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Improvement in glucose biosensing response of electrochemically grown polypyrrole nanotubes by incorporating crosslinked glucose oxidase

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ABSTRACT

In this paper a novel enzymatic glucose biosensor has been reported in which platinum coated alumina membranes (Anodisc™s) have been employed as templates for the growth of polypyrrole (PPy) nanotube arrays using electrochemical polymerization. The PPy nanotube arrays were grown on Anodisc™s of pore diameter 100 nm using potentiostatic electropolymerization. In order to optimize the polymerization time, immobilization of glucose oxidase (GO_x) was first performed using physical adsorption followed by measuring its biosensing response which was examined amperometrically for increasing concentrations of glucose. In order to further improve the sensing performance of the biosensor fabricated for optimum polymerization duration, enzyme immobilization was carried out using cross-linking with glutaraldehyde and bovine serum albumin (BSA). Approximately six fold enhancement in the sensitivity was observed in the fabricated electrodes. The biosensors also showed a wide range of linear operation (0.2–13 mM), limit of detection of 50 μM glucose concentration, excellent selectivity for glucose, notable reliability for real sample detection and substantially improved shelf life.

1. Introduction

Since the discovery of enzyme electrodes for glucose detection by Clark and Lyons in 1962, stimulated research augmented by increasing demand of portable glucose biosensors for treatment of diabetes have been carried out worldwide [1–3]. Though non-enzymatic biosensors are also being investigated, however, such biosensors suffer from poor selectivity due to large interferences from easily oxidizable electroactive species present in the blood. On the other hand, extraordinarily superior specificity and sensitivity of enzymes towards specific substrate molecules has encouraged accelerated research in the field of enzymatic glucose biosensors for various applications such as glucose monitoring for diabetes care, beverage and food industry, bioprocess monitoring, and drug discovery and analysis [4,5].

Conceptually, a biosensor incorporates two important components: a bio-recognition element (such as enzymes) and a transducer adjacent to it. The role of transducer is to sense the changes in the system as a consequence of the interaction between the substrate (analyte) and bio-recognition element (enzyme) [5]. Enzyme immobilization is an indispensable step in order to enable prolonged and repetitive use of biosensor with consistent biosensing response [6]. In this respect, as a transducer, conductive polymers (CPs) are materials of choice due to their excellent electronic properties and biocompatibility [4,7]. Among various CPs, one of the most studied polymers is polypyrrole (PPy) [8,9]. Due to high electronic conductivity, high environmental stability and inherent biocompatibility it provides a very suitable microenvironment for enzyme immobilization and signal transduction [8,10]. Owing to excellent solubility of pyrrole monomer in a broad range of aqueous as well as non-aqueous solvents and its low oxidation potential for electro-oxidation, electrochemical polymerization has been adopted as one of the most preferred methods for obtaining PPy thin films on conductive substrates [9,11–13].

CP nanostructures exhibit much higher specific surface area than their bulk counterparts leading to significant enhancement in their electrochemical activity [13–16]. Hence, manifold enrichment in the enzyme loading and subsequent interactions of enzyme with substrate molecules leads to improved sensitivity of biosensors [14,15,17]. Some of the well-known techniques for enzyme immobilization are physical adsorption, co-entrapment, cross-linking, covalent binding etc., each holding its own merits [5,18]. In our recent research work, PPy nanotubes had been grown over Pt coated Anodisc™s with 200 nm pore diameter, in which physical adsorption was employed for immobilization of GO_x [17]. However, it suffered from poor stability. Due to the involvement of weak Van der Waal forces, the enzymes immobilized via physical adsorption are very much prone to leach out of the support matrix [19]. Improvement in the shelf life of biosensor is of critical importance towards the realization of a practically useful biosensor [7,10]. Another popular method of immobilization is co-entrapment, which requires the enzyme molecules to be mixed with monomer solution. At a pH greater than its iso-electric point, enzyme bears a net negative charge and gets attached with the polycationic backbone of the growing

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polymer film [20–22]. However, a major drawback of immobilization by co-entrapment is that it is very much sensitive to pH of the monomer solution and requires huge amounts of enzyme molecules for efficient enzyme loading [7]. Also due to most of the enzyme molecules are located deep in the polymer matrix, delayed interaction between enzyme and substrate molecules may lead to unfavorably large response times [20]. Immobilization by covalent binding provides efficient binding between enzyme and immobilizing material. However, it requires modification of sensor surface to acquire a reactive group for attachment with enzyme [23]. In contrast, immobilization by crosslinking of enzyme with the support matrix via some bi-functional crosslinking agent such as glutaraldehyde is a separate process similar to physical adsorption and a relatively small amount of enzyme is required as compared to co-entrapment. At the same time sensitivity and storage stability of the biosensor can be extended up to several times [24].

Template based method invented by Martin et al. has received considerable attention over the recent years mainly owing to its simplicity, controllability and widespread applications [25,26]. In the past decade, anodically oxidized alumina membranes (Anodisc™) have been extensively used as templates for the growth of nanotube and nanowire structures. The experimental parameters viz. monomer and supporting electrolyte concentrations, applied potential or current density, polymerization duration etc. determine whether nanotube or nanowire arrays are grown along the porous walls of the Anodisc™. Xiao et al. emphasized that low monomer concentration and high polymerization potential are crucial for the growth of polymer nanotubes [27]. Anodisc™s are commercially available with three standard pore diameters viz. 20, 100 and 200 nm. In order to get the benefit of quantum effects, it is highly desirable to choose as small dimension as possible. 20 nm pore diameter is not suitable for loading of GO_x due to restrictions imposed by the dimension (6.0 nm × 5.2 nm × 7.7 nm) of enzyme molecule [28,29]. Therefore, in this work, Anodisc™s with 100 nm pore diameter were employed in order to grow PPy nanotube arrays using potentiostatic electrochemical oxidation of pyrrole monomer. The fabricated electrodes were used as supporting matrix for GO_x immobilization. Polymerization time was optimized for the highest sensitivity for the fabricated biosensors. Realization of a novel biosensor with high sensitivity, selectivity, reproducibility and long storage life was the main objective of the presented work. In this regard, effect of two different immobilization techniques on the sensing response, selectivity towards glucose detection, shelf life and various other parameters has been studied for the optimum polymerization time for the fabricated biosensors using amperometric detection technique.

2. Experimental

2.1. Reagents

All required chemicals including pyrrole monomer, lithium perchlorate ($LiClO_4$), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), D-(+)-glucose, uric acid, L-ascorbic acid, ethanol, glucose oxidase from *Aspergillus niger* (E. C. 1.1.3.4), hydrogen peroxide (H_2O_2), glutaraldehyde, and bovine serum albumin (BSA) were used as obtained from Sigma-Aldrich. All the chemicals were of analytical grade. Anodisc™s (pore diameter: 100 nm) were purchased from Whatman. Deionized (DI) water of 18 MΩ resistivity was used for the preparation of aqueous solutions. A 50 mM phosphate buffer solution (PBS) of pH 6.8 was prepared using NaH_2PO_4, Na_2HPO_4 and DI water.

2.2. Apparatus

All electrochemical experiments including activity assay, electropolymerization and response current measurements were performed at room temperature in a three electrode electrochemical cell using electrochemical station (Autolab PGSTAT302N). Platinum foil and Ag/AgCl were used as counter and reference electrodes, respectively. The morphological characterization of the produced Pt and PPy films was performed using Field Emission Scanning Electron Microscope (FESEM, Carl Zeiss SUPRA55). The morphology of nanotubes was also studied by using Transmission Electron Microscope (TEM, FEI's Tecnai G^2 S-TWIN model) operated at 200 keV. In order to quantify the amount of enzyme loaded onto different PPy nanotube array electrodes, fluorescence emission spectra were recorded using Fluoromax-4p spectrofluorometer from Horiba Jobin Yvon (Model: FM-100). Structural analysis of native GO_x as well as GO_x immobilized using physical adsorption and cross-linking, was performed using Fourier Transform Infra-Red (FTIR) spectroscopy. In order to record the IR spectra, Tensor 27 (from Bruker) spectrometer was operated in the wave number range from 4000 to 500 cm^{-1}. Each spectrum was the resultant of average of 16 scans collected with 2 cm^{-1} resolution. Image processing tool of MATLAB (© 1984–2011 The MathWorks, Inc.) was employed to compute the surface porosity of Pt coated electrode before and after polymerization for different durations. Pore volumes of PPy nanotubes were also experimentally determined for different electrodes using Brunauer–Emmett–Teller (BET) analysis. Nitrogen adsorption–desorption isotherms were measured at 77 K using volumetric gas adsorption station (Quantachrome Autosorb 1C TCD Analyzer, Model: ASIC-X-TCD6).

2.3. Fabrication of electrodes

The schematic diagram shown in Fig. 1(a) illustrates the important steps followed for the fabrication of biosensors. Pt/Anodisc™ electrodes were obtained by depositing Pt on the masked Anodisc™s using direct current magnetron sputtering system operated at base pressure of 2×10^{-7} mbar. Pt was sputtered on the substrates rotated at a speed of about 30 rotations per minute (rpm) under the argon gas pressure of 5×10^{-3} mbar and a deposition rate of 1 Å-s^{-1}. During deposition argon gas flow rate was maintained at 20 cm^3-min^{-1}. Subsequently, electropolymerization was carried out under potentiostatic condition (1.8 V) in aqueous solution containing freshly distilled pyrrole monomer (25 mM) and $LiClO_4$ (100 mM). As a final step, the immobilization of GO_x resulted into a functional glucose biosensor. Fig. 1(b) shows the cross-sectional view of GO_x/PPy/Pt/Anodisc™ electrode. Nanoporosity of electrodes is the key requirement to realize significant improvements in the performance of the biosensor by means of enhancement in the surface-to-volume ratio [30]. Hence, the optimization of Pt thickness is critical in this regard. Optimum thickness of Pt for producing conducting surface and walls of template without hurting the porous nanostructures was obtained to be 50 nm. Polymerization duration was varied from 3 to 70 s for different samples.

2.4. Enzyme immobilization

2.4.1. Physical adsorption and quantification

Immobilization of enzymes to the surface of transducer is an essential step in order to localize them in the supporting matrix (which was the electrochemically grown PPy in our case) so as to extend their life for repetitive use. As required by the optimization of the polymerization time, enzymes were first immobilized by physical adsorption method. This simple method binds enzymes with a transducer via electrostatic interactions [18]. GO_x solution (10 mg-ml^{-1}) was prepared in 50 mM PBS (pH 6.8). Enzyme immobilization was carried out by placing 10 μl aliquot from GO_x solution onto PPy coated surface (PPy/Pt/Anodisc™) for physical adsorption and electrode was kept overnight in incubator at 4 °C. Before using the electrode for amperometric biosensing, it was washed carefully in PBS to do away with loosely adhered enzyme molecules. Electrodes were stored in incubator when not in use.

Polymerization time plays an important role in controlling (i) the coverage of Pt surface by PPy and (ii) porosity of the electrodes, which in turn affect the subsequent enzyme loading. The polymerization time corresponding to the highest enzyme loading indicates a perfect balance between the Pt surface coverage by PPy and electrode porosity