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DEVELOPMENT OF VOLTAMMETRIC MELANIN SENSOR WITH 2,5-DIMETHYLFURAN MODIFIED PLATINUM ELECTRODE

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In this study, an electrochemical sensor was developed for the electrochemical determination of melanin. For this purpose, phosphate buffer salt (PBS, pH 7.00) solution was used as an electrolyte, 2,5-dimethylfuran (2,5-DMF) as a monomer, Pt wire as the auxilary electrode, Ag/AgCl electrode as the reference electrode and a platinum electrode as the working electrode. The modification of the electrode was performed by polymerization of 2,5-DMF at the bare Pt electrode surface using the cyclic voltammetry method. Parameters such as film thickness, scan rate and pulse amplitude with prepared 2,5-DMF modified platinum electrode (*p*(2,5-DMF)/PtE) were optimized by square wave voltammetry (SWV) in PBS buffer containing melanin. In optimum conditions, the anodic peak height showed a linear increase with the melanin concentration. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated to be 0.088 mmol/l and 0.292 mmol/l, respectively, with melanin concentrations ranging from 0.10 to 2.20 mmol/l and the correlation coefficient (R^2) was calculated as 0.9985. Stability of the prepared sensor was found to be 99.63% by using the reproducibility experiments (n = 15).

Keywords: square wave voltammetry; melanin; sensor; 2,5-dimethylfuran

РАЗВОЈ НА ВОЛТАМЕТРИСКИ СЕНЗОР ЗА ОПРЕДЕЛУВАЊЕ МЕЛАНИН СО УПОТРЕБА НА ПЛАТИНСКА ЕЛЕКТРОДА МОДИФИКУВАНА СО 2,5-ДИМЕТИЛФУРАН

Во овој труд е развиен електрохемиски сензор за волтаметриско определување на меланин. За таа цел, како основен електролит е употребен раствор на фосфатен пуфер со pH = 7,00, 2,5-диметилфуран е користен како мономер, платинска жива служеше како помошна електрода, Ag/AgCl електрода е користена како референтна, додека Pt електрода е употребена како работна електрода. Модификувањето на работната електрода е извршено со полимеризација на 2,5-диметилфуран на површината од Pt електрода со примена на циклична волтаметрија. Оптимизирањето на параметрите како што се дебелината на филмот, брзината на скенирање и квадратно-брановата амплитуда е извршено со употреба на квадратно-бранова волтаметрија во фосфатен пуфер што содржи меланин. При услови што беа определени како оптимални, постои линеарна зависност помеѓу анодната струја на волтаметрискиот пик и концентрацијата на меланин. Границата на детекција и границата на квантификација на меланин соодветно изнесуваа 0,088 mmol/l и 0,292 mmol/l, определени при концентрации на меланин во подрачје од 0,10 до 2,20 mmol/l, со коефициент на корелација R^2 = 0,9985. Стабилноста на овој волтаметриски сензор изнесуваше 99,63%, што е пресметано преку експерименти на репродуцибилност (n = 15).

Клучни зборови: квадратно-бранова волтаметрија; меланин; сензор; 2,5-диметилфуран

1. INTRODUCTION

Dark brown melanin is synthesized by certain fungal species. Although melanin is generally found in the cell wall, some fungal melanin is found in the medium in the form of granules in certain fungus species [1]. Fungal melanins with high molecular weight are also negatively charged and hydrophobic pigments. These fungal pigments are formed by oxidative polymerization of phenolic and indolic compounds such as glutaminyl-3,4-dihydroxybenzene (GDHB), catechol, 1,8-dihydroxynaphthalene (DHN) and 3,4-dihydroxyphenylalanine (DOPA). Some fungi belonging to the *Phylum ascomycota* synthesize DHN-melanin from the polyketide synthase pathway. A few species of fungi also synthesize melanin from L-DOPA, which is the synthesis route in mammals. Melanin is produced in special cells called melanocytes [2, 3]. Melanin pigments are produced by the oxidation and polymerization reactions of the amino acid tyrosine, called melanogenesis. These pigments, which are not necessary for the growth of fungi, protect microorganisms from harmful environmental conditions. That's why it has been reported to act as "fungal armor". Fungi that do not produce melanin are more sensitive than fungi that produce melanin. Melanin-producing fungi are resistant to antimicrobial drugs, lytic enzymes, heavy metals, UV rays and free radicals. Especially fungi with melanin such as *Cladosporium* and *Alternaria* are resistant to heavy metal and unsaturated hydrocarbon pollution.

The functions of melanin in living things are different. Some types of melanin are synthesized by many cephalopods as a defense mechanism against predators. Melanin pigment has many properties such as thermoregulatory, photoprotective, antimicrobial, antiviral, cytotoxic, anti-inflammatory and immunomodulator. Therefore, in recent years it has been used in many fields such as medicine, cosmetics, materials science and nanotechnology [4, 5]

There are three basic types of melanin called eumelanin, pheomelanin and neuromelanin: The most common type is eumelanin. Pheomelanin is a derivative of cysteine that is largely responsible for the color of red hair. Neuromelanin is found in the brain. Eumelanin has excellent physical and chemical properties, such as broad band absorbance, free radical scavenging, as well as photo and electrical conductivity [6, 7].

Since melanin is a biologically functional and organic material, this pigment is of great interest for many biotechnological applications in the cosmetic, pharmaceutical, electronics and food processing industries [8–10].

Pigments synthesized from microorganisms have many advantages over animal and plant pigments. Their production is economical, they are easily produced in media, and they do not require large production areas such as agricultural land [11, 12].

Other microorganisms such as fungi also produce a wide variety of biologically important pigments other than melanin. Microorganisms act as microbial cell factories that produce these pigments. These are pigments such as carotenoids, melanins, flavins, phenazines, quinones, monascins, violacein and indigo [13, 14].

There are several methods for assessing the amount of melanin in biological samples. One of these is electron spin resonance spectrometry (ESR). Electron spin resonance signals are measured over free radicals derived from melanin. This technique is quite specific, but not sensitive. High performance liquid chromatography (HPLC) is also used to quantify melanin. The most widely used method for measuring melanin is absorption spectroscopy. The absorbance of melanin in the cells is measured and compared with the data obtained with a standard synthetic melanin curve. Although it is the most popular method for melanin, this method has its disadvantages; especially, this method does not always provide precision. Therefore, there is a need for reliable, simple and economical alternative methods for melanin quantification [15].

On the other hand, melanin is a promising semiconductor biomacromolecule for application as a pH sensor [16]. The melanin pigment is one of a few bio-macromolecules that display electrical and photo-conductivity in the solid-state. Melanin is mechanically flexible and biocompatible. For potential in vivo applications, bioelectronic sensing devices based on melanin may be more useful as opposed to many other conductive materials whose performance declines with hydration [17].

Some metabolites produced by microorganisms have the ability to detect a variety of substrates compared to mammalian cells. These products belonging to microorganisms have many advantages such as low cost, mass production, and easy genetic modification. These products can often be used as biosensors.

Biosensors are usually analytical devices that identify and recognize a particular component. These biosensors combine a biological structure or product with a physicochemical detector for the detection of a chemical [18–20]. Biological structures such as tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc. are biomimetic structures that recognize the target, react with it, and bind or recognize it.

Biological products, materials or structures, act as a biosensor designed to detect an effect that can be measured by the transducer and react with the relevant material. There are many biomolecules that can be used as biosensors. These are antibody/antigens [21], enzymes/ligands, nucleic acids/DNA, cellular structures/cells, or biomimetic materials [22, 23].

In recent years, many biomaterials have been used as a substrate for the production of biodegradable and biocompatible electronics [5, 8]. Melanin is a polymer obtained from natural sources and therefore has good biocompatibility and biostability. For example, when fungal melanins were added to cell culture or injected directly into organisms, various side effects such as cytotoxic effects and any antigenic response were not observed [9–11, 12]. In addition to the easy and cheap availability of melanin, it also has many other properties such as scavenging free radicals, UV-visible and infrared radiation absorption, and hybrid ionic-electronic conductivity [13, 14, 18]. In terms of these properties, melanin is a specific biomaterial for biotechnological applications. [19, 20].

Determination of melanin is also important due to the above mentioned features. There are no methods to accurately determine the proportion of various units contained in melanins [24], although the determination of melanin is mostly conducted by spectroscopy, electron paramagnetic resonance (EPR) spectroscopy [25], photoacoustic spectroscopy [26], HPLC [24], and electrochemical anaylsis methods [27, 28]. There are not many studies on this subject in the literature. Morever, previous methods used for quantification of melanins in pigmented tissues required the isolation of melanins [24]. Sensors, especially, have made great progress in the determination of biologically important compounds such as antioxidants or drug active substances in recent years [29–34].

Electrochemical sensors are a crucial alternative for making analysis more accurate, faster and reproducible at lower costs. Especially, with specially modified electrodes, very low concentrations and highly reproducible results can be obtained in a very short time.

Electrochemical techniques most frequently encountered in biomedical investigations are potentiometry and voltammetry. Voltammetric techniques are recognized as cheap, simple, and powerful tools provided by commercial electrochemical instrumentation.

Voltammetry is used in many laboratories dealing with chemical, biochemical, environmental and physical analysis. It is particularly important for the detection and quantification of biochemical and physiologically active compounds and is a technique underlying the operation of many biochemical sensors.

Since square wave voltammetry (SWV), one of the voltammetric techniques, is considered to be the most advanced member of the family of pulsed voltammetric techniques, it is stated in the literature that special attention has been paid to this technique [35, 36].

SWV has been widely used in the development of electrochemical sensors and biosensors in recent years due to its high selectivity and sensitivity [37]. Therefore, it is frequently applied for the quantitative determination of various compounds such as drugs, biomolecules, environmental pollutants, antioxidants, etc. Besides the obvious sensitivity, SWV is considered a fast electroanalytical method, often requiring a very short analysis time.

In the last decade, in many cases, SWV has emerged as a viable and cheap alternative to other more expensive and time-consuming techniques (such as chromatography, for example) for the determination of inorganic and biologically important compounds. All these advantages make SWV a technique of choice for the quantification of important classes of compounds [36].

On the other hand, the modification of electrode surfaces with various conductive polymers seems inevitable for many electrode-analyt systems to achieve better compatibility. It is possible to develop miniature voltammetric sensors/biosensors by modifying the working electrode surface [35].

To contribute to the literature, in this study, an electrochemically modified Pt electrode with 2.5-DMF was used for the determination of melanin. In this way, modified electrodes as melanin sensors were developed for fast, sensitive, accurate and selective determination of melanin. To determine the stability, selectivity and specificity of modified electrodes, parameters such as polymer scan rate and thickness of the film layer were analyzed using methods such as cyclic voltammetry (CV) and SWV. In conclusion, we believe that a developed sensor for determination of melanin in this way will be of great interest and will contribute to the literature.

2. EXPERIMENTAL

2.1. *Apparatus and chemicals*

Electrochemical behavior of melanin was investigated using two different voltammetric methods, CV and SWV. CV was used for preliminary studies such as determining the redox potential of melanin and polymerization of bare PtE.

SWV was used for subsequent optimization of electrochemical parameters. All voltammetric experiments were performed using an Ivium Vertex One brand potentiostat. The potentiostat device was controlled by IviumSoft ™ software, which is used for both data acquisition and data analysis and includes many electrochemical techniques.

Melanin MP was supplied from Biomedicals. Alpha lipoic acid, Aniline, 3-Methylthiophene, Acetonitrile (AcN) and 2,5-dimethylfuran were obtained from Sigma-Aldrich. Citric acid, NaCl, KCl, Na_2HPO_4 and KH_2PO_4 were purchased from Merck and used without any purification. All chemicals used were of analytical purity. Melanin stock solution was prepared in dilute ammonia. 2,5-DMF solution was prepared in AcN containing 0.1 M sodium perchlorate. PBS (Phosphate Buffered Saline) solution was prepared to contain 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4 and 0.0018 M KH_2PO_4 [38].

Voltammetric measurements were carried out in 10–20 ml pyrex cells, which were placed in BASi brand C3 (cell stand) cage. A Faraday cage was used to protect the electhrochemical measurement cell system from magnetic and electrical effects in the external environment. In all voltammetric experiments, Pt wire as the auxiliary electrode (BASi brand, MW-1032), bare and modified Pt electrodes (BASi brand, MF-2013) and Ag/AgCl (3 M NaCl) as the reference electrodes (BASi brand, MF-2052 RE-5B) were used in the electrochemical measurement cell system. The reference electrode was washed with ultrapure water after each use and then stored in a 3 M NaCl solution saturated with AgCl. The auxiliary electrode was cleaned by washing with ultra pure water after burning it for certain intervals on a naked fire in order to remove organic impurities. Cleaning of the Pt working electrodes was carried out as specified in [37].

For cleaning, cells and other glass materials were first washed with detergent and then rinsed with distilled water (Millipore brand Elix 20). Afterwards, they were soaked in 6 M HNO_3 solution, preferably overnight, for at least one hour, then rinsed with distilled water at least three times and finally rinsed with ultra pure water and dried in the oven.

Thermo Scientific STAR A-111 pH-meter was used for pH measurements and Weightlab Instrument brand WSA-224 model scales were used for weighing chemicals. Branson brand 3510 model ultrasonic bath was used for cleaning of electrodes.

Ultra pure water was supplied from Millipore brand Elix 20.

2.2. *Determination of peak potential*

The CV of the background (PBS pH = 7.00 buffer solution) in the potential range (–1.2) – (1.2) V was taken (Fig.1a). Then, 2 mmol/l melanin stock solution was added into it and CV was taken at the same interval (Fig. 1b). As seen in Figure 1, a small peak of melanin oxidation was observed around 0.03 V. It was understood that this peak belongs to melanin from the fact that it was not observed in the background solution. The melanin oxidation reaction occurring in pH 7.00 PBS buffer solution was considered to be an irreversible reaction as no peaks were observed in the reverse scanning, i.e., in the reduction direction. According to this result, we determined the potential range in our next studies. We have also shown that melanin is an electroactive molecule.

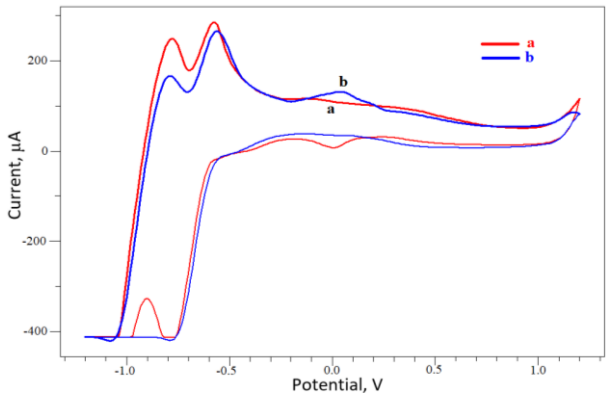

Fig. 1. Cyclic voltammogram of (a) background (PBS, pH = 7.00) and (b) 2 mmol/l melanin in PBS (pH = 7.00)

2.3. *Determination of monomer type*

The effect of each polymer electrode on melanin responses was investigated using the SWV technique. For this purpose; alpha lipoic acid (Ala), aniline (An), 3-Methylthiophene (3MTF), 2,5-dimethylfuran (2,5-DMF) and citric acid (CA) monomer solutions, each of which had a concentration of 5 mM, were prepared and 4-cycle thickness polymers were coated with a scanning speed of 100 mV/s in the potential range (–1.2) – (0.8) V by dipping the Pt electrode in this solution. Then, for the melanin, SWV response of each of the prepared polymer electrodes were taken in PBS (pH 7.00) background solution.

As a result, from electrochemical responses compared to other modified electrodes, it was determined that the best responsive electrode was the *p*(2,5-DMF) coated platinum electrode (Figure 2).

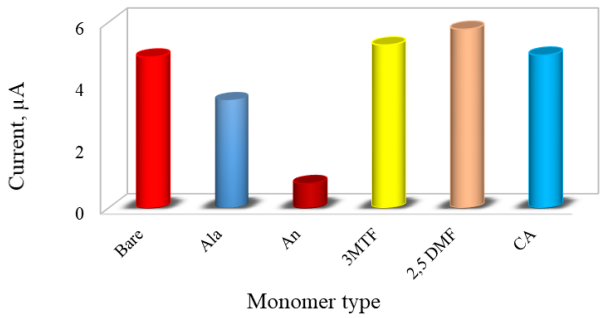

Fig. 2. The effect of bare electrode and coated electrodes with Ala, An, 3-MTF, 2,5-DMF, CA on melanin response

2.4. *Effect of the monomer concentration*

2,5-DMF monomer solutions at different concentrations (3, 5, 8, 10, 12 and 14 mmol/l) were prepared in AcN containing 0.1 M sodium perchlorate solution. Electrochemical polymerizations in the potential range of (–1.2) – (0.8) V were carried out on the bare Pt electrode using the CV method in all these solutions separately.

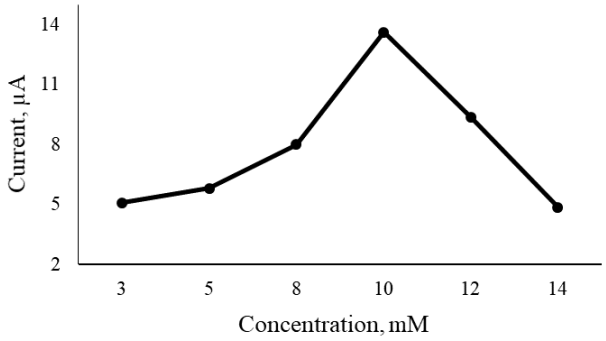

Fig. 3. Effect of the monomer concentrations on melanin SWV response of the modifed electrodes. (Conditions: film thickness: 4-cycle, scan rate: 100 mV/s, solution medium: 0.1 M PBS pH 7.00, potential step: 10 mV, pulse amplitude: 10 mV, frequency: 10 Hz)

The effect of *p*(2,5-DMF) coated Pt electrodes prepared at different concentrations on melanin oxidation peak height was investigated using the SWV method. According to Figure 3, the best response was obtained at 10 mmol/l monomer concentration.

2.5. *Effect of the film thickness*

The thickness of the polymer film on the electrode surface was controlled by adjusting the number of cycles by the CV method during the electropolymerization process. When the melanin responses of the modifed electrodes obtained at different polymer film thicknesses were examined, it was seen that the response obtained with 4-cycle film thickness electrodes was the highest (Figure 4). As the number of cycles increased further, thicker films were formed and the melanin responses decreased. Therefore, in subsequent studies, film thickness was used as 4 cycles.

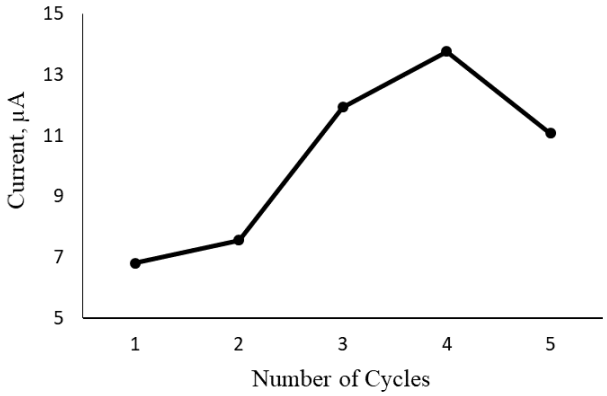

Fig. 4. Effect of the polymer film tickness on melanin SWV response of the modifed electrodes. (Conditions: scan rate: 100 mV/s, solution medium: 0.1 M PBS pH 7.00, potential step: 10 mV, pulse amplitude: 10 mV, frequency: 10 Hz)

2.6. *Effect of the scan rate*

The effect of 2,5-DMF polymerization scan rate on melanin peak height was investigated using the CV method in AcN containing 0.1 M sodium perchlorate on a bare Pt electrode in the range of 25–125 mV/s. When the melanin SWV responses of the modified electrodes obtained at different scan rates were examined in 0.1 M PBS (pH 7.00), it was clearly seen that the highest response occurred at 75 mV/s scan rate (Fig. 5). As a result, the scan rate was chosen as 75 mV/s for electrode modifications.

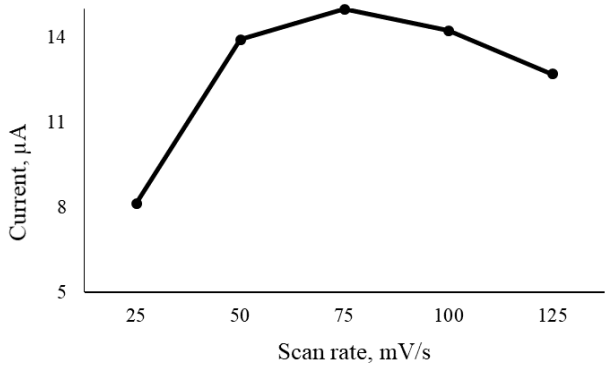

Fig. 5. Effect of the polymerization scan rate, (SWV responses of prepared modified electrodes with different scan rate 2,5-DMF for 2 mmol/l melanin in 0.1 M PBS (pH 7.00, film thickness: 4 cycle, potential step: 10 mV, pulse amplitude: 10 mV, frequency: 10 Hz).

2.7. *Effect of the pulse amplitude*

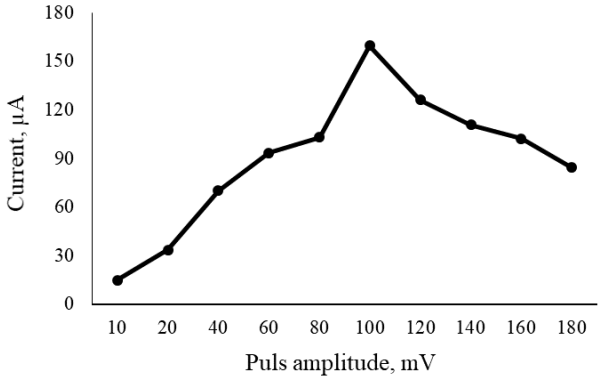

Fig. 6. Effect of the pulse amplitude on 2 mmol/l melanin SWV response. (Conditions; scan rate: 100 mV/s, solution medium: 0.1 M PBS pH 7.00, potential step: 10 mV, frequency: 10 Hz)

The effect of pulse amplitude on melanin peak current in 0.1 M pH 7.00 PBS buffer was studied in the range of 10–180 mV (10, 20, 40, 60, 80, 100, 120, 140, 160 and 180 mV) by the SWV method. As can be seen from Figure 6, it is understood that the optimum pulse amplitude is 100 mV.

An AFM image of the modified Pt electrode with 2,5-dimethylfuran substrate was taken under optimum conditions (Figure 7). According to Figure 7, there was a remarkable increase in the surface area of the electrode. The increase in this surface area resulted in an increase in the melanin peak current. As a result, compared to the melanin response obtained on the bare electrode, a much higher melanin signal was obtained with the electrode modified with 2,5-dimethylfuran.

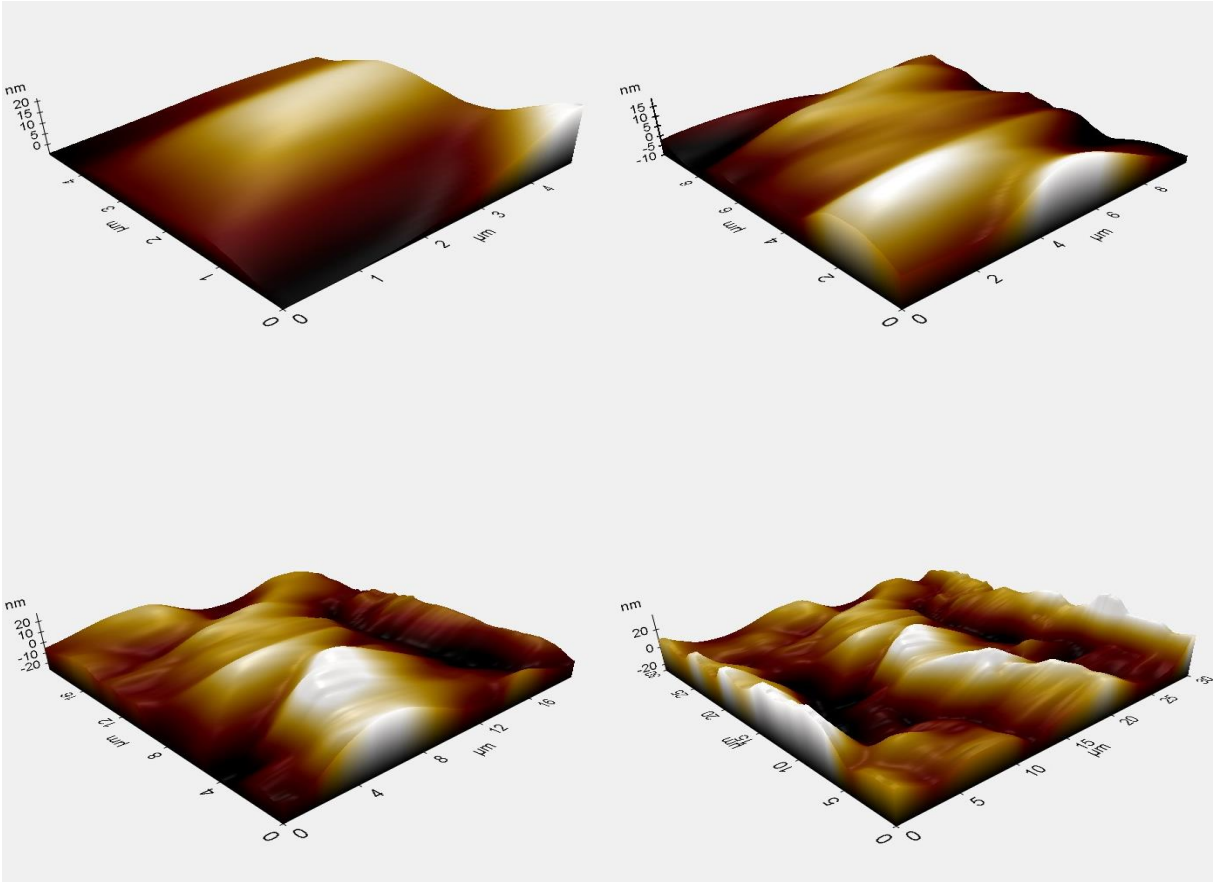

Fig. 7. AFM image of 2,5-dimethylfuran modified Pt electrode at 5, 10, 20 and 30 µm magnifications

2.8. *Effect of melanin concentration*

Square wave voltammograms at different melanin concentrations under optimum experimental conditions are shown in Figure 8A and background, the lowest and highest concentration, in Figure 8B. As can be seen from Figure 8, background, the lowest and highest peak heights are 6,892, 17,547 and 179,713 µA respectively. After subtracting the background peak current, the lowest and highest peak currents are 8,855 and 172,821 µA. As a result, peak currents increased in proportion to the increase in melanin concentration.

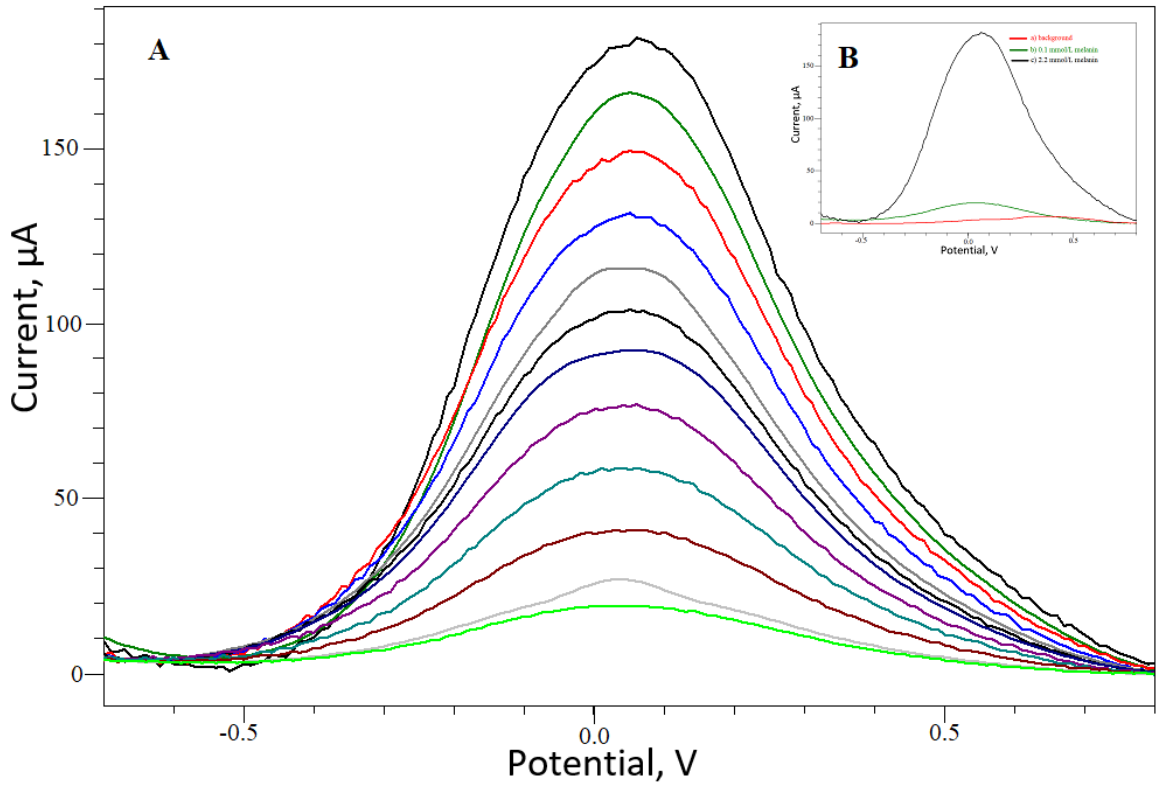

Fig. 8. Square wave voltammograms of *p*(2,5-DMF) coated Pt electrode with increasing amounts **(A)** of melanin (0.10–2.20 mM), **(B)** and 2.2 mmol/l in 0.1 M PBS (potential step: 10 mV, pulse amplitude 100 mV, frequency: 10 Hz, pH: 7.00, scan rate:100 mV/s, film thickness: 4 cycle, and background correction is applied to voltammograms)

The electrochemical performance of the mentioned modified electrode provided a sensitive determination of melanin in a wide linear range of 0.10–2.20 mmol/l with a correlation coefficient of 0.9985. From the calibration curve in Figure 9, the limit of determination (LOD) with the formula 3*s*/*m*, and the quantitation limit (LOQ) with the formula 10s/m, are 0.088 mmol/l and 0.292 mmol/l respectively. (*s* is the standard deviation; *m* is the slope of the calibration curve).

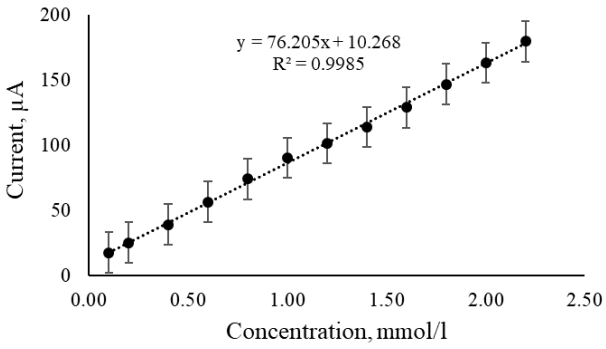

Fig. 9. Calibration curve for detection of melanin with the *p*(2,5-DMF) coated Pt electrode

2.9. *Determination of reproducibility*

The stability and reproducibility of the *p*(2,5-DMF) coated Pt electrode for detection of melanin was examined by measuring the SWV peak hight at 0.20 mmol/l concentration in 0.10 M PBS solution at pH 7.00 (Fig. 10).

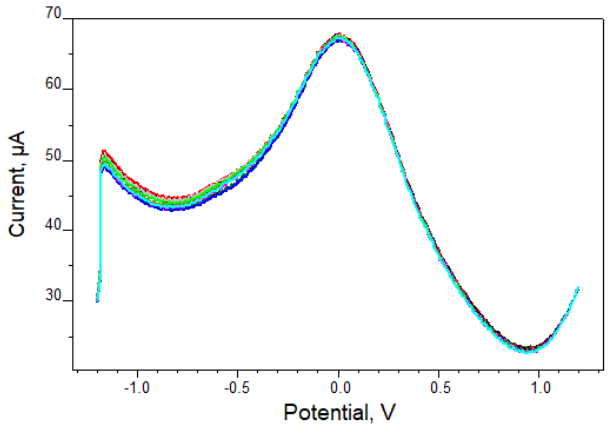

Fig. 10. Reproducibility of the SWV response of 0.20 mmol/l melanin on *p*(2.5-DMF)/PtE in pH 7.00 PBS buffer (n = 15). (Background correction is applied to voltammograms). (Potential step: 10 mV, pulse amplitude: 100 mV, frequency: 10 Hz)

The modified Pt electrode by *p*(2,5-DMF) was prepared daily and dried in air. The modified electrode showed a standard deviation of 0.13 and an RSD of 0.37 % in the peak currents of melanin after fifteen measurements (Fig. 11).

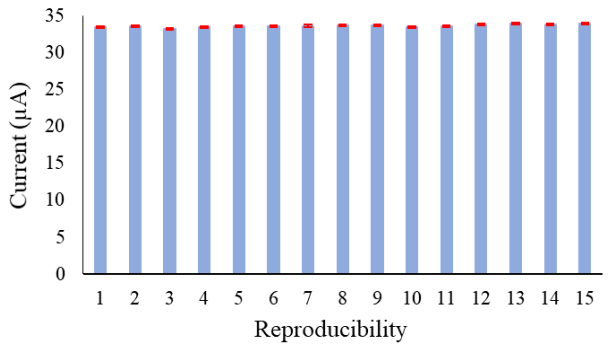

Fig. 11. Reproducibility of the *p*(2,5-DMF) coated Pt electrode

3. CONCLUSION

In this study, polymer film modified electrodes with different structures were prepared for the rapid, effective, and reproducible determination of melanin. In order to prepare polymer film modified electrodes with different chemical structures, five different monomers were electropolymerized separately on the electrode surface by the CV method. By comparing the melanin responses of the obtained modified electrodes, 2,5-DMF was chosen as the most suitable monomer type for electrode modification. Parameters such as monomer type, monomer concentration, scan rate, film thickness and pulse amplitude for the 2,5-DMF based modified electrodes were optimized. Then, modified electrodes were prepared by making electropolymerization with 4 cycled film thickness. The oxidation peak hight of melanin with prepared modified electrodes increased linearly with the increase of melanin concentration.

In this study, for the first time, a sensor was developed for voltammetric determination of melanin by using *p*(2,5-DMF) film. This melanin sensor was prepared by a simple CV method and has a wide 0.10–2.20 mmol/l linear range with a low as 0.088 mmol/l detection limit, short response time, and high stability (99.63 %). When the responses of the developed sensor are compared with those of unmodified electrodes, the sensor has been proven to show higher stability and reproducibility. As a result of the work done, a new voltammetric melanin sensor with low cost, fast response, high sensitivity, and reproducibility has been developed.

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