



Enzymatic polymerization of polythiophene by immobilized glucose oxidase



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ABSTRACT

In this study ‘green’, environmentally friendly enzymatic reaction-based synthesis of conducting polymer polythiophene (PTP) is proposed. Glucose oxidase (GO_x) was shown as an effective catalyst, which, in the presence of glucose, produces hydrogen peroxide suitable for the oxidative polymerization of PTP under ambient conditions at neutral pH. Enzymatically induced formation of the PTP layer over GO_x -modified graphite rod electrode (GRE) was demonstrated and evaluated amperometrically and by attenuated total reflectance – Fourier transform infrared (ATR-FTIR) spectroscopy. Surface morphology of GO_x - and PTP-modified GR electrodes was characterized by atomic force microscopy. It was clearly shown that the apparent kinetic Michaelis constant ($K_{M(app.)}$) of GO_x /PTP-modified GRE increased by increasing the duration of polymerization reaction. Therefore, enzymatic polymerization could be applied in adjustment and/or tuning of $K_{M(app.)}$ and other kinetic parameters of GO_x -based electrodes used in biosensor design.

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1. Introduction

In recent decades there has been a tremendous interest in the use of conducting polymers (CPs) in various applications because of attractive electrical, electrochemical, and optical properties as well as their good stability [1]. The CPs can be characterized by the physicochemical properties of both organic polymers (different permeability of various substances, viscoelasticity) and semi-conductors (conductivity) [2]. The conductivity of polymers consisting of heterocyclic monomers connected to the conjugated system after the attachment of different redox groups to the monomer backbone could increase from semi-conductor to almost metal level [3]. Moreover, the CPs can be used as an immobilization matrix for biologically active molecules in biosensor and biofuel cell constructions [4,5]. The proper immobilization of biomolecules, e.g., enzymes, within CP layer provides many advantages such as

enhanced biocatalytic stability, easy protection against the negative environmental impact, e.g., contamination by microbes and undesirable electrochemical interactions, and allows the application of modified electrodes in a wider range of experimental conditions (pH and temperature) [6]. The usage of different kinds of monomers and special conditions during CP polymerization enables to form polymer layers with different permeability and electrochemical properties, which lead to slowing or, on the contrary, enhancing electron transfer. This routine can be used to modulate bioelectrokinetic parameters, e.g., Michaelis constant and maximal rate of biochemical reactions in a certain range [6,7]. These kinetic parameters are very important in biosensor design. For such purpose polypyrrole, polyaniline, and polythiophene are most commonly used [8]. Monomers, constituting these CPs, are usually conjugated, small and flat heterocyclic compounds, which during the polymerization process form stable conducting conjugated systems. Regarding these CPs, polythiophene (PTP) compounds have been often considered as a model for the study of charge transport in CPs [9]. Moreover, the environmental stability of both doped and undoped states of PTP, together with its structural versatility have led to multiple developments in various fields

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ranging from bioelectronics to biotechnology, where PTP could be applied as a matrix for cell cultivation, etc [10]. PTP is composed of thiophene molecules, which are 5-membered heterocyclic compounds with sulphur as a heteroatom. Investigation related to the preparation of PTP compounds and the characterization of their electrochemical properties has intensified only during the last few decades [11,12].

PTP can be synthesized by means of photoinduced [13], chemical [14] or electrochemical [15] synthesis. In electrochemical polymerization, a potential is applied across a certain electrolyte solution containing thiophene, producing a conductive PTP film on the anode. During electrochemical synthesis thin and predictable polymer layer forms on the electrode surface but the conditions of the reaction (strongly acidic solution, high monomer concentration, high potential of polymerization reaction initiation) might have a negative influence on the enzyme conformation, which is entrapped within the PTP layer, formed during this polymerization reaction [16,17]. On the other hand, chemical synthesis is superior owing to a wider choice of monomers and using certain catalysts, as it is possible to synthesize less branched polymers. Some examples of oxidizers initiating polymerization of PTP are FeCl_3 , H_2O_2 , CuClO_4 , etc. [18,19]. It is possible to entrap a variety of molecules into the PTP-based layer in order to change its parameters, such as electrical conductivity [20]. Owing to the diffusional permeability of the polymer, a decent substrate and product mobility towards/outwards the enzyme is retained. Consequently, the resulting PTP polymer layers serve as an excellent basis/matrix to design stable and electrochemically effective biosensors or biofuel cells. However, it is impossible to obtain reproducible thin polymer layers by chemical synthesis, since during this process amorphous polymer suspension is formed [21].

In order to avoid problems, which occur during electrochemical and chemical PTP synthesis, an excellent alternative method is enzyme catalysed polymerization. This simple technique does not require a highly acidic medium, strong oxidizing agent and, the enzyme acts as a catalyst for the polymerization reaction. Enzymatic synthesis also allows the control of regioregularity and stereochemistry of the resulting polymer [22]. Enzymatic polymerization has been proven by polypyrrole and polyaniline synthesis using horseradish peroxidase [23–26], laccase [27,28], soybean peroxidase [29], royal palm tree peroxidase [30] and glucose oxidase [6,31], enzymes, which mostly catalyse the generation of respective monomer free radicals. These free radicals undergo coupling to produce dimers. Successive oxidation and coupling reactions lead to the polymer formation. In our previous study we took an advantage of the enzymatically by glucose oxidase (GO_x) produced hydrogen peroxide to polymerize aniline and pyrrole in a broad pH conditions ranging from 2.0 to 9.0 [6,7,31–34]. GO_x is an oxidoreductase that catalyses the oxidation of β -D-glucose into D-glucono-1,5-lactone, which is later hydrolysed in aqueous media to gluconic acid, and reduction of molecular oxygen to hydrogen peroxide. GO_x is a very important enzyme, which is usually employed in glucose biosensors.

In this study a self-encapsulation of glucose oxidase from *Aspergillus niger* within PTP layer was investigated. The polymerization of thiophene was initiated by hydrogen peroxide produced in GO_x catalysed glucose oxidation reaction. Hydrogen peroxide acted as an oxidizer generating free radicals, which initiated the polymerization reaction. The influence of PTP layer on GO_x bioelectrocatalytic activity and stability was determined by the evaluation of amperometric signal. The formation of PTP was confirmed by attenuated total reflectance–Fourier transform infrared (ATR–FTIR) spectroscopy and atomic force microscopy (AFM) based investigations.

2. Experimental

2.1. Materials

All commercial chemicals were of analytical grade or better, and were used as received. The glucose oxidase (GO_x) from *A. niger* (EC 1.1.3.4.) of 295 U/mg enzymatic activity and 25% glutaraldehyde (GA) were purchased from AppliChem GmbH (Darmstadt, Germany). Thiophene monomer was received from Merck (Boston, US). D-(+)-glucose was obtained from Carl Roth GmbH & Co (Karlsruhe, Germany). 90% phenazine methosulfate (PMS), 95% ethanol and other chemicals were purchased from Sigma–Aldrich (Berlin, Germany). The 1.0 M solution of glucose was prepared in distilled water (with resistance greater than 18.0 M Ω cm) at least 24 h before use to allow glucose to mutarotate and to reach equilibrium between α - and β -forms. When needed, GO_x solution was freshly prepared from lyophilized enzyme powder in mixed 0.05 M sodium acetate–phosphate buffer (A-PBS), pH 6.0, with 0.1 M KCl.

2.2. Electrode pretreatment

In order to obtain a smooth and clean surface of the graphite rod electrode (GRE), the electrode was hand-polished using fine emery paper. Prior to the immobilization of GO_x all GREs were washed with ethanol and purified water and, then thoroughly dried at room temperature.

2.3. Electrode modification with GO_x

Basic method suitable for rapid preparation of enzyme-modified GREs was applied for the immobilization of GO_x [35]. In order to prepare GO_x -coated GREs, 3 μl of 40 mg/ml GO_x solution were deposited on the electrode surface, later allowing the water to evaporate at room temperature. Afterwards, electrodes were stored for 20 h over the 5% solution of GA at +4 °C in a closed vessel as described in our previous studies [36] to cross-link enzymes adsorbed on the electrode surface. Before the experiments, the GO_x -modified electrodes were washed with purified water to remove non-cross-linked enzyme molecules and the side surface of the electrodes was sealed into epoxy to prevent its contact with the solution.

2.4. Coating of the GO_x -modified electrode by PTP layer

To prepare GO_x -modified GREs covered with additional PTP layer, the electrodes were immersed into polymerization solution, i.e., 0.05 M A-PBS, pH 6.0, containing 200 mM of dispersed thiophene and 50 mM of dissolved glucose at room temperature, for a definite period of time. Additionally, control electrodes were prepared keeping the GO_x -modified electrodes in A-PBS non containing one of the components, i.e., glucose or thiophene, which are essential for PTP polymerization process. The first control electrode (control electrode I) was incubated in A-PBS containing 50 mM of glucose ('control solution I') and the second control electrode (control electrode II) A-PBS containing 200 mM of thiophene ('control solution II'), respectively. Prior to amperometric measurements, the GO_x and GO_x /PTP-modified GREs, further in the text denoted as GO_x - and GO_x /PTP-modified electrodes, respectively, were thoroughly washed with purified water.

2.5. Electrochemical detection of analytical signal

All amperometric measurements were performed using a potentiostat/galvanostat Autolab PGSTAT 30 (Utrecht, Netherlands)

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