UDC 620.3; 53:002; 577.15; 543.6; 543.9; 543.55
DOI https://doi.org/10.32782/2450-8640.2021.1.4

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IMPROVEMENT OF LACCASE-BASED AMPEROMETRIC BIOSENSOR WITH TITANIUM DIOXIDE NANOPARTICLES

Abstract. The development of new approaches for monitoring of the dangerous substances in environment is a topical present problem to improve human life quality. In this point of view, electrochemical biosensors have received increased attention due to their high specificity, sensitivity, reliability, portability and simplicity in operation. Nowadays, there is a rapid growth in the use of semiconductor nanoparticles (NPs) in fabricating electrochemical sensors, stimulated with the unique properties of such nanomaterials as large surface area and good biocompatibility. The NPs-modified laccase based biosensors are very promising in quantifying phenolic compounds with good precision and accuracy.

Here, we described construction of biosensor for phenols analysis based on laccase combined with commercial TiO_2 nanoparticles incorporated in Nafion® polymer. The constructed bioelectrodes have demonstrated improving the operational parameters compare with the bioelectrodes without usage of TiO_2 that make them more promising for the phenols analysis in the real samples of wastewater and ground water.

Keywords: TiO_2, Nafion®, laccase, ABTS, amperometric biosensor.

INTRODUCTION

Technogenic pressure on the environment significantly affects the pollution of water resources, resulting that the one of the most important problems is the contamination of soil, water and air by toxic chemicals. Due to extremely rapid development of the industrial sphere and wide use of plastic, detergents, pharmaceuticals, pesticides in agriculture, the level of environmental pollution is growing up extremely fast that causes a serious threat to human life and health. Some compounds, such as polycyclic aromatic hydrocarbons, pentachlorophenols, polychlorinated biphenyles, benzene, toluene and phenol are accumulated in the environment. These pollutants join to natural water streams along with industrial effluents of chemical-related sector, such as coal refineries, pharmaceutical manufacturing, production of resins, paints, wood processing, textiles, petrochemicals, and pulp, including the manufacturing of phenols causes carcinogenic and mutagenic effects on living organisms [1; 2]. Therefore, reliable determination of phenols is important for control of wastewater purification in the environmental safety.

During the past two decades, bioelectrochemistry has received increased attention between the other bioanalytical techniques. A biosensor is an integrated biological component probe with an electronic transducer, converting a biochemical signal into a quantifiable electrical response that detects, transmits and records information regarding biochemical or physiological change. They are specific, sensitive, reliable, portable and simple in operation.

Laccase (EC: 1.10.3.2, benzenediol: oxygen oxidoreductase) is widely studied in biosensors in order to detect the various phenolic compounds and amides. This, a multicopper oxidase enzyme, catalyzes oxidation of the target substrates with electrons transfer to the trinuclear copper cluster, where molecular oxygen serves as a final acceptor and is reduced to water. The enzymatic active site is formed with a copper type I, type II, and type III. The type I copper center consists of a single copper atom that is ligated to two histidine residues and a single cysteine residue. The type III copper center consists of two copper atoms that each possess three histidine ligands and are linked to one another via a hydroxide bridging ligand. The final copper center is the type II copper center, which has two histidine ligands and a hydroxide ligand. The type II together with the type III copper center forms the tricopper ensemble, which is where dioxygen reduction takes place [3] (Fig. 1). Because of laccase does not require the addition exogenous cofactors or mediators in the electron transfer reactions and forms a safe byproduct of reaction (water), it is a very promising tool in modern biosensorics [2].

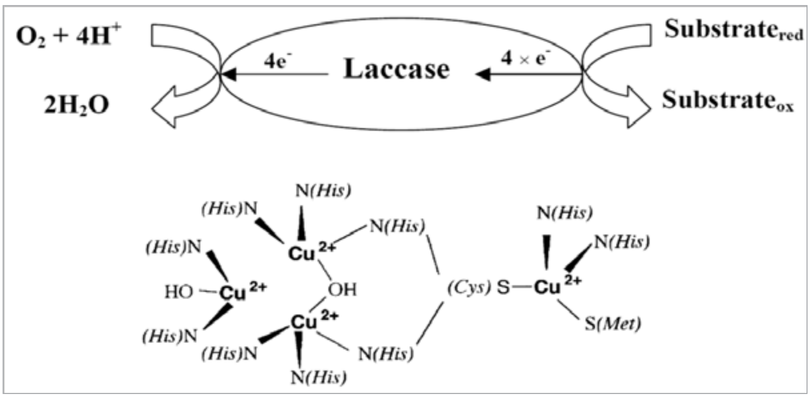

Fig. 1. Typical catalytic reaction of laccase according to Yashas et al. [2]

Nowadays, nanotechnology approaches have been successfully used for improvement of functional properties of the enzymatic sensors. The integration of micro- and nanotechnologies seems to be very promising in further development and production of such biosensors due to the unique combination of chemical inertness, surface chemistry, size- and shape-dependent electrochemical and optical properties. Recently, a number of innovative amperometric biosensors based on laccase and different types of NPs have been constructed and successfully tested for monitoring the level of wastewater pollution. It was shown a positive effect on the biosensor parameters of NPs based on carbon [4], noble metals [5; 6], iron oxide [7], and transition elements [8-10]. Among the transition metals and their compounds, much attention has been paid to titanium dioxide (TiO_2) an inexpensive amphoteric semiconductor material with a high surface reactivity, in particular, to the adsorption of biomolecules [11].

The optimization of the co-immobilization procedure for laccase and NPs to exhibit a maximum of the enzymatic activity in the sensor's microenvironment, simultaneously ensuring good diffusional properties of the formed bionanocomposite membrane, is a critical point in such biosensors' construction [12; 13]. In this point of view, the physical entrapment with highly permeable membranes looks to be very promising among the known immobilization techniques due to its simplicity and a minimal effect on the structural and conformational changes of the incorporated/held bio-nano molecules [14]. In the current work, we propose to use an effective non-covalent co-immobilization of TiO_2 and laccase from *Trametes versicolor* by means of Nafion® polymer, forming a high stable bio-nano-membrane on the carbon electrode surface.

MATERIALS AND METHODS

Materials. Laccase enzyme (E.C. 1.10.3.2) from *Trametes versicolor* with activity of ≥10 $U·mg^{-1}$, 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 99%, Nafion®, and other reagents and buffer compounds were purchased from Sigma-Aldrich (Darmstadt, Germany). AEROXIDE®TiO2 P25 was obtained from (Evonik industries AG, Essen, Germany). All chemicals and reagents were prepared using triple distilled water. Prior to analysis, the raw wastewater sample was filtered through a Millipore 0.45 µm filter.

Formation of bio-nanocomposite membrane of the biosensor. The formation of bio-nanocomposite membrane of the biosensor was performed as follows. 10 µL of enzyme solution (with a concentration of 1 $mg·mL^{-1}$ and a volumetric activity of 13.6 $U·mL^{-1}$, in 50 mm acetate buffer, pH 4.5) was mixed with 5 µL of a colloidal solution of commercial nanoparticles AEROXIDE®TiO2 P25 (1 $mg·mL^{-1}$) in 1 % aqueous solution of Nafion®. The formed mixture was dropped onto the 3.05 mm diameter carbon rod working electrode, with the next step of drying the resulting mixture (nanoparticles + enzyme + polymer) for 10 min occurring in air at room temperature. After drying, mechanically strong TiO_2-enzyme-Nafion biocomposite membrane was formed on the surface of the carbon rod electrode. The prepared bio-nanofunctionalized electrodes were rinsed with 50 mM acetate buffer, pH 4.5 and kept at 4°C till usage.

Construction of amperometric biosensor. The amperometric biosensors were constructed in a three-electrode configuration to be used for constant-potential amperometry. The biosensor configuration included a Pt counter electrode, Ag/AgCl/KCl(3M) reference electrode, and a graphite-rod (type RW001, 3.05 mm) from Ringsdorff Werke (Bonn, Germany) as a working electrode. Before usage, the working electrodes were polished with P2000 emery paper. The amperometric analysis was carried out using a potentiostat CHI 1200A (IJ Cambria Scientific, Burry Port, UK) in an electrochemical cell at room temperature, avoiding direct light.

The amperometric measurements were performed in a glass electrochemical cell with a working volume of 50 mL, filled with 20 mL acetate buffer, pH 4.5 at room temperature, avoiding direct light. For chronoamperometric analysis, the bio-nano-modified electrodes were placed in a vigorously stirred solution and, after setting the base signal at an operating potential of -150 mV *vs* Ag/AgCl, increasing aliquotes of laccase substrate (ABTS) were added to the measuring cell.

RESULTS AND DISCUSSION

The commercial titanium dioxide nanoparticles marked as AEROXIDE®TiO_2 P25 (TiO_2) were used in the work. These TiO_2 are characterized with average diameter 25.4 nm; specific surface area (BET) 35-65 m^2/g; composition: 90 wt% anatase and 10 wt% rutile. To form the bio-nanorecognizing layer of the biosensor on the surface of the carbon working electrodes, it is necessary to immobilize all components of the bio-recognizing film (TiO_2 and laccase) while maintaining their electrochemical and catalytic properties. For this purpose, Nafion® was used. Nafion is a synthetic polymer based on tetrafluoroethylene with ionic properties.

The commercial laccase (EC 1.10.3.2 *p*-diphenol:oxygen oxidoreductase from *Trametes versicolor*) a multicopper-containing enzyme, was used for the biosensor construction. In a typical laccase reaction, the phenolic substrate is subjected to one-electron oxidation to form an aryl radical, which in the next stage of the enzymatic reaction is converted to quinone. The operation of the laccase-based amperometric biosensor is based on the reduction of the oxidized products generated by the laccase-catalyzed reaction, which correspond to the following transformations: for ABTS → cation radicals $ABTS^{+\bullet}$ → $ABTS^{(2+\bullet)}$ (Fig. 2).

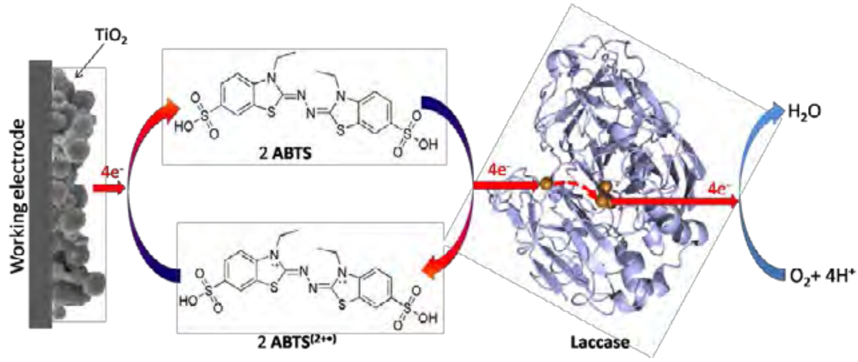

Fig. 2. Schematic representation of the electron transfer pathway in the biorecognition layer of an amperometric lassase sensor based on Nafion-TiO_2 bio-nanocomposite membrane using ABTS as a model substrate

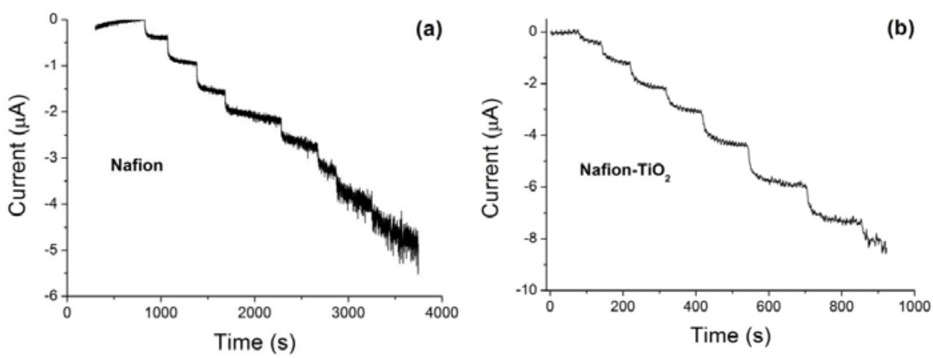

Fig. 3. The typical chronoamperometric current response for laccase-based carbon electrodes modified by Nafion only (a) and with Nafion-TiO_2 (b) upon subsequent additions of ABTS. *Conditions*: working potential -150 mV *vs* Ag/AgCl in 50 mM acetate buffer, pH 4.5 at room temperature at continuous stirring.

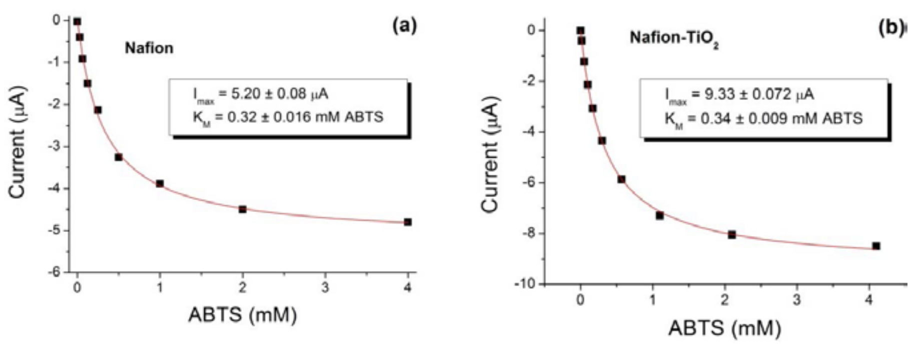

Fig. 4. The typical calibration curves for laccase-based carbon electrodes modified by Nafion only (a) and with Nafion-TiO_2 (b) upon subsequent additions of ABTS. *Conditions:* working potential -150 mV *vs* Ag/AgCl in 50 mM acetate buffer, pH 4.5 at room temperature at continuous stirring

As the operating potential for the biosensor was chosen -150 mV *vs* Ag/AgCl/KCl (3M) reference electrode as commonly used potential for numerous developed laccase biosensors [15; 16]. Such relatively low operating potential makes the biosensor more selective, avoiding the interfering response of electroactive chemicals, contained in real samples, which can be auto-oxidized or auto-reduced at extreme operating potential values.

The analogues electrodes based on laccase immobilized by means of Nafion avoiding the addition of TiO_2 were used as control (Fig. 3).

The results of Fig. 3 are indicated of a smaller noise and wider output scales of chrono-amperometric response for Nafion-TiO_2 formed bioelectrodes. The corresponding calibration curves for both types of laccase bioelectrodes presented in Fig. 4.

Evaluation of the dependence of the operating parameters of Nafion-TiO_2-modified laccase bioelectrodes relative to the control (without using TiO_2) was performed according to three main parameters: I_{max} – the maximal response of the biosensor at substrate saturation; K_M^{app} – the apparent Michaelis-Menten constant; and sensitivity. It was demonstrated that the Nafion-TiO_2 based bioelectrodes are characterized with increasing of I_{max} twice comparing with control electrodes (9.33 ± 0.07 *vs* 5.20 ± 0.08 μA). The value of K_M^{app} toward ABTS for both types of bioelectrodes was the same (0.34 ± 0.01 *vs* 0.32 ± 0.02 mM ABTS) (Fig. 4). The same affinity of different types of laccase bioelectrodes indicates any interfering effect of TiO_2 onto the laccase catalysis. Thus, demonstrating a high biocomparability of the TiO_2 with biomolecules. The sensitivity of both types of bioelectrodes was calculated taking into account that the geometrical surface area of the working electrode was equal to 7.3 mm^2 (Fig. 5).

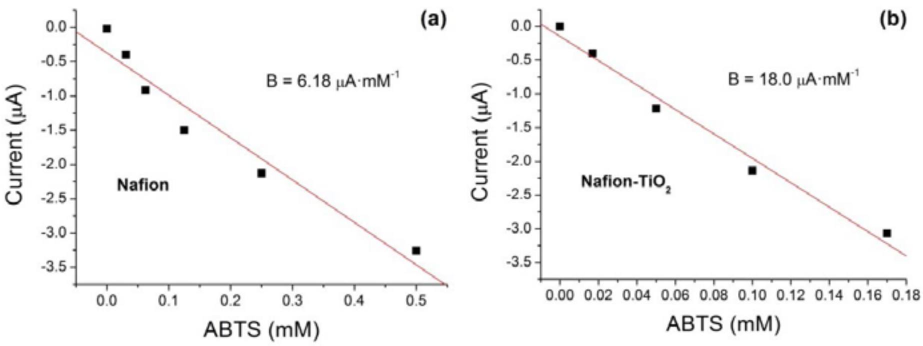

Fig. 5. Analysis of the sensitivity of the laccase-based carbon electrodes modified by Nafion (a) and with Nafion-TiO_2 (b) upon subsequent additions of ABTS. Abbreviation: B – slope of the curve.

Compared with the control bioelectrodes (without of TiO_2 nanoparticles), the Nafion-TiO_2-modified bioelectrode was characterized by increasing of sensitivity in three times (2466 *vs* 847 $A\cdot M^{-1}\cdot m^{-2}$) (Fig. 5). The increasing of the biosensor sensitivity makes it more promising for precision analysis of toxic phenols of the real samples where their content is very low (e.g., drinking water). But it should be mentioned that even very small concentration of phenols has a greatly negative impact for the human health, so the analysis of trace amount of these xenobiotics are very actual taking to attention of ongoing increasing the technogenic pressure on the environment.

CONCLUSIONS

In this paper, we have described a positive impact of commercial titanium dioxide NPs onto the operational parameters of laccase-based biosensor. It has been demonstrated that modification of the carbon rod electrode with TiO_2 and laccase incorporated into Nafion film, significantly improves two main sensor's characteristics: the maximal response of the biosensor at substrate saturation (twice) and sensitivity (three times) compared with the control bioelectrodes (without usage of TiO_2). The increasing of the biosensor sensitivity makes it more promising for precision analysis of toxic phenols of the real samples where their content is very low (e.g., drinking water).

However, despite the fact that the ABTS analysis as a model substrate with the developed biosensor was successful, the real motivation of the study was in establishing the new biosensor platform using TiO_2 (amphoteric semiconductor) and Nafion polymer for efficient immobilization of enzyme and NPs. Based on the obtained results, the next step in the project will include TiO_2 doping with different amount of sulfur, construction of bioelectrodes and their testing with different laccase substrates (ABTS, catechol, phenol) and real wastewater samples. We believe, that presence of sulfur in the TiO_2 should effects on copper clusters and cysteine residues (*see* Fig. 1), affecting on the active center of laccase, resulting in an increase in the affinity of the formed bio-nanocomposite to target analytes (phenols). This more sophisticated biosensor system is presently in the study and the results will be published elsewhere soon.

ACKNOWLEDGEMENTS

This work was supported in part by the Ministry of Education and Science of Ukraine (projects Nos. 0121U109539 and 0121U109543), National Academy of Sciences of Ukraine in the framework of the Scientific-Technical Program "Smart sensor devices of a new generation based on modern materials and technologies" (projects Nos. 13 and 10/3), and National Research Foundation of Ukraine (projects Nos. 2020.02/0100 "Development of new nanozymes as catalytic elements for enzymatic kits and chemo/biosensors" and 2021.01/0010 "Development of an enzymatic kit and portable biosensors for express-analysis of creatinine, a marker of acute functional disorders of the kidneys"). T.K. also acknowledges the SAIA (Slovak Academic Information Agency) in the framework of the National Scholarship Programme of the Slovak Republic.

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АНОТАЦІЯ

УДОСКОНАЛЕННЯ АМПЕРОМЕТРИЧНОГО БІОСЕНСОРА НА ОСНОВІ ЛАККАЗИ З НАНОЧАСТИНКАМИ ДІОКСИДУ ТИТАНУ

Розробка нових підходів до моніторингу небезпечних речовин у навколишньому середовищі є актуальною проблемою сучасності для покращення якості життя людей. З цієї точки зору електрохімічним біосенсорам приділяється підвищена увага через їхню високу специфічність, чутливість, надійність, портативність і простоту в експлуатації. У наш час спостерігається швидке зростання використання напівпровідникових наночастинок (НЧ) у виготовленні електрохімічних сенсорів, стимульованих унікальними властивостями таких наноматеріалів, як велика площа поверхні та хороша біосумісність. Біосенсори на основі лаккази, модифіковані НЧ, є дуже перспективними для кількісного визначення фенольних сполук з хорошою надійністю та точністю. Тут ми описали конструкцію біосенсора для аналізу фенолів на основі лаккази в поєднанні з комерційними наночастинками TiO_2, включеними у полімер Nafion®.

Показано, що модифікація графітового стрижневого електроду TiO_2 і лакказою, вбудованими у плівку Nafion, значно покращує дві основні характеристики сенсора: максимальну реакцію біосенсора при насичення субстрату (вдвічі) і чутливість (у три рази) порівняно з контрольними біоелектродами (без використання TiO_2). Підвищення чутливості біосенсора робить його більш перспективним для точного аналізу токсичних фенолів реальних зразків, де їх вміст дуже низький (наприклад, питної води). Однак, незважаючи на те, що аналіз ABTS як модельного субстрату з розробленим біосенсором був успішним, справжньою мотивацією дослідження було створення нової біосенсорної платформи з використанням TiO_2 (амфотерного напівпровідника) та полімеру Nafion для ефективної іммобілізації ферменту. Виходячи з отриманих результатів, наступним кроком буде легування TiO_2 різним вмістом сульфуру, конструкція біоелектродів та їх випробування різними субстратами лаккази (ABTS, катехол, фенол) та реальними зразками стічних вод. Ми вважаємо, що наявність сульфуру в TiO_2 повинно впливати на кластери міді та залишки цистеїну, впливаючи на активний центр лаккази, що призводитиме до збільшення спорідненості утвореного біонанокомпозиту до цільових аналітів (феноли). Ця більш складна біосенсорна система зараз знаходиться у дослідженні, і результати будуть опубліковані в іншому місці незабаром.

Сконструйовані біоелектроди продемонстрували покращення експлуатаційних параметрів у порівнянні з біоелектродами без використання TiO_2, що робить їх більш перспективними для аналізу фенолів у реальних зразках стічних та підземних вод.

Ключові слова: TiO_2, Nafion®, лакказа, ABTS, амперометричий біосенсор.