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Layer-by-layer self-assembly of functionalized graphene nanoplates for glucose sensing *in vivo* integrated with on-line microdialysis system

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ABSTRACT

In this work, a novel amperometric biosensor for hydrogen peroxide was fabricated through the layer-by-layer (LBL) self-assembling of amine-terminated ionic liquid (IL-NH_2), and sulfonic acid (SO_3^-) functionalized graphene by covalent bonding. The modification of the two functionalities introduced positive and negative charge onto the surface of graphene respectively, thus facilitating the formation of a multilayer film denoted with {IL-RGO/S-RGO}$_n$ through electrostatic interaction and further immobilization of glucose oxidase (GOx). The resulting {IL-RGO/S-RGO}$_n$/GOx/Nafion biosensor displayed an excellent response to glucose at a potential of −200 mV. Combined with on-line microdialysis system, the glucose biosensor in the on-line system showed good linear range from 10 μM to 500 μM with the detection limit of 3.33 μM (S/N = 3). Consequently, the basal level of glucose in the striatum of anesthetic rats was calculated to be 0.376 ± 0.028 mM (mean ± s.d., n = 3). The {IL-RGO/S-RGO}$_n$/GOx/Nafion biosensor was further applied for *in vivo* sensing of the glucose level in the striatum when rats received intraperitoneal (i.p.) injection of 30 μL insulin, which resulted in an obvious decrease in the extracellular concentration of glucose within 30 min. The method was proved to be sensitive and reproducible, which enabled its promising application in physiology and pathology.

1. Introduction

Graphene, a counterpart of graphite with well separated 2-D aromatic sheets composed of sp^2-bonded carbon atoms, has attracted extensive interest since its discovery in 2004 (Muszynski et al., 2008). Due to its unique properties, such as high surface area, high electrical conductivity, exceptional thermal properties and strong mechanical strength (Stankovich et al., 2006a,b; Lee et al., 2008; Zeng et al., 2010), immense interest has been indeed spurred in designing novel graphene-based materials for a variety of technological applications such as nanoelectronics, biosensing, electromechanical resonators, H_2 production and storage, batteries, biofuel cells, drug delivery and catalysis (Allen et al., 2010; Stoller et al., 2008; Subbiah et al., 2010; Guo et al., 2010; Stankovich et al., 2006a,b; Zhang et al., 2010). To produce single- or few- or multi-layered graphene nanosheets, various approaches have been established which include chemical vapor deposition (CVD) of methane gas (Reina et al., 2009; Li et al., 2009), graphite oxide reduction (Tung et al., 2009), carbon nanotube unzipping (Kosynkin et al., 2009), and epitaxial growth of graphene resulted from the high temperature reduction of silicon carbide (Berger et al., 2006). Among them, chemical conversion from graphite powder to graphite oxide (GO) and further reduction to graphene is more attractive due to its economic feasibility and massive scalability.

However, these unique properties are associated with individual sheets, which put forwards the importance of surmounting its inherent disadvantages, such as hydrophobicity and easy-to-aggregation in aqueous solution. This challenge of obtaining well-dispersed graphene has been well addressed through covalent and uncovalent functionalization methods. Xu et al. have synthesized uncovalent functionalized graphene with a water-soluble pyrene derivative, 1-pyrenebutyrate (PB-) (Xu et al., 2008). An et al. have reported the graphene has been achieved by noncovalent functionalization of graphene with 1-pyrenecarboxylic acid in a polarity-controlled combination (An et al., 2010). Graphene has been covalent modified with a long-chain alkylamine by Niyogi et al. (2006) group. Shan's group has presented functionalization of graphene with biocompatible poly-L-lysine as a linker through a covalent amide group (Shan et al., 2009). However, noncovalent

Abbreviations: LBL, layer-by-layer; IL-NH_2, amine-terminated ionic liquid; SO_3^-, sulfonic acid; S-RGO, sulfonic acid (SO_3^-) functionalized graphene; IL-RGO, amine-terminated ionic liquid functionalized graphene; Gox, Glucose oxidase; AA, ascorbic acid; DA, dopamine; UA, uric acid.

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functionalization of graphene sheets is through π–π interactions and vander Waals interaction, which is less stable compared with the covalent modified graphene through covalent interactions (Liu et al., 2009; Xu et al., 2008; Shan et al., 2009). Our methodology introduces sulfonic acid groups and the amine-terminated ionic liquid (IL-NH_2) in graphene in a simple way (Yang et al., 2009a,b; Si et al., 2008). The charged -SO_3^- units prevent the graphitic sheets from aggregating in solution after the final reduction stage of the graphene oxide, thereby yielding isolated sheets of lightly sulfonated graphene with improved water solubility. The cations of IL-NH_2 contribute to a well dispersible graphene-based material with good stabilization *via* electrostatic repulsion. Consequently, it makes it a possible route to harnessing excellent properties of graphene sheets for applications through incorporating them into composite materials (Kosynkin et al., 2009).

Recently, considerable efforts have been made to fabricate different graphene-based nanocomposites and explore their applications in various fields (Zhang et al., 2011; Bai et al., 2011). Layer-by-layer assembly method is of intrinsical advantages to assemble ultrathin films of a variety of organic and inorganic compounds in a simple and inexpensive manner, with thickness controllable in the nanometer range (Zhu and Tour, 2010). However, the formation of graphene-based LBL films has been much less discussed in the literature, especially fabrication of the two carbon-based nanomaterials (Kong et al., 2009; Yu and Dai, 2010). Herein, to fabricate the chemically self-assembled graphene thin films, the positively charged IL-RGO are alternately complexed with the negatively charged S-RGO through an electrostatic LBL process. Simply by varying the number of LBL depositions, the physical properties of the constructed thin films, such as film thickness or optical transparency, can be readily manipulated (Park et al., 2011).

In this work, a simply sequential immersion method was employed to fabricate a novel biosensor by {IL-RGO/S-RGO}$_n$ composite material, whose unusually electronic properties make it suitable for electrochemical sensing. The modified procedure was shown in Scheme 1 (supporting information). Except to overcome the unsatisfying hydrophobicity of pure graphene, the IL-RGO/S-RGO films can not only enhance the electrochemical response with its good ionic conductivity, but also sustain most of the continuous π-electronic structure of graphene sheets and introduce a positive and negative charge in aqueous solution, which realized the immobilization of glucose oxidase *via* electrostatic interaction under mild conditions. Herein, we also demonstrated an excellent electrocatalytic activity toward H_2O_2 at {IL-RGO/S-RGO}$_n$ modified electrode. What is more, a third generation glucose biosensor based upon {IL-RGO/S-RGO}$_n$ films was constructed with GOx as the biological recognition element. Because of the synergetic affects of the two conductive graphenes, the direct electron transfer can be efficiently achieved between electrode surface and the immobilized GOx. The mediatorless on-line detection of glucose was realized continuously at a potential of −200 mV in the striatum of rat with on-line microdialysis system. The construction of on-line microdialysis system and the performance of the glucose biosensor with respect to sensitivity, selectivity and the detection limit *etc.* were presented and discussed in this work. Finally, the prepared biosensor was applied to monitor the variation of glucose concentration in rat's striatum stimulated by intraperitoneal (i.p.) injection of 30 μL insulin injection.

2. Experimental

2.1. Materials

Graphite powder (GO, 99.5%, 325 mesh) and 1-Methylimidazole (>99%) were purchased from TCI Development Co., Ltd (Shanghai, China). 2-Bromoethylamine hydrobromide (99%) and Hydrazine solution (85%) were obtained from Shanghai Darui Finechemical Co., Ltd (Shanghai, China). Unless otherwise stated, other reagents were of at least analytical grade and used as received. All aqueous solutions were prepared with distilled water.

2.2. Fabrication of {IL-RGO/S-RGO}$_n$/GOx/Nafion biosensor through layer-by-layer self-assembly

The glassy carbon (3-mm diameter, CHI Company) electrode (GCE) was used in both off-line and on-line electrochemical experiments. Prior to surface modification, the GC electrode was polished with 0.05 μm alumina followed by successive sonication in acetone, HNO_3 (1:1, v/v), NaOH (50%, w/w) and pure distilled water. Afterward, the clean GCE was immersed into IL-RGO dispersion (5 mg/mL) for 30 min to adsorb a positively charged layer, then, washed with distilled water and dried under ambient condition. The IL-RGO modified electrode was sequentially immersed into the prepared anionic S-RGO dispersion (2 mg/mL) for 30 min again, on which a negatively charged film was adsorbed onto the IL-RGO-GCE, followed by the same washing and drying procedure. This affords one bilayer of IL-RGO and S-RGO with a notation of {IL-RGO/S-RGO}$_1$. Multilayer films were fabricated by alternating the deposition between IL-RGO and S-RGO until desired number of bilayers was achieved. For co-fabrication of glucose oxide (GOx) onto the surface of (IL-RGO/S-RGO)$_n$, an additional immersion in the IL-RGO suspension was firstly performed to introduce the positive charge. After drying and washing, it was then soaked in 10 mg/mL GOx solution (0.1 M phosphate-buffered saline, pH 7.4) for 24 h at 4 °C. At this pH, GOx (pI = 4.5) bears a net negative charge, thus allowing electrostatic attraction with positively charged IL-RGO film. Finally, a thin film of 0.5% Nafion solution was coated. The resulting {IL-RGO/S-RGO}$_n$/GOx/Nafion electrode was stored at 4 °C when not in use.

2.3. Animal experiments

All procedures involving animals were conducted with the approval of the Animal Ethics Committee in ECNU, China. Male Sprague–Dawley rats (weight ranges from 200 to 250 g) were purchased from Shanghai SLAC Laboratory animal Co. Ltd and acclimatized for 4 days. Then the rats were anesthetized with chloral hydrate (initial dose of 300 mg/kg (i.p.) with additional doses of 50 mg/kg (i.p.) as needed to maintain anesthesia) and wrapped in a homeothermic blanket (Beijing Tide-Gene Biotechnology Development Center). The rats were placed in a stereotaxic frame (Beijing Tide-Gene Biotechnology Development Center) with the incisor bar set at 5 mm above the interaural line and appropriately placed holes were drilled through the skull. The microdialysis probe (CMA/110/111 Tub) was implanted in the striatum at the site of 2.5 mm anterior to bregma, 2.5 mm lateral from midline, and 7.0 mm below dura. In order to reduce the injury to the rat, the microdialysis probe should be implanted into the striatum of rats slowly within 30 min with special care.

2.4. In vivo experimental design

The *in vivo* experiment was performed as previously reported (Shi et al., 2003; Yu et al., 2011a,b). Firstly, aCSF solution in one syringe was pumped into the on-line microdialysis system at 2.0 μL/min and flowed to the thin-layer electrochemical flow cell. After a stable baseline was achieved, the syringe selector was then switched to another syringe. The current started to increase slowly and reached a plateau within several minutes, which was ascribed to the biosensing of glucose in the striatum of rat. At last, 30 μL