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Optical Biosensor for Permanent Monitoring of Phenol Derivatives in Water Solutions

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Phenolics are part of a large group of pollutants, widely expanded throughout the medical, food and environmental matrices. They are main contaminants in ground and surface water. In view of their toxicity and persistence in the environment, the identification of phenolic compounds becomes an important matter. Enzymatic biosensors represent potential options to detect these species especially in case of *in situ* techniques. Particularly, biosensors based on polyphenol oxidase as well as tyrosinase or laccase have been developed for the determination of phenol derivatives. Due to the fact, here is reported a ceramic-based biosensing system fabricated through the immobilization of laccase in an electrochemically synthesized polymer - poly[2,7-bis(carbazole)-*N*-hexylacridone], based on low temperature co-fired ceramics technology (LTCC). This setup can be adequate for the permanent monitoring of aqueous solutions (i.e. presence of phenolics). The enzyme was immobilized on the surface of polymer film covering the microreaction chamber. The work of the biosensing system was estimated in the presence of ABTS (ammonium salt of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)), used as an enzymatic assay substrate. The setup revealed clear catalytic activity, and the analyte was optically determined. Due to the fact, the designed catalytic system showed sufficient efficiency, the obtained results suggest that, the phenoloxidase immobilization possesses potential to fabricate the bioelectronic devices.

1. Introduction

Phenols or phenolics are a wide group of species of natural and anthropogenic source (Stoytcheva et al., 2014). The rough release of wastes generated during dyes, chemicals, textiles, resins, and plastics fabrication, as well as during the processes of wood preservation, and petroleum processing generates phenolic species pollution (Michalowicz et al., 2007). When phenolics are persistent in the environment and highly toxic, they are included in the EPA list of priority pollutants (Code to Federal Regulations, CFR). When consumed, phenol-laced water may cause lung, liver, kidney, and genitourinary system damage in both humans and wildlife (Gupta et al., 2008).
Phenols and its derivatives have been detected by chromatographic, spectroscopic, and other methods (Meulenberg, 2009). Nevertheless, some of these techniques are expensive, with poor sensitivity and usually require time-consuming sample preparation. Moreover, these analyses may not be suitable for *in situ* monitoring.
Enzyme-based sensing systems represent potential alternatives to these techniques. In particular, biosensors based on laccase as well as tyrosinase (Cabaj et al., 2010) have been developed for the detection of phenolic compounds (Tuncagil et al., 2010). The biosensors based on laccase are of great meaning because the enzyme is more sensitive to chlorinated organic species, what is valid in respect to the environment. The substrate specificity of laccases is wide, and includes phenols, polyphenols, methoxy-substituted phenols, aromatic amines, anilines and organic and inorganic metal compounds. Moreover, the list of compounds that can be oxidized by laccase can be increased using redox mediators, i.e. ABTS (Munteanu et al., 1998).

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Next important point connected with engineering of enzyme-based sensor is use of conducting polymers for the fabrication of various biosensors, what has recently been studied extensively in order to their redox, optical, mechanical and electrical character. Conducting polymers are considered as one of the most convenient materials for biosensors (Ucan et al., 2014)**.** Sensor efficiency depends mainly on the surface character, interaction among the enzyme and electrode surface and protection of three dimensional structure of protein. Therefore, conducting structures have occurred as elements of the most eligible transducers (in order to their simple fabrication). Biosensors based on conducting polymer give simple, accurate, reliable and reasonable-cost determination of different analytes, and operate as an effective tool in the monitoring of food processes as well as in medicinal and environmental diagnostics.

By continuing the investigations in the field of protein sensitive systems, a novel optical sensor for phenolic compounds detection has been reported. The sensor is engineered with LTCC (low temperature co-fired ceramics) technology. This is well-established technique, which has been utilized in electronics, telecommunication and the automotive industry for the last 35 years (Jedrychowska et.al, 2015). The LTCC material is high-temperature and pressure resistant. A wide group of solvents, bases and moderately concentrated acids are inert to the fired LTCC material (Thelemann et al., 2007). Moreover, different 3D structures (i.e. channels, cavities) can be created inside the LTCC substrate using laser, hot embossing or mechanical milling (Barlow et al., 2009). The LTCC technology is cost-effective, easy, and the possibility for the integration of microfluidic species and electronic elements in a single LTCC substrate is the great profit of LTCC (Vasudev et al., 2013).

The reported LTCC-based biosensor for the monitoring of phenolics in water solutions consists of a microfluidic chip and chip holder with integrated optoelectronic parts. The idea of the biosensor operation is based on the measurement of optical absorbance. The concentration of the analyte is determined according to the well-known Lambert-Beer's law.

Reported here ceramic biosensor was built with novel semiconducting polymer - poly[2,7-bis(carbazole)-*N*-hexylacridone]. Carbazole derivatives represent a class of compounds which are characterized by facility of preparation, stability under ambient atmosphere and relatively high conductivity (Świst et al., 2014). The direct charge transfer between redox enzyme and electrode is usually forbidden due to the donor– acceptor distance as a main element controlled the electron transfer rates. The most redox enzymes have the redox centers deeply settled and consequently electrically insulated. In this situation, redox enzymes that generate a donor–acceptor pair with an electrode support lack electrical contact with the solid support. In these setups the biofunctions of the enzymes are electrochemically induced by electron transport (Jędrychowska et al., 2014). The electrical contact may be improved by the application of i.e. conductive polymers. Modification of platinum electrodes with the semiconducting polymer before deposition of the biocatalyst generates a strong immobilization of protein to support, much stronger than the adsorption of the biocatalyst on the bare solids (Jędrychowska et al., 2014).

Conducting polymers such as poly[2,7-bis(carbazole)-*N*-hexylacridone] have an electronic structure, which allows the transfer of the electric charge generated as a result of protein interactions with the substrate/analyte. π electrons, within the heterocyclic units are mobile charges that may be transferred along the backbone in polymers (Gerard et al., 2002). This type of electron mediators facilitate the transport of the electric charge to the transmitter and extend the length of time of catalytic activity of immobilized enzymes. It enhances the generated sensor signal, and makes shorter the tool response time.

2. Materials and methods

2.1. Reagents and materials

The convenient immobilization of the enzyme on solid substrates is vital for the investigation of the biosensing tool. The structure of the layer utilized for laccase immobilization should mainly preserve enzyme functionality. In this report, a polymer built of 2,7-bis(carbazole)-*N*-hexylacridone (Figure 1) was electrosynthesized in the presence of 0.1M TBA-TFB. Monomer – derivative of acridone was synthesized according to previous experience (Świst et al., 2014). The electropolymer occurred in the conducting oxidized state. The total charge of the polymer was neutral in order to the doping anions, which were included into the polymer matrix during the electropolymerization.

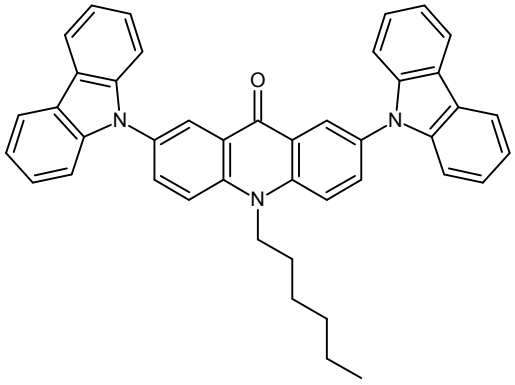

Figure 1: Structure of monomer - 2,7-bis(carbazole)-N-hexylacridone.

Due to generate the conducting polymer film on the Pt electrode, the cyclic voltammetry technique was utilized. The optimal potential range for polymer deposition was found to be 0–1600 mV, and the polymerization lasted for 10 cycles. The polymer synthesized under the conditions were of a constant approximate thickness of 500 nm. The thickness of the polymer was estimated by means of the tapping mode AFM. Figure 2 presents the results of the electrochemical polymerization of 2,7-bis(carbazole)-*N*-hexylacridone. The graph demonstrates the creation of the electroactive polymer on the Pt electrode. The plot displays the growth of peak currents during subsequent scans, which suggests the formation of the electroactive polymer.

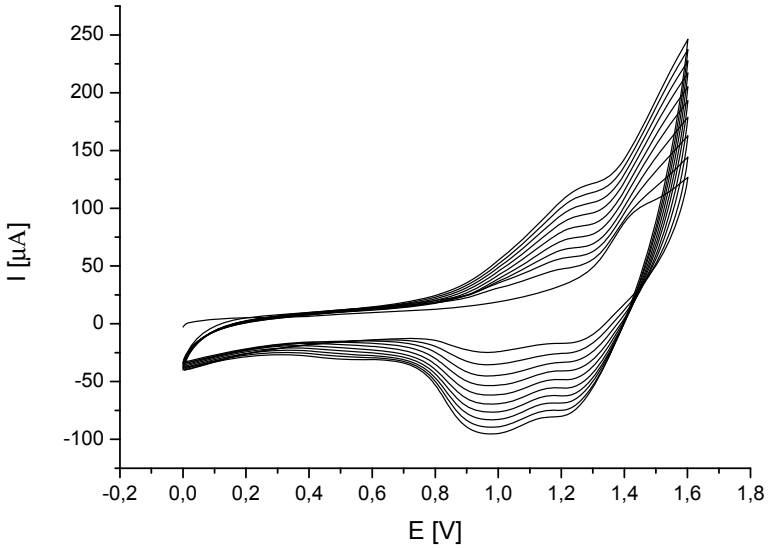

Figure 2: Cyclic voltammograms of investigated monomer; (2,7-bis(carbazole)-N-hexylacridone - 1mM) in 0.1 M TBA-TFB. Measurement conditions: scan rate 50 mV s^{-1}, Ag/AgCl – reference electrode, 10 cycles.

The immobilization of laccase was processed in a one-step procedure. It was carried out for 120 minutes, under standard physical adsorption conditions, in temperature 298 K. The optimal conditions were estimated by the consideration of a high signal-noise ratio, and the stability of the biosensor response.

The practicability of the voltammetric determination of the interaction of laccase and the phenolic analyte (catechol, Figure 3) implies the following estimations. The well-defined and stable redox peaks for Pt/polymer/laccase arrangement were recorded at potentials -100-700mV, which are possibly imputed to the direct electron transfer between the enzymatic active center and the electrode surface. As it is presented in

Figure 3, the redox process is reversible. The well-seen anodic and cathodic peak potentials are located at 420 and 200 mV, respectively (vs. Ag/AgCl). The formal potential (E^0) for laccase was calculated from the average value of the anodic and the cathodic peak potentials, and the value obtained was 310 mV. This value is quite close to the E^0 values for T1 copper of laccase (originating from *Rhus vernicifera* and *Melanocarpus albomyces*) (Brodani et al., 2013).

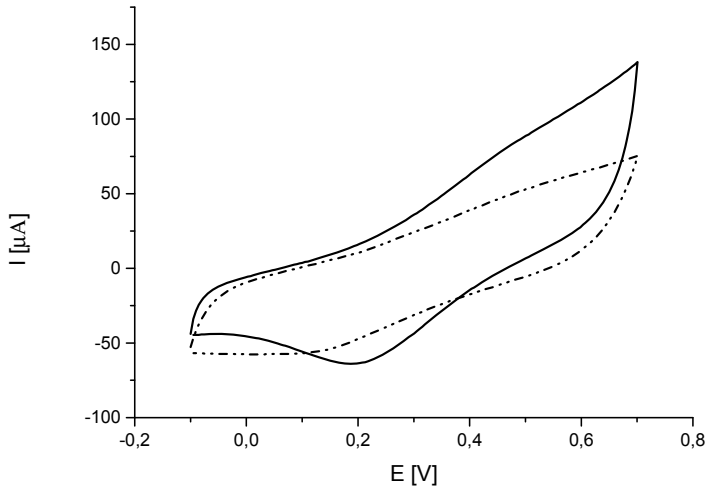

Figure 3: Electrode responses recorded in 0.2 mM catechol, pH 5.2; solid line – Pt electrode modified with polymer and laccase, dotted line – Pt electrode modified with polymer. Measurement conditions: scan rate 100 mV s^{-1}, Ag/AgCl – reference electrode.

2.2. Sensor construction

The system consisted of the microfluidic chip with integrated optical fibers, and the chip holder with integrated optoelectronic and passive components, as well as thick-film conductive lines. Both parts of the setup were fabricated using LTCC (low temperature co-fired ceramics) technology similar as reported earlier (Jędrychowska et al., 2015).

2.3. Principle of detection

The catalytic activity of the immobilized laccase and the accuracy of the microfluidic chip were verified by optical surveys. The researches were performed with the classic laccase assay substrate – ABTS.
The enzyme-mediated reactions were carried out in ABTS solutions at pH 5.2. In order to evaluate the linear range of the designed setup, its response for various concentrations of ABTS was measured. The linear range is the range of analyte concentrations, in which the sensor response changes linearly with the concentration. The developed LTCC-based laccase sensor has a linear response to ABTS in the range 60 µM – 180 µM, which is similar to results reported by Roy et al., 2005.

3. Results and discussion

3.1. Sensor parameters - characterization

The presented setup was studied to determine the effect of its procedure condition flow rate on its sensitivity. In order to this sensor responses for different concentrations of the analyte, and for various values of the flow rate (25 µl/min, 50 µl/min and 100 µl/min) were measured. In systematic 1 minute intervals, the concentration of ABTS was altered with the buffer from 60 µM to 180 µM. The LTCC-based sensing system output signals for three various flow rates are presented in Figure 4. When the sensitivity of the system can be estimated by the slope of the calibration curve, the scores were estimated based on the variations of the absorbance according to the analyte concentration. According to obtained results, the sensitivity of the biosensor grows with the reducing values of the flow rate. The coefficient of the determination for the linear relationship of absorbance versus substrate concentration was equal to 0.96, 0.94 and 0.93, for flow rates equal to 25 µl/min, 50 µl/min and 100 µl/min, respectively. Then, the most precise outcomes were achieved for the lowest flow rate (25 µl/min).

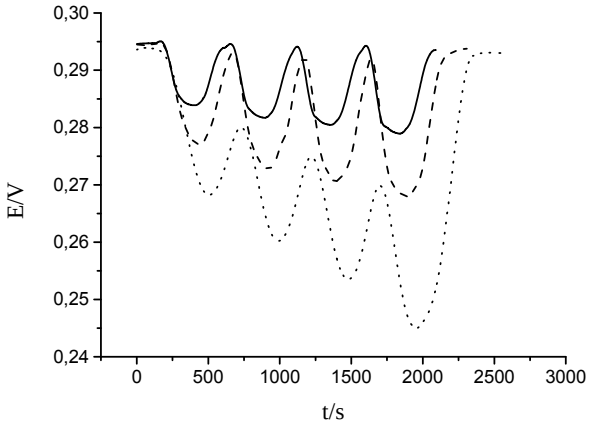

Figure 4: The output signal of the produced biosensor for different flow rates: 150 µl/min – solid line, 75 µl/min – dashed line and 25 µl/min – dotted line

The detection limit, at a signal-to-noise ratio of 3, was found as 6.25 µM, 8.0 µM, and 10.0 µM for flow rates: 25 µl/min, 50 µl/min and 100 µl/min, respectively. The detection limit recorded for slower motions of the analyte (less than 120 µl/min) are lower than the maximum concentration of i.e. phenols allowed in waste water by the European Union (10.6 µM, European Community, 1991), which means that the presented system may be used in detection of the pollutants in urban water.

Selectivity is a next valid feature of biosensor. By recording the studied values for ABTS before and after adding an interferent, respectively. It was found, that the input of the substrate toward the sensor response for ABTS was ≤3.5%, indicating negligible interference. Due to the fact, the reported biosensor revealed sufficient selectivity for ABTS.

It was found that the response time reduces, when the flow rate of the substrate is higher. The relationship between these two variables is logarithmic. The fastest analysis ($t_{0.9}$ = 2 min) was obtained for flow rates equal to 100 µl/min, which is a sufficient effect paralleled to the other reported optical laccase biosensors (i.e. 10 min for laccase in a chitosan film (Abdullah et al., 2007)).

When the most vital points for an enzyme immobilization procedure are both to avoid leaking of the enzyme to the solution and to maintain its catalytic effect, the stability of the presented setup was estimated by recording the consecutive surveys in the same conditions. The repeatability of the outcomes obtained by the sensor was developed by performing optical measurements at 180 µM of ABTS, at a flow rate of 50 µl/min, and LED current of 15 mA (Figure 5). The setup retained its first current response; the distinction between each absorbance was less than 1%. The stability of the output signal from the sensor prepared by physical adsorption of the enzyme onto the electrode surface was sufficient. These surveys signify that laccase was immobilized in a biocompatible surroundings.

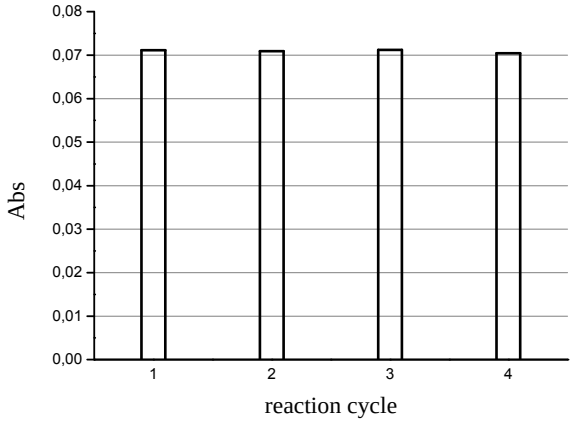

Figure 4: Absorbance recorded in consecutive measuring cycles, measurement conditions: flow rate – 50 µl/min, LED current: 15 mA, ABTS concentration: 180 µM

4. Conclusions

We reported here procedure for the fabrication of the flow-through optical phenolics biosensor based on laccase. The poly[*N*-hexyl-2,7-bis(carbazole)acridone] layer revealed to be a proper matrix for the immobilization of enzyme - laccase. The biocatalyst was highly attached to the solid and maintained its high catalytic effect. Optical study (by fabricated biosensor) signified that the engineered setup acted with high reliability. The main benefits of the reported sensor are: sensitivity, precision, linearity and simplicity of construction. This type of setup made continuous analysis possible. The outcomes of the studies may contribute, in the future, in the design of a new class of bioelectronic tools.

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