Colloids and Surfaces B: Biointerfaces 112 (2013) 74–80

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

A novel promising biomolecule immobilization matrix: Synthesis of functional benzimidazole containing conducting polymer and its biosensor applications

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ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form 26 July 2013
Accepted 28 July 2013
Available online 2 August 2013

Keywords:
Conducting polymer
Biosensor
Enzyme immobilization
Low detection limit
Glucose level

ABSTRACT

In order to construct a robust covalent binding between biomolecule and immobilization platform in biosensor preparation, a novel functional monomer 4-(4,7-di(thiophen-2-yl)-1*H*-benzo[*d*]imidazol-2-yl)benzaldehyde (BIBA) was designed and successfully synthesized. After electropolymerization of this monomer, electrochemical and spectroelectrochemical properties were investigated in detail. To fabricate the desired biosensor, glucose oxidase (GOx) was immobilized as a model enzyme on the polymer coated graphite electrode with the help of glutaraldehyde (GA). During the immobilization step, an imine bond was formed between the free amino groups of enzyme and aldehyde group of polymer. The surface characterization and morphology were investigated to confirm bioconjugation by X-ray photoelectron spectroscopy (XPS) and transmission electron microscopy (TEM) at each step of biosensor fabrication. The optimized biosensor shows good linearity between 0.02 mM and 1.20 mM and a low limit of detection (LOD) of 2.29 μM. Kinetic parameters K_m^{app} and I_{max} were determined as 0.94 mM and 10.91 μA, respectively. The biosensor was tested for human blood serum samples.

1. Introduction

Conducting polymers (CPs) with their fascinating electronic, electrochemical and optical properties have crucial contribution to material science. Conjugated polymers have high electron affinity, low ionization potential, low energy for optical transitions which makes them useful materials for technological developments [1]. Thus, these multifunctional materials have the wide range of promising applications in the fields of energy storage [2,3], electrocatalysis [4,5], electrochromic devices [6,7], sensors [8,9] and molecular electronic devices [10,11].

Integration of biomolecules such as enzymes, antibodies and amino acids with CPs provides improvement of many electrochemical, electronic and optical biosensors [12,13]. A biosensor mainly consists of a biological recognition element and a transducer which transforms the signal resulting from reaction between biomolecule and its analyte into a measurable signal. The most significant challenge for the construction of a biosensor is to achieve a robust attachment between transducer and protein molecule. Among well-known immobilization techniques, covalent binding is remarkably powerful method for bioconjugation [14]. This easily applicable technique allows fabricating new generation biosensors with high activity, stability, sensitivity and faster response time in detection [15]. Since enzymes are huge molecules, immobilization platform should be well organized to promote interaction with biomolecule. Increase in the functional groups on the surface provides the maximum enzyme loading with high stability. For this purpose, functionalized conducting polymers are favorable materials and can be used as conjugation matrices for biomolecules [16]. Since polybenzimidazoles are biocompatible molecules and widely used in biological applications, aldehyde functionalized donor acceptor donor type benzimidazole containing polymer was utilized as a bioconjugation platform in this study [17].

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http://dx.doi.org/10.1016/j.colsurfb.2013.07.049

Glucose oxidase (GOx) has high resistance against unfavorable microenvironment conditions such as acidic media and denaturing agents. Since it is also commercially available with low price and has two flavine adenine dinucleotide (FAD) units, this enzyme is widely used in many analytical detection studies [18,19]. During the detection of glucose, β-D-glucose is oxidized to gluconolactone by GOx and gluconolactone is hydrolyzed to gluconic acid. As a second step, reduced GOx is reoxidized in the presence of molecular oxygen [20]. In amperometric studies, consumption of oxygen or formation of H_2O_2 resulting from reduction of oxygen are monitored.

In this report, we highlight design and synthesis of a novel functionalized conducting polymer for biomolecule immobilization. In this context, the electronic and optoelectronic properties of this polymer were examined in detail. To investigate the biological matrix properties of this system, firstly, corresponding monomer was electropolymerized on graphite electrode. Then, GOx as a model enzyme was covalently immobilized on the polymer coated electrode. Meanwhile, imine bonds were achieved between free amine groups of enzyme and aldehyde groups of polymer structure. Furthermore, glutaraldehyde was used as the crosslinking agent providing a compact structure for enzyme on electrode surface. Thus, bioconjugation was successfully performed with the formation of desired robust binding. The new biosensor was optimized for parameters like scan number in polymerization, amount of loaded enzyme, glutaraldehyde amount and pH of the buffer solution used in the amperometric measurements. Optimum biosensor was characterized in terms of kinetic parameters, K_M^{app} and $I_{max.}$ During the detection of glucose, amperometric studies were carried out at −0.7 V to monitor the consumption of molecular oxygen. Surface morphology and characteristic were explored via TEM and XPS. Finally, accuracy of fabricated biosensor was tested for glucose amount in human blood serum samples.

2. Materials and methods

2.1. Materials

All chemicals used in monomer synthesis were commercially obtained and used without any purification. THF was distilled over sodium and benzophenone. 4-(4,7-Dibromo-1*H*-benzo[*d*]imidazol-2-yl)benzaldehyde, 4-(4,7-di(thiophen-2-yl)-1*H*-benzo[*d*]imidazol-2-yl)benzaldehyde and tributyl(thiophene-2-yl)stannane were synthesized. All chemicals used in the synthesis of the monomer (bromine, bromic acid, dichloromethane (DCM)) were purchased from Sigma except for thiophene which was purchased from Acros Organics (Geel, Belgium). Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, 21 200 units/g) from *Aspergillus niger*, D-glucose, sodiumborohydride were purchased from Sigma (St. Louis, USA). Acetonitrile, hydrochloric acid, sodium hydroxide were purchased from Merck (Darmstadt, Germany). All chemicals used for electropolymerization were purchased from Aldrich. All other chemicals were analytical grade. Real human serum samples were obtained from Middle East Technical University (METU) Medical Center from patients volunteered for that matter.

2.2. Apparatus

All experiments were carried out in pre-dried glassware (1 h, 150 °C) under argon atmosphere. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on Bruker Spectrospin Avance DPX-400 spectrometer and the chemical shifts were expressed in ppm relative to DMSO-d_6 (d 2.50 and 39.52 for ^{1}H and ^{13}C NMR, respectively) as the internal standard. Amperometric and cyclic voltammetry measurements were carried out using Palm Instrument (PalmSens, Houten, The Netherlands, www.palmsens.com) with a conventional three electrode configuration. As the working electrode, graphite electrodes (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) were used. Ag/AgCl (3 M KCl saturated with AgCl) and a Pt electrode (Metrohm, Switzerland, www.metrohm.com) were used as the reference and counter electrodes, respectively. XPS (X-ray Photoelectron Spectroscopy) was carried out on a PHI 5000 Versa Probe (*Φ* ULVAC-PHI, Inc., Japan/USA) model X-ray photoelectron spectrometer instrument with monochromatized Al Kα radiation (1486.6 eV) as an X-ray anode at 24.9 W. The pressure inside the analyzer was maintained at 10^{-7} Pa. The binding energy scale was referenced by setting the C—H peak maximum in the C 1s spectrum to 285.0 eV and the atomic composition was estimated using Multipak software. Transmission electron microscope (CTEM) images were recorded using FEI Tecnai G^2 Spirit BioTwin CTEM Microscope.

3. Experimental

3.1. Synthesis of the monomer

3.1.1. Synthesis of 4-(4,7-dibromo-1H-benzo[d]imidazol-2-yl) benzaldehyde

3,6-Dibromobenzene-1,2-diamine was synthesized as reported in literature [21]. After the bromination of 2,1,3-benzothiadiazole in the presence of HBr/Br_2, reduction of 4,7-dibromobenzo[*c*][1,2,5]thiadiazole was performed with $NaBH_4$ in ethanol. 3,6-Dibromobenzene-1,2-diamine (8.50 mmol, 2.30 g), terephthalaldehyde (34 mmol, 4.56 g) and $ZrCl_4$ (0.43 mmol, 97.75 mg) were dissolved in acetonitrile (ACN) (150 mL) and the mixture was stirred at room temperature for 24 h. After the solid product was filtered, 150 mL ACN was added and the mixture was heated overnight under reflux conditions [22]. Afterwards the purified product was filtered as a pale yellow solid (5.1 mmol, 1.53 g, 60%). ^{1}H NMR (d-DMSO): δ 10.10 (s, 1H), 8.56 (d, 2H, J = 8.26 Hz), 8.10 (d, 2H, J = 8.36 Hz), 7.43 (s, 2H). ^{13}C NMR (d-DMSO): 1927. 1517. 1 37 1.1 34.1. 1298. 1280. 1268, 1267, 126.6.

3.1.2. Synthesis of 4-(4,7-di(thiophen-2-yl)-1H-benzo[d]imidazol-2-yl)benzaldehyde

Tributyl(thiophene-2-yl)stannane was synthesized according to a previously described method [23]. 4-(4,7-Dibromo-1*H*-benzo[*d*]imidazol-2-yl)benzaldehyde (0.70 g, 1.70 mmol) and tributyl(thiophen-2-yl)stannane (3.18 g, 8.50 mmol) were dissolved in anhydrous THF (50 mL) and dichlorobis(triphenylphosphine)-palladium(II) (50 mg, 0.05 mmol) was added at room temperature. The mixture was refluxed for 12 h under argon atmosphere. Solvent was evaporated under vacuum and the crude product was purified by silica gel column chromatography as a yellow solid. (eluent: DCM) (0.60 g, 45%). ^{1}H NMR (d-DMSO): δ 12.89 (s, 1H), 10.12 (s, 1H), 8.62 (d, 2H, J = 8.23 Hz), 8.26 (d, 1H, J = 3.56 Hz), 8.11 (d, 2H, J = 8.22 Hz), 7.72–7.65 (m, 4H), 7.40 (d, 1H, J = 7.85 Hz), 7.28 (dt, 1H, J = 4.74 and 3.91 Hz), 7.25 (dt, 1H, J = 4.76 and 3.91 Hz). ^{13}C NMR (d-DMSO): δ 192.2, 150.8, 140.1, 139.1, 138.4, 136.2, 134.3, 132.3, 129.2, 127.8, 127.5, 127.1, 126.1, 125.8, 125.5, 123.5, 122.8, 118.9, 117.6. HRMS: Calculated $[M]^+$ 387.0626, Measured $[M]^+$ 387.0631.

3.2. Amperometric measurements

All measurements; generation of a calibration curve and determining glucose concentration in samples, were achieved at −0.7 V versus Ag/AgCl electrode in 50 mM sodium acetate buffer, pH 5.5, while mildly stirring the medium at room temperature. Hence, current difference between baseline and steady state (before and after