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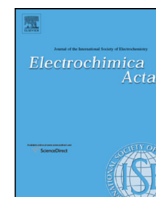

The use of different glucose oxidases for the development of an amperometric reagentless glucose biosensor based on gold nanoparticles covered by polypyrrole

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The amperometric glucose biosensors based on adsorbed electron transfer mediator (ETM) tetrathiafulvalene (TTF) or 1,10-phenanthroline-5,6-dione (PD) and glucose oxidase (GOx) from *Aspergillus niger* ($GOx_{A.niger}$), *Penicillium adametzii* ($GOx_{P.adametzii}$) or *Penicillium funiculosum* ($GOx_{P.funiculosum}$) cross-linked with glutaraldehyde were investigated. ETM and enzyme were immobilized layer by layer on bare graphite rod electrode (GR) premodified with gold nanoparticles (AuNP) of (i) 3.5 nm ($GOx/ETM/AuNP_{3.5}/GR$), (ii) 6.0 nm ($GOx/ETM/AuNP_{6.0}/GR$) and (iii) 13.0 nm ($GOx/ETM/AuNP_{13.0}/GR$) size. The amperometric signals for all the developed biosensors were higher using PD in comparison with TTF. The biosensor based on $GOx_{P.funiculosum}$ showed higher analytical signal to glucose in a comparison to biosensors based on $GOx_{A.niger}$ and $GOx_{P.adametzii}$. The registered current to glucose using $GOx_{P.funiculosum}/PD/AuNP_{3.5}/GR$ electrode was linear in the glucose range from 0.1 to 10.0 mmol L^{-1} and the limit of detection was 0.024 mmol L^{-1}. Enzymatical synthesis of polypyrrole (Ppy) layer on the electrode was applied in order to expand the linear glucose detection range. After 22 h of polymerization the amperometric signal was linear in the glucose concentration range from 0.1 to 25.0 mmol L^{-1}, while after 69 h this rage was increased up to 50.0 mmol L^{-1}. Additionally Ppy layer on the electrode surface reduced the influence of interfering species on the amperometric signal. The performance of developed biosensor was investigated in human serum samples.

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1. INTRODUCTION

Glucose biosensor-related research has tremendous interest from the introduction of the first glucose biosensor based on glucose oxidase (GOx) in 1962 [1]. The electrochemical GOx-based biosensors are still among the most widely used, although many improvements have been added since the 1960's. Currently, these biosensors are applied in different areas such as food and pharmaceutical industry and particularly in clinical diagnostics because diabetes is a worldwide public health problem [2–5]. A glucose biosensor with a commercial success is likely to be small, not expensive and portable to meet the interest of millions of diabetic patients which daily need to perform glucose test in a simple way and everywhere. The biosensor should display high storage and operational stability, enjoy a simple and stable calibration. Real challenge is to minimize blood volume and design an alternative system to avoid painful sampling [6].

Over the last ten years glucose biosensors based on nanomaterials with enhanced performance were widely designed. The application of nanomaterials in biosensor design allows developing a biosensor with a commercial success, such as sensitivity, stability, miniaturisation, continuous and *in situ* monitoring in a complex matrix [2]. High biocompatibility, nanometric scale, long shelf-life, perfect optical properties and simple preparation make gold nanoparticles (AuNP) one of the most attractive and widely studied nanomaterial [7–9]. AuNP provide many advantages and

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new possibilities for enzymes immobilization on the surface of electrode [10]. Various size AuNP have been used in glucose biosensors design, but small nanoparticles appeared the most suitable for enzyme immobilization, because they could bind with enzymes without unfolding of their structure [11,12].

A lot of methods are used for the deposition of AuNP on the surface of electrode, but non-electrochemical methods are the most attractive and usable [13–15]. Chemically immobilized AuNP thin film is stable [10] and it improves conductivity, analytical sensitivity and selectivity of the electrode, as well as facilitates the electron transfer [16–19]. Novel amperometric glucose biosensor based on the immobilization of GOx and 12.0 nm AuNP on a glassy carbon electrode by a Nafion film was developed by Zhao et al. [20]. Hoshi et al. [21] proposed glucose sensors based on multilayers consisting of layer by layer deposited GOx and AuNP (5.0, 10.0, or 50.0 nm) on sensor substrates, such as a platinum electrode and a quartz glass plate. Another group of scientists developed a novel way to fabricate glucose biosensor by covalent attachment of GOx to a 2.6 nm size AuNP monolayer modified gold electrode [22]. Liu and Ju described the direct electrochemistry of GOx adsorbed on a 24.0 nm AuNP modified carbon paste electrode [23]. A group of scientists proposed a novel method to fabricate glucose biosensor by immobilization of GOx on 11.0 nm AuNP, which had self-assembled on gold electrode modified with thiol-containing three-dimensional network of silica gel [24]. A feasible approach to constructing multilayer films of GOx and 12.0 nm AuNP on gold electrode surfaces using cysteamine as a covalent cross-linker was described by Yang et al. [25]. Due to large specific surface area and high surface free energy AuNP can adsorb biomolecules strongly. The AuNP are biocompatible and can retain biological activity of adsorbed biomolecules. That plays an important role in the immobilization of biomolecules and construction of biosensors [12].

Many advantages and new possibilities are provided by π–π conjugated polymers. In designing biosensors they can be used as a matrix for physical adsorption or covalent enzyme immobilization. Due to excellent conductivity and electroactivity, these polymers can act as mediators and facilitate the electron transfer to the electrode. One of the most widely used π–π conjugated polymer is polypyrrole (Ppy). Ppy is chemically stable on various substrate materials and can be synthesized by different electrochemical, chemical oxidative and enzymatic polymerization techniques [26–28]. Ppy is often used as a matrix for the incorporation of metal particles and for the immobilization of enzymes [29,30].

The aim of this research was to evaluate the efficiency and the applicability of GOx from *Aspergillus niger* ($GOx_{A.niger}$), *Penicillium adametzii* ($GOx_{P.adametzii}$) and *Penicillium funiculosum* ($GOx_{P.funiculosum}$), and AuNP of different size for amperometric glucose biosensors design. Tetrathiafulvalene (TTF) and 1,10-phenanthroline-5,6-dione (PD) immobilized on the electrode were used as electron transfer mediators (ETM). The influence of enzymatically synthesized Ppy layer on the linear glucose detection range was tested. The performance of the developed biosensor and influence of interfering species on the amperometric signal were investigates in human serum samples.

2. Experimental

2.1. Chemicals

$GOx_{A.niger}$, type VII was purchased from Sigma–Aldrich (Buchs, Switzerland). TTF and PD were purchased from Sigma–Aldrich (Buchs, Switzerland). D-(+)-glucose, tetrachloroauric acid ($HAuCl_4 \times 3H_2O$) and tannic acid were obtained from Carl Roth GmbH&Co (Karlsruhe, Germany), $NaH_2PO_4 \times 2H_2O$ and $Na_2PO_4 \times 12H_2O$ – from Reachim (Saint Petersburg, Russia). CH_3COONa was purchased from Penta (Praha, Czech Republic), HCl – from Acta Medica (Hradec Kralove, Czech Republic), KCl – from Lachema (Neratovice, Czech Republic), acetonitrile – from Carl Roth GmbH&Co (Karlsruhe, Germany), pyrrole (Py) – from Sigma–Aldrich (Steinheim, Germany), glutaraldehyde – from Fluka Chemie GmbH (Buchs, Switzerland). All other chemicals used in the present study were either analytical pure or of highest quality.

$GOx_{P.adametzii}$ and $GOx_{P.funiculosum}$ were received from Institute of Microbiology, National Academy of Science (Minsk, Belarus). Enzyme preparations from active strain-producer of extracellular $GOx_{P.adametzii}$ LF F-2044.1 [31] and GOx $_{P.funiculosum}$ 46.1 [32] were produced by sequential ultrafiltration [33,34]. The method for the determination of GOx activity is based on enzymatic conversion of 1.4-benzoquinone into hydroquinone and the measurement of hydroquinone formation rate at 290 nm [35–37]. The unit of GOx activity (U) was defined as the amount of enzyme sufficient to catalyze transformation of 1 mmol L^{-1} 1.4-benzoquinone into hydroquinone during 1 min at 25 °C. Enzyme activity is expressed in U mL^{-1}. Glucose oxidases derived from *Penicillium* fungi are distinguished by lower specific activities, but their glucose binding efficiencies are higher [33,34].

Pyrrole was purified by passing 1.5 mL aliquots through a neutral Al_2O_3 column (5.0 cm length and 0.4 cm diameter) to remove all coloured components. Before investigations glucose solution was allowed to stay overnight to reach equilibrium of the α and β optical isomers. 0.05 mol L^{-1} sodium acetate-phosphate buffer solution (A-PBS) pH 6.0 with 0.1 mol L^{-1} KCl and other solutions were prepared in deionised water purified with water purification system Millipore S.A. (Molsheim, France). Graphite rods (3.0 mm diameter, 150 mm length, 99.999% purity, low density) were purchased from Sigma–Aldrich (St. Louis, USA).

2.2. Synthesis of AuNPs

AuNP of different size (3.5, 6.0 and 13.0 nm) were synthesized reducing $HAuCl_4 \times 3H_2O$ by sodium citrate in the presence of tannic acid. An aqueous solution of tetrachloroauric acid (81 mL of 0.01% [w/v] $HAuCl_4 \times 3H_2O$) was brought to boiling in an Erlenmeyer flask on a magnetic stirrer with electric heating. Sodium citrate solution (4 mL of 1% [w/v]) and tannic acid in deionised water (5.0, 0.5 and 0.05 mL of 1% [w/v] solution for 3.5, 6.0 and 13.0 nm AuNP, respectively) were added to the flask and heated up to 60 °C stirring rapidly. After preheating solutions were mixed, heated up to 98 °C and kept at this temperature for 3 min to yield solution of AuNP. The reaction duration was 10 min and the mixing speed 1000 rpm. Then 5 mL of 25 mmol L^{-1} Na_2CO_3 was added for the neutralization of the solution containing 3.5 nm size AuNP. The size of AuNP was measured by atomic force microscope (AFM). The average size of AuNP distributed on the surface of 8 Å SiO_2 substrate received from AIXTRON AG (Aachen, Germany) was evaluated from the height-distribution histograms of AFM images. Formed AuNP are nearly monodispersed since distribution in diameter of 13.0, 6.0 and 3.5 nm AuNPs is narrow, within the range of 12–16, 5–7 and 2–5 nm, respectively [29]. The initial concentration of gold in all used solutions was the same – 0.058 mg mL^{-1}. Solutions of AuNP were stored in dark glass flasks at +4 °C [38].

2.3. Pre-treatment of the working electrodes

Graphite rod was cut and polished on fine emery paper. After this the surface of electrodes was rinsed with distilled water, dried at 20 ± 2 °C and sealed into silicone tube in order to prevent contact of the electrode side surface with the solution. The working surface area of GR electrodes was 0.071 cm^2.

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