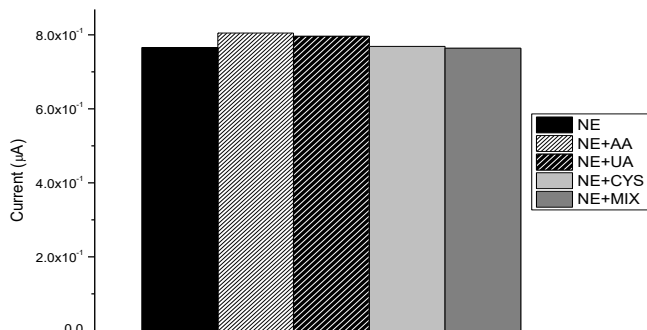


Fig. 5. Effect of interfering substances (50 μM) on NE determination

All presented interfering species were added in concentration equals 50 μM to neurotransmitter samples to check the influence of these compounds in the high excess, equilibrium and deficiency of them. Each tested reagent has an insignificant effect (<5.2%) on the peak current of the samples compared to the blank. Presented results (Fig. 5) confirm, negligible impact on the selectivity of fabricated setups, and prove that existing interference does not interrupt the prominence of the proposed NE test. The biosensor exhibit an adequate selectivity for such catecholamine determination.

IV. CONCLUSIONS

Due to the global demand for quick and cheap analytical methods, the research which was carried out in this paper aimed at intensifying the work on the design of a new generation of miniature diagnostic devices with wide application possibilities. In summary, a rapid, sensitive, selective and simple biosensor system for the detection of norepinephrine has been developed using Pt electrode modified with 9-nonyl-2,7-di(selenophen-2-yl)-9H-carbazole and laccase. The electrochemical measurements were carried out employing the cyclic voltammetry method. The biosensing assay demonstrates an exquisite electrocatalytic activity over a linear concentration range (0.1 – 200 × 10⁻⁶ M) with a detection limit equal to 240 nM. Obtained LOD value is comparative with a wide range of described electrochemical biosensor for norepinephrine detection, which proves that electrode modified with carbazole derivative in combination with laccase create very good material for neurotransmitters investigation. In addition, the detection biosystem proved, that all examined interfering substances have a slightly effect on the signal during amperometric measurements (≤ 5.2 %). All characteristics made for biosensor establish a convenient, stable, simple and long-term technique for neurotransmitters detection and recommend it as an excellent bio-tool for diagnostic. Our preliminary studies presented here, may lead to the creation of a sensitive, fast, biocompatible and selective bio-device suitable for single-use, disposable *in vitro* or *in situ* applications.

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