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Layer-by-layer assembly of functionalized reduced graphene oxide for direct electrochemistry and glucose detection

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ABSTRACT

We report an electrochemical glucose biosensor made with layer-by-layer (LbL) films of functionalized reduced graphene oxide (rGO) and glucose oxidase (GOx). The LbL assembly using positively and negatively charged rGO multilayers represents a simple approach to develop enzymatic biosensors. The electron transport properties of graphene were combined with the specificity provided by the enzyme. rGO was obtained and functionalized using chemical methods, being positively charged with poly(diallyldimethylammonium chloride) to form GPDDA, and negatively charged with poly(styrene sulfonate) to form GPSS. Stable aqueous dispersions of GPDDA and GPSS are easily obtained, enabling the growth of LbL films on various solid supports. The use of graphene in the immobilization of GOx promoted Direct Electron Transfer, which was evaluated by Cyclic Voltammetry. Amperometric measurements indicated a detection limit of 13.4 $\mu mol \cdot L^{-1}$ and sensitivity of 2.47 $\mu A \cdot cm^{-2} \cdot mmol^{-1} \cdot L$ for glucose with the $(GPDDA/GPSS)_1/(GPDDA/GOx)_2$ architecture, whose thickness was 19.80 ± 0.28 nm, as determined by Surface Plasmon Resonance (SPR). The sensor may be useful for clinical analysis since glucose could be detected even in the presence of typical interfering agents and in real samples of a lactose-free milk and an electrolyte solution to prevent dehydration.

1. Introduction

The incidence of diabetes will double by 2030, according to the World Health Organization [1], thus requiring massive investment in prevention research, including development of sensitive devices capable of detecting glucose from several intake sources such as food and medicines. Efforts have been made in this direction, with enzymes being used to provide high specificity to sensors [2]. Since the first enzyme biosensor for detecting glucose by Clark [3], considerable advances have been achieved in methodologies for immobilizing biomolecules. The key to successful enzyme immobilization is the preservation of its biocatalytic activity in the recognition of a specific element, which is provided by electron transfer reactions from the redox process involving the analyte. Therefore, the structure of the biomolecule should be preserved after the immobilization process.

The layer-by-layer (LbL) technique is a relatively simple, versatile, robust and low-cost method for fabricating ultrathin films [4], which can be used to functionalize surfaces for several applications, including sensing and biosensing. It has been shown to be effective with several types of materials such as enzymes [5], dendrimers [6], polypeptides [7], nucleic acids and DNA [8], proteins [9], virus [10], conducting polymers [11], inorganic materials [12], nanoparticles [13], nanotubes [14] and nanowires and nanosheets [15]. It is widely used for immobilizing biomolecules [16–18] as the electrochemical activity is preserved due to water entrapment in the LbL film structure [19], minimizing protein denaturation for long time periods [20].

While devising a biosensor made with LbL films, one has to consider not only the choice of a matrix material capable of preserving the activity of biomolecules but also the need to have efficient charge transport. In this context, graphene is unique in that a single sheet free of defects exhibits high electrical conductivity [21], with electrons moving at nearly the speed of light (~10^6 m/s) [22]. With such properties, graphene has been used in electrochemical sensors, for detecting reduction of bromate [23], single nucleotide polymorphisms of DNA [24], leukemia [25,

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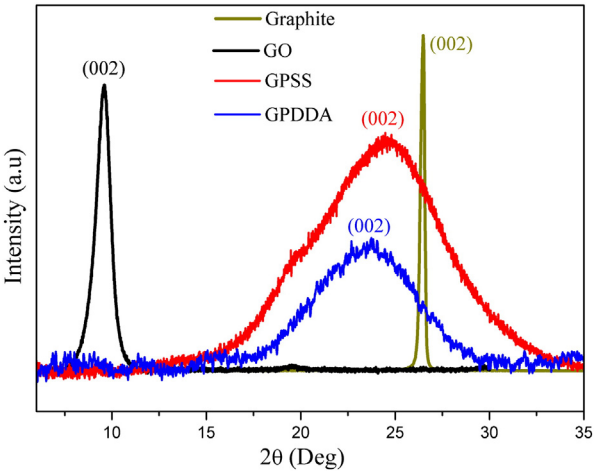

Fig. 1. XRD patterns of graphite, GO, GPDDA and GPSS powder, including the crystalline orientation of the XRD peaks.

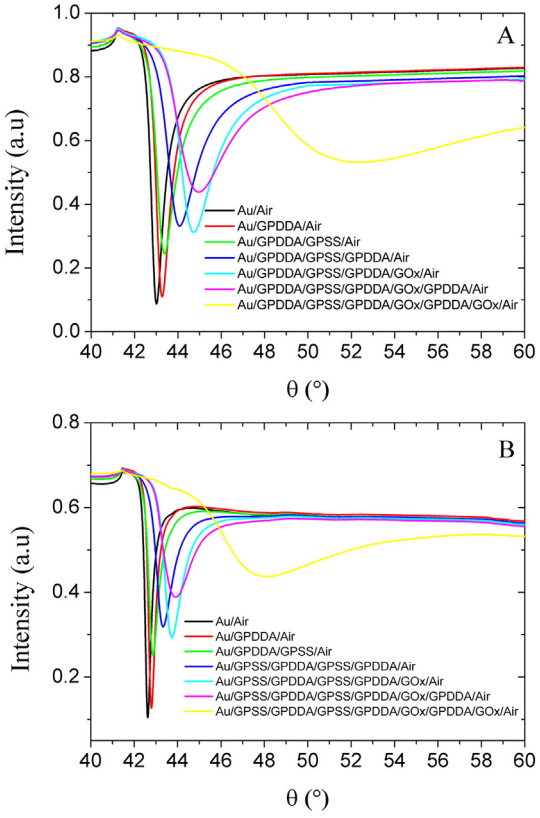

Fig. 2. SPR angular spectra for gold sensor during deposition of (a) $(GPDDA/GPSS)_1/(GPDDA/GOx)_2$ film excited by λ = 670 nm laser and (b) λ = 785 nm laser.

26] and dopamine [15]. However, graphene is highly hydrophobic and forms clusters when dispersed in water [27]. To increase applicability in LbL assemblies, graphene nanosheets are normally functionalized with stabilizers for aqueous dispersions. LbL films fabricated with functionalized rGO, for instance, feature tunable film thickness [28], with electrons from protein active sites permeating the protective shell for the LbL film, thereby improving the analytical performance of a biosensor [29]. The use of graphene in the immobilization of glucose oxidase (GOx) has promoted Direct Electron Transfer (DET) [30–34], which is advantageous for sensing for several reasons, including reagent-less detection, low operation potential and high selectivity [29].

In this work, graphene nanosheets were chemically synthesized from graphite and stabilized in positively charged PDDA and negatively charged PSS, thus producing, respectively, rGO stabilized in PDDA (GPDDA) and rGO stabilized in PSS (GPSS). Aqueous stable dispersions of the functionalized rGO nanoplatelets were used for producing LbL films alternated with GOx. We have chosen the LbL technique owing to its suitability to immobilize enzymes with their structure and biocatalytic activity preserved. The architecture $(GPDDA/GPSS)_1/(GPDDA/GOx)_2$ was self-assembled onto an ITO substrate and successfully tested for the electrochemical determination of glucose. Amperometry was used for glucose detection and different interferents were tested.

2. Materials and methods

2.1. Materials

Graphite powder with 98% purity, 95% H_2SO_4, Na_2HPO_4, 99% $KMnO_4$, 99% $K_2S_2O_3$, P_2O_5, 30% H_2O_2, hydrazine sulfate ($H_6N_2O_4S$) and NaH_2PO_4 were purchased from Synth. Glucose Oxidase (GOx) from *Aspergillus niger* (138,800 units·g^{-1}), 99.5% D-(+)-glucose, poly(styrene sulfonic acid) sodium salt (PSS; Mw = 70,000) and poly(diallyldimethylammonium chloride) (PDDA; 20 wt.% in H_2O) were purchased from Sigma Aldrich. All reagents were obtained as analytical grade and used without further purification.

2.1.1. Instruments

X-ray diffraction (XRD) studies were performed with a XPERT-PRO MPD (PANalytical) diffractometer using Cu Kα radiation (λ = 1.544 Å). UV-Vis spectroscopy was carried out using Genesys 6 UV–visible spectrophotometer (Thermo Fischer). To analyze the LbL film growth, the SPR Navi 200 Surface Plasmon Resonance (SPR) analyzer (BioNavis, Finland) was used, with a *p*-polarized laser light beam at two wavelengths, viz. λ = 670 nm and λ = 785 nm. Previously cleaned gold covered glass slides (BioNavis, Finland) were used as substrates for the SPR experiments. Fourier Transform Infrared (FTIR) spectroscopy was carried out using a Nexus 470 (Thermo Nicolet). The films were assembled onto silicon substrates and the pure components were compressed in KBr pellets. Circular dichroism (CD) spectroscopy was carried out on a J-815 spectrometer (JascoInc) with a bandwidth of 1 nm, a response time of 0.5 s and a scanning rate of 100 nm.min^{-1}. The GOx solution was analyzed using a quartz cell of 1 mm optical path and the films were analyzed on quartz substrates, with the optical path given by the film thickness. Chronoamperometry was performed on a potentiostat/galvanostat (Autolab PGSTAT 30 — Echochemie). A conventional three-electrode system was used with a working electrode of $(PDDA/GPSS)_1/(GPDDA/GOx)_2$ film onto ITO, a platinum plate as counter electrode, and a saturated calomel electrode (SCE) as the reference electrode. All electrochemical measurements were acquired in 10 mmol·L^{-1} and phosphate buffer pH 6.3.

2.1.2. Synthesis of GPSS and GPDDA

Graphite was pre-oxidized as reported by Kovtyukhova [35]. 15 mL of H_2SO_4 were mixed with 5 g of $K_2S_2O_8$ and 5 g P_2O_5 kept at 80 °C, then 10 g of graphite powder were added to the mixture, which was cooled to room temperature, carefully diluted and filtered. Graphite oxide was prepared from graphite powder using a modified Hummers method [36]. Briefly, 10 g of pre-oxidized graphite were mixed in 230 mL concentrated H_2SO_4 kept in an ice bath at 0 °C and 30 g of $KMnO_4$ were added gradually to the mixture. This mixture was stirred for 2 h at 35 °C and diluted gradually with 460 mL of ultrapure water. After 15 min, the mixture was diluted with 1.4 L of ultrapure water and treated with 30 mL 30% H_2O_2 (the color of the mixture turned to