Bioelectrochemistry 99 (2014) 46–52

Contents lists available at ScienceDirect

Bioelectrochemistry

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

A new self-assembled layer-by-layer glucose biosensor based on chitosan biopolymer entrapped enzyme with nitrogen doped graphene

Madalina M. Barsan [a], Melinda David [a,b], Monica Florescu [c], Laura Ţugulea [b], Christopher M.A. Brett [a,*]

[a] Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3004-535 Coimbra, Portugal
[b] Facultatea de Fizica, Universitatea din Bucuresti, Magurele 077125, Romania
[c] Facultatea de Medicina, Universitatea Transilvania din Brasov, Brasov 500019, Romania

ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form 13 June 2014
Accepted 13 June 2014
Available online 21 June 2014

Keywords:
Layer-by layer
Quartz crystal microbalance
Nitrogen doped graphene
Enzyme
Chitosan

ABSTRACT

The layer-by-layer (LbL) technique has been used for the construction of a new enzyme biosensor. Multilayer films containing glucose oxidase, GOx, and nitrogen-doped graphene (NG) dispersed in the biocompatible positively-charged polymer chitosan ($chit^+$(NG + GOx)), together with the negatively charged polymer poly(styrene sulfonate), PSS^-, were assembled by alternately immersing a gold electrode substrate in $chit^+$(NG + GOx) and PSS^- solutions. Gravimetric monitoring during LbL assembly by an electrochemical quartz microbalance enabled investigation of the adsorption mechanism and deposited mass for each monolayer. Cyclic voltammetry and electrochemical impedance spectroscopy were used to characterize the LbL modified electrodes, in order to establish the contribution of each monolayer to the overall electrochemical properties of the biosensor. The importance of NG in the biosensor architecture was evaluated by undertaking a comparative study without NG in the chit layer. The GOx biosensor's analytical properties were evaluated by fixed potential chronoamperometry and compared with similar reported biosensors. The biosensor operates at a low potential of −0.2 V vs., Ag/AgCl, exhibiting a high sensitivity of 10.5 μA cm^{-2} mM^{-1}, and a detection limit of 64 μM. This study shows a simple approach in developing new biosensor architectures, combining the advantages of nitrogen-doped graphene with the LbL technique for enzyme immobilization.

1. Introduction

One of the key issues in developing new biosensors with improved sensitivity and stability is effective immobilization of the recognition element, e.g. the enzyme. Among the enzyme immobilization methods, such as covalent linkage [1,2], sol–gel entrapment [3,4], adsorption [5], etc., layer-by-layer (LbL) self-assembly is a simple and powerful method, efficient because protein denaturation is minimized since the films are produced under mild conditions, based on the adsorption of macromolecules from aqueous solution onto solid supports [6]. LbL films have unique mechanical properties, uniformity and stability [7], the technique having the advantage of allowing the construction of thin multilayer films, based mainly on electrostatic interactions in between layers, which require a very small amount of material, therefore being a cost-effective preparation method for enzyme biosensors.

One of the problems to be overcome in an enzyme biosensor is the slow electron transfer between the enzyme redox center, which is usually buried in a hydrophobic cavity formed by polypeptide, and the electrode surface. Nanomaterials, such as graphene, carbon nanotubes (CNTs), metal nanoparticles, etc., are advantageous in increasing the possibility of direct electron transfer between the enzyme active sites and the electrode, acting as electrical bridges [8–10]; however, direct electron transfer between enzymes and carbon nanomaterials is not always the mechanistic basis of the substrate detection [11]. Nanomaterials can also bring benefits for immobilizing enzymes since they maintain enzyme bioactivity due to their microenvironment [12,13]. Among the above-mentioned materials, graphene is a 2D plane sheet with an open structure and both sides of graphene could be utilized for enzyme immobilization, unlike 1-D CNTs, which are more difficult to be controllably assembled [14].

LbL formation of multilayer films with incorporation of graphene in one of the components of the self-assembly process combines the excellent electrochemical properties of graphene and the versatility of LbL assembly, showing great promise for highly efficient sensors and advanced biosensing systems. The excellent conductivity and small band gap of graphene are favorable for conducting electrons from the biomolecules [15]. Although, by using different types of intermolecular interactions, LbL structures are able to incorporate diverse molecules as building blocks, it is still a challenge to include certain kinds of molecules, especially hydrophobic species, into LbL films [16].

Graphene and nitrogen-doped graphene (NG) have been successfully dispersed in chitosan and used as substrate for the immobilization of

* Corresponding author. Tel.: +351 239854470; fax: +351 239827703.
E-mail address: cbrett@ci.uc.pt (C.M.A. Brett).

http://dx.doi.org/10.1016/j.bioelechem.2014.06.004
1567-5394/

enzymes [9,17]. The N-doping of graphene has been done by thermal annealing in the presence of ammonia, the nitrogen atom in the graphene framework existing in "graphitic", pyridinic or pyrrolic forms, which are beneficial for the electric conductivity of the material [18]. The biopolymer chitosan (chit) is often employed for enzyme immobilization, through covalent linkage, when the polymer is chemically modified to allow crosslinking with enzyme amino acids [19], and by electrostatic interaction in LbL films [20], in this case usually combined with carbon nanotubes, redox mediators or metal nanoparticles, due to its relatively poor conductivity [10,21].

In the LbL enzyme immobilization study presented here, the positively-charged chitosan layer contains the enzyme glucose oxidase (GOx) together with dispersed NG, chit$^+$(GOx) or chit$^+$(NG + GOx), and the negatively charged layer is poly(styrene sulfonate), PSS$^-$. Self-assembled adsorption of the multilayers on Au substrates was monitored by using an electrochemical quartz crystal microbalance (EQCM), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The influence of NG and of each monolayer on the electrochemical properties of the LbL biosensor was analyzed, together with biosensor sensitivity after addition of each enzyme layer, in order to determine the best biosensor architecture.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and were used without further purification. N-doped graphene was prepared according to the procedure described in [18] and was a gift from Prof. X. Sun, University of Western Ontario, Canada. Chitosan (low molecular weight), minimum 85% degree of deacetylation, monobasic and dibasic sodium phosphate, and sodium polystyrene sulfonate (NaPSS) were from Sigma-Aldrich, Germany. The deacetylated chitosan used in this study was chosen due to its higher positive charge density. Glucose oxidase (GOx) from *Aspergillus niger* (24 U mg^{-1}) was from Fluka, Germany.

Experiments were performed in neutral sodium phosphate buffer 0.1 M NaPB pH = 7.0.

Millipore Milli-Q nanopure water (resistivity ≥ 18 MΩ cm) was used for the preparation of all solutions. All experiments were performed at room temperature (25 ± 1 °C).

2.2. Instrumentation

Gravimetric measurements were performed with an electrochemical quartz crystal microbalance eQCM 10 M, Gamry Instruments, containing an Au quartz crystal (AuQC) with 10 MHz central frequency.

Electrochemical experiments were performed in a three-electrode cell, containing the AuQC (area 0.205 cm^2) as working electrode, a Pt wire counter electrode and an Ag/AgCl (3.0 M KCl) reference electrode, using a μ-Autolab potentiostat/galvanostat (Metrohm-Autolab, Netherlands).

Electrochemical impedance spectroscopy (EIS) experiments were carried out with a potentiostat/galvanostat/ZRA, (Gamry Instruments, Reference 600). An rms perturbation of 10 mV was applied over the frequency range 65 kHz–0.1 Hz, with 10 frequency values per frequency decade.

The pH measurements were carried out with a CRISON 2001 micro pH-meter (Crison Instruments SA, Barcelona, Spain) at room temperature (25 ± 1 °C).

2.3. Preparation of the LbL biosensors

NG was used as received and was dispersed together with the enzyme GOx in 1% (w/v) chitosan dissolved in 1% (v/v) acetic acid, to obtain a final solution containing 0.05% NG and 1% GOx (24 U in 100 μL of enzyme solution). This solution had a pH of 4.0, just below the isoelectric point of GOx, which is 4.2 [22], so that both chitosan and enzyme were positively charged. The AuQC was always previously cleaned with acetone and placed in the AuQCM cell, which is a small chamber of 200 μL liquid capacity with the AuQC on the cell bottom and into which the counter and reference electrodes are inserted. For the gravimetric studies, 200 μL of water was first placed in the chamber by micropipette to allow the stabilization of frequency in aqueous media. Then the water was removed and 200 μL of the solution containing the NG + GOx or GOx, i.e. chit$^+$(NG + GOx) or chit$^+$(GOx) was placed in the chamber and left for 60 min, followed by rinsing with water and drying with a nitrogen stream, during 2–3 min. Following this, the chamber was filled with 200 μL of negatively charged 1% NaPSS solution and left during 20 min, then again rinsed with water and dried with a nitrogen stream, during 2–3 min. This procedure was repeated for further bilayers. In this way, biosensors AuQC/{chit$^+$(NG + GOx)/PSS$^-$/chit$^+$(NG + GOx)}$_n$ (n = 1, 2) or AuQC/{chit$^+$(GOx)/PSS$^-$/chit$^+$(GOx)}$_n$ (n = 1, 2) were obtained.

3. Results and discussion

3.1. Gravimetric monitoring of the chit$^+$(NG + GOx)/PSS$^-$ and chit$^+$(GOx)/PSS$^-$ self-assembly on AuQC

The QCM is an excellent tool of monitoring the dynamics of the adsorption process during LbL self-assembly. The frequency variation with time can be used to determine the deposited mass by using the Sauerbrey equation [23], for the specific case of rigid films:

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\mu_q \rho_q}}\Delta m$$

where f_0 is the resonant frequency (Hz), Δf is the frequency change (Hz), Δm is the mass change (g), A is the piezoelectrically active crystal area, ρ_q is the density of quartz (g cm^{-3}) and μ_q is the shear modulus of quartz for AT-cut crystals (g cm^{-1} s^{-2}). In the specific case of the AuQCM employed in this study, the conversion factor, $-\Delta f/\Delta m$, is 226.0 Hz per 1 μg.

The AuQCM cell utilized is a small chamber of capacity 200 μL of liquid. Water was first placed in the chamber in order to stabilize the crystal resonant frequency in liquid, was emptied by using a micropipette, and dried with a nitrogen stream. Self-assembly then began with chit$^+$(NG + GOx) solution as described in Section 2.3.

As shown in Fig. 1A, red line, the AuQC frequency immediately decreased when it contacted for the first time with the chit$^+$(NG + GOx) solution, indicating fast adsorption of the molecules on the clean AuQC surface. The overall shift in frequency for this first step was Δf_1 = 0.93 kHz, corresponding to a deposited mass of m_1 = 850 ng. The deposition vs. time profile of PSS$^-$ followed an exponential decrease, of Δf_2 = 3.41 kHz, a film of m_2 = 2.06 μg being deposited. An exponential decrease in frequency was also observed for the second immersion of the AuQC in the chit$^+$(NG + GOx) solution Δf_3 = 8.45 kHz, with a fast and sharp decay during the first 30 min, of 7.96 kHz, and slower for the next 30 min, corresponding to 1.30 kHz. This indicates that fast adsorption of molecules occurs in the first 30 min, the film formed being of mass 7.26 μg, only 1.19 μg being deposited afterwards.

The total decrease in frequency was Δf_{tot} = 13.61 kHz, corresponding to a deposited film of m_{tot} = 12.36 μg.

A AuQC/{chit$^+$(GOx)/PSS$^-$/chit$^+$(GOx)} was also constructed, this time the chitosan solution containing only the enzyme GOx, without dispersed NG. The frequency and mass shift during the LbL process are presented in Fig. 1(A) and (B), blue line. As can be observed, the deposition profile during the first two self-assembly steps is similar to that obtained for AuQC/{chit$^+$(NG + GOx)/PSS$^-$/chit$^+$(NG + GOx)}, but there is a significant difference in the last LbL deposition step, when only a small shift in frequency is recorded when NG is not present.