Glucose oxidase and graphene bionanocomposite bridged by ionic liquid unit for glucose biosensing application

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A B S T R A C T

Ionic liquid functionalized graphene (IL-graphene) composite was synthesized successfully through an epoxide ring-opening reaction between graphene oxide (GO) and amine-terminated ionic liquid (NH2-IL). The IL-graphene exhibited good electronic conductivity, stability and electrocatalytic activity towards the reduction of O2 and H2O2. Then negatively charged glucose oxidase (GOD) was immobilized onto the composite matrix simply by ionic exchange. The ionic liquid here could improve the dispersibility of graphene and provide a favorable conductive microenvironment for the immobilized GOD, thus promote its direct electron transfer at the GC electrode. This novel IL-graphene–GOD bionanocomposite could act as a biosensor towards the detection of glucose with a linear response up to 16 mM. In this report, the method for immobilizing GOD by ionic interaction is of universality and has widespread use, even in other biological systems, which brings a forceful combination between GOD and IL-graphene. Besides, the biosensor is easy to prepare, have good stability, and will have potential application in glucose detection.

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1. Introduction

Graphene, a novel kind of two-dimensional single-atom carbon sheet with large surface-to-volume ratio, high conductivity, has attracted tremendous research interest from both the theoretical and experimental scientists recently [1,2]. With a special structure of a single layer of carbon atoms in a closely packed two-dimensional lattice, graphene has many unusual properties, such as high carrier transport mobility, superior mechanical flexibility and excellent thermal/chemical stability. Owing to these properties, graphene has many potential applications in supercapacitors [3], optoelectronic devices [4], nanocomposites [5], and biosensors [6,7]. However, a great obstacle in the synthesis of bulk-quantity graphene sheets is aggregation, which limits many of their applications, because most of their remarkable performances are based on individual sheet. As for this, many efforts have been addressed to prevent graphene sheets from aggregating through covalent [8,9] or noncovalent functionalization [10].

Glucose has always been a concentrated analyte because of its vital effect in the treatment of diabetics. Tremendous interests have been focused on the chemical sensor to detect the concentration of glucose in vitro or in vivo [11–13]. Enzymeless glucose sensors based on carbon materials [14], heavy metals [15], and transition metals [16] have long-term stability, but usually the sensitivity and selectivity are not satisfying. As a result, more efforts have been contributed to glucose biosensors based on glucose oxidase. For instance, many researchers used carbon nanotubes (CNTs) as electron transfer relay between GOD and electrode substrate to construct glucose biosensors [17,18]. Investigations of glucose biosensors based on graphene materials are booming recently. Considering the large specific surface area, unusual electronic conductivity, and compatibility with biomolecules of graphene, it is reasonable for us to suppose that graphene-based materials can be a good choice for constructing glucose biosensor.

Herein, as a part of our ongoing development for functionalization of graphene sheets and applying them in electroanalytical chemistry, we synthesized an ionic liquid-functionalized graphene nanocomposite. Then a novel bionanocomposite of graphene and GOD bridged by ionic liquid unit was obtained by ionic exchange. Due to its widespread use in biological systems, ionic interaction acted as a simple, but powerful approach to guarantee efficient bindings. Ionic liquid in this experiment plays three important roles: firstly, the dispersibility of graphene is improved by the electrostatic inter-sheet repulsion of ionic liquid; secondly, it is a bridge to connect graphene and the GOD forcefully; thirdly, it is also an electron transfer mediator between GOD component and electrode substrate. The unique properties of both graphene and ionic liquid make this novel nanocomposite a promising platform.
for the construction of mediator-free enzymatic biosensors. This paper proposes a novel method for fabricating glucose sensors by ionic interaction. This method provides an alternative to the usual physical adsorption which is weak and unstable, and to the covalent combination which is complicated and uncontrollable in the preparation.

2. Experimental

2.1. Reagents

Graphite powders (320 meshes) were of spectroscopic purity and were purchased from Shanghai Chemicals, China. Hydrazine solution (50 wt.%) and ammonia solution (28 wt.%) were obtained from Sinopharm Chemical Reagent Co., Ltd. 3-Bromopropylamine hydrobromide (98%) and 1-methylimidazole were obtained from Aldrich. And 1-methylimidazole was distilled at reduced pressure before use. Hydrogen peroxide solution (30%) was purchased from Beijing Chemical Reagent (Beijing, China), and a fresh solution of \( \text{H}_2\text{O}_2 \) was prepared daily. Glucose oxidase (EC 1.1.3.4, Type X-S, lyophilized powder, 100 units/mg, from Aspergillus niger) and D-(-)-glucose (>99.5%) was obtained from Sigma. Glucose stock solution was stored overnight at room temperature before use. Unless otherwise stated, other reagents were of analytical grade and used as received. Aqueous solutions were prepared with double-distilled water from a Millipore system (>18 MΩ cm).

2.2. Instruments

Cyclic voltammetric (CV) measurements were performed with a DyneChem Electrochemical Analyzer (DyneChem, China) in a conventional three-electrode system with bare or modified glassy carbon electrode (GCE, \( d = 3 \text{ mm} \)) as working electrode, a platinum wire as the counter electrode and Ag/AgCl (saturated KCl) as reference electrode, respectively. Transmission electron microscopy (TEM) micrographs were obtained using a JEOL 2000 transmission electron microscopy operating at 200 kV. AFM images were obtained with a Digital Instruments nanoscope IIIa (Multimode, Veeco), operating in tapping mode. X-ray photoelectron spectroscopy (XPS) analysis was carried out on an ESCALAB MK II X-ray photoelectron spectrometer. Fourier transform infrared spectroscopy (FTIR) was recorded on a CaF\(_2\) substrate with a Bruker Tensor 27 Spectrometer.

2.3. Preparation of graphene and ionic liquid composite (IL-graphene) and pure graphene

Graphene oxide was prepared using a modified Hummers’ method which was originally presented by Kotovnykhova and colleagues [19,20]. 1-(3-Aminopropyl)-3-methylimidazolium bromide (NH\(_2\)-IL) was prepared according to our previous work [21]. IL-graphene was also synthesized according to the previous report in our group [22]. In general, NH\(_2\)-IL (15 mg) was added into 15 mL GO aqueous dispersion (0.5 mg/mL). Then KOH (15 mg) was added into the above vessel and the obtained turbid mixture was subjected to ultrasonication until it was transformed into transparent and homogeneous dispersion. The resulting solution was vigorously stirred at 80°C for 24 h. The synthesized IL-graphene was subjected to centrifugation, washed with ethanol and water alternately, and finally air-dried. Graphene, which was used in control experiments, was prepared according to a typical procedure of hydrazine reduction in alkaline solution [2].

![Fig. 1. Illustration of the ionic exchange process and construction of the IL-graphene-GOD bionanocomposite modified glassy carbon electrode.](image)

2.4. Preparation of IL-graphene-GOD-modified GCE

The GCE was polished with 1.0, 0.3, and 0.05 μm α-alumina powders successively, and thoroughly rinsed by ultrasonication with deionized water between each polishing step and then dried under N\(_2\) before use. Then 10 μL of homogeneous IL-graphene dispersion (0.05 mg/mL) was dropped onto the surface of the GCE. After dried in air for 12 h, the IL-graphene-modified GCE (GCE/IL-graphene) was obtained. The as-prepared modified GCE was soaked in phosphate-buffer saline (PBS, 0.05 M, pH 7.4) containing GOD (2.5 mg/mL) at 4°C for 24 h to obtain the IL-graphene-GOD modified electrode (GCE/IL-graphene-GOD). GOD, with its isoelectric point \( (p_I) \) at about 4.5, was negatively charged at this pH, and it was favorable for negatively charged GOD to exchange with Br\(^-\) in this condition. The prepared GCE/IL-graphene-GOD was thoroughly rinsed with PBS for three times to remove the unwanted GOD by weak physical absorption before use. The IL-graphene-modified and graphene-modified electrode used in the control experiments were prepared similarly by dropping 10 μL of IL-graphene and 10 μL graphene dispersion of the same concentration onto the GCE.

3. Results and discussion

3.1. Characterization of GO and IL-graphene

The illustration of the ionic exchange process and the conformation of the modified electrode were showed in Fig. 1. Fig. 2a was a typical AFM image of the diluted graphene oxide dispersion on a newly cleaved mica surface. The AFM analysis revealed that the interlayer spacing of exfoliated GO obtained in this work was ca. 1 nm (height profile, Fig. 2b), indicating that exfoliation of graphite down to individual GO nanosheets was indeed achieved. The planar GO sheets with rippled structure were shown by TEM in Fig. 2c.

TEM was employed to characterize the prepared IL-graphene (Fig. 3). Different from the GO images, IL-graphene displayed a flake-like shape with a wrinkled structure due to the presence of lattice defects after the chemical reaction. The prepared GO, IL-graphene, and graphene in this work can form stable and homogeneous aqueous dispersion after ultrasonic treatment, as shown in Fig. 3. After storing for a longer time (three months), however, the graphene dispersion tended to agglomerate while the IL-graphene remained good dispersion because of the stabilization of ionic liquid (Fig. S1).

GO and IL-graphene were characterized by XPS in Fig. 4. It was obvious that GO and IL-graphene had similar absorbance peaks, except that after reacting with ionic liquid, the peak at ∼286.7 eV (C—O—C in epoxy/ether) was greatly decreased. Besides, there was also an additional peak at ∼285.9 eV in IL-graphene, which accompanied the notable decrease in epoxy groups. The new component can be attributed to the combination of the C—N bond which newly formed after reaction, and the C—N bond from the imidazolium ring of the ionic liquid.
FTIR was performed to characterize the resulting GO, IL-NH2, and IL-graphene (Fig. 5). The peaks at 1258 cm$^{-1}$ and 873 cm$^{-1}$ were ascribed to the epoxy groups in GO. The CH$_3$(N) stretching, CH$_2$(N) stretching, and ring in-plane asymmetric stretching arising from imidazolium ring were observed at ca. 1170 cm$^{-1}$, which were characteristic in IL-NH$_2$. In the IL-graphene, the characteristic peak of epoxy group in GO and primary amine in IL-NH$_2$ decreased and a new peak at 1350 cm$^{-1}$ appeared, corresponding to the N connected to C in conjugated system. The FTIR spectra together with the XPS spectra accorded well with our previously reported works [22,23], indicating the covalent reaction between amidocyanogen of IL-NH$_2$ and the epoxy group in GO sheets occurred successfully.

### 3.2. Electrocatalytical property of O$_2$ and H$_2$O$_2$ at the IL-graphene-modified electrode

The IL-graphene-modified electrode exhibited excellent electrocatalytical property towards the reduction of O$_2$ and H$_2$O$_2$. Fig. S2a showed the CVs at the IL-graphene-modified, graphene-modified and bare electrode in N$_2$ saturated and O$_2$.
saturated PBS solution (0.05 M, pH 7.4). An obvious reduction peak of O₂ was observed at ca. −0.4 V at graphene-modified electrode. The IL-graphene-modified electrode had more obvious catalysis effect with the peak potential at about −0.3 V, and the onset potential was more positive than that at the graphene-modified electrode. Besides, both of them were much more positive than that at the bare GCE. The electrocatalytic performance of the GCE/IL-graphene towards the reduction of H₂O₂ was also researched, as shown in Fig. S2b. The reduction potential was around −0.4 V, with its onset potential more positive than 0 V. In contrast, the GCE/graphene was not so active as that at the GCE/IL-graphene, and the difference here might be attributed to the existence of ionic liquid. The above results indicated that the IL-graphene composite had admirable electrocatalytical property, which was in favor of further application for detecting glucose. It is reasonable to conclude that the good electrocatalysis of the IL-graphene-modified electrode is owing to the extraordinary electron transfer property of the unique two-dimensional carbon nanostructure of graphene. The ionic liquid here has no detectable electrocatalytic activity for H₂O₂ and O₂ itself, but it plays an enhanced electrocatalytic effect to graphene for the improved dispersibility and facilitated electron transfer of ionic liquid to graphene.

3.3. Direct electrochemistry of GOD in the bionanocomposite

Fig. 6a showed the CVs of IL-graphene–GOD, IL-graphene modified electrode and bare electrode in N₂-saturated PBS solution (0.05 M, pH 7.4). A pair of well-defined redox peaks was observed at the GCE/IL-graphene–GOD (Fig. 6a, solid). The formal potential (E°) calculated by averaging the cathodic and anodic peak potentials was found to be ca. −0.45 V, which was close to the standard electrode potential of GOD [24]. The ratio of the anodic peak over the cathodic peak was nearly to 1, and the peak-to-peak separation (ΔEₚ) was about 49 mV. From the characteristic redox waves of the GCE/IL-graphene–GOD, we could conclude that the direct electron transfer of GOD had been achieved. In the control experiment, however, the GCE/IL-graphene did not show such redox peaks (Fig. 6a, dashed). This indicated that the redox waves should be ascribed only to GOD. These features were the characteristic of the reversible electron transfer process of the active redox center (flavin adenine dinucleotide, FAD) in glucose oxidase [25]. With the advantages of both graphene and ionic liquid, IL-graphene here can provide a favorable and conductive microenvironment for the immobilized GOD and thus promote its direct electron transfer at the GCE. For comparisons, CVs of graphene–GOD modified GCE were also measured (Fig. S3). It was found that graphene–GOD modified GCE could also obtain direct electron transfer of GOD, however, the current decreased evidently after thoroughly wash as GOD was adsorbed to graphene by weak physical adsorption, and no immobilizing agent was used.

The influence of the scan rate on the cyclic voltammetric performance of the GCE/IL-graphene–GOD was also investigated (Fig. 6b). The curves had a strict linear relationship (up to 300 mV s⁻¹), and the peak-to-peak separation was small, indicating that the redox process of the modified electrode was a reversible and surface-confined process.

It is well known that the biocatalytic reaction of glucose oxidase to glucose involves reduction of the flavin group (FAD) imbedded in the enzyme by reacting with glucose to give the reduced form of the enzyme (FADH₂), followed by reoxidation of the flavin by equal molecular oxygen to regenerate the oxidized form of the GOD (FAD) [11]. The process can be illustrated as follows:

\[
\text{GOD(FAD) + glucose} \rightarrow \text{GOD(FADH}_2\text{)} + \text{gluconolactone}
\]  

(1)

\[
\text{GOD(FADH}_2\text{)} + \text{O}_2 \rightarrow \text{GOD(FAD)} + \text{H}_2\text{O}_2
\]  

(2)

In general, the amperometric detection of hydrogen peroxide can be carried out either by oxidation or reduction. However, in the practical clinic analysis which is conducted by the oxidation method, some endogenous reducing species, such as ascorbic acid (AA) and uric acid (UA) and some drugs (e.g., acetaminophen), are also electroactive [26], and can be oxidized during the oxidation process, which may interfere with the detection of glucose. The reduction method was undertaken to avoid interference of these molecules in our experiment.

3.4. Performance of the IL-graphene–GOD-based glucose sensor

Fig. 7 shows the CVs of the IL-graphene–GOD-modified electrode in PBS (0.05 M, pH 7.4) containing various concentrations of glucose under the condition of air saturation. The peak current originating from reduction of O₂ decreased linearly with the increase of the glucose concentration. The amperometric response decreased linearly with the concentration of glucose ranging from 2 to 16 mM (correlation coefficient, \( R = 0.997 \)). The amperometric response was taken at the potential −0.48 V instead of the potential of O₂ reduction (at ca. −0.35 V) as the response here was more obvious. It is well known that the normal range of blood glucose concentration is 80–120 mg/dl (4.4–6.6 mM) [11], so our glucose nanobiosensor is adequate to practical application for detecting blood sugar concentration. And the detection based on of O₂ reduction can
Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACCU-CHEK Active (mM)</th>
<th>Sensor proposed (mM)*</th>
<th>RSD (%) (n = 5)</th>
<th>Glucose added (mM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.8</td>
<td>4.02</td>
<td>3.8</td>
<td>0.5</td>
<td>102</td>
</tr>
<tr>
<td>2</td>
<td>4.4</td>
<td>4.26</td>
<td>3.9</td>
<td>0.5</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td>5.3</td>
<td>5.35</td>
<td>4.2</td>
<td>0.5</td>
<td>103</td>
</tr>
<tr>
<td>4</td>
<td>7.8</td>
<td>7.68</td>
<td>4.7</td>
<td>0.5</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>6.2</td>
<td>6.17</td>
<td>3.6</td>
<td>0.5</td>
<td>95</td>
</tr>
<tr>
<td>6</td>
<td>6.7</td>
<td>6.66</td>
<td>4.0</td>
<td>0.5</td>
<td>106</td>
</tr>
</tbody>
</table>

* Average of five determinations.

To avoid the interference of some coexisting biomolecules, such as AA and UA. Nowadays, diabetes has become one of the leading causes of death and disability in the world, so fast and accurate detection of glucose by electrochemical glucose sensor is of great importance. And our method of using IL-graphene–GOD as a biobiosensor is of facility and universality. The linear range in this method is much wider than many reported methods, such as 0.04–0.28 mM for GOD immobilized on the colloidal gold modified carbon paste electrode [27], 0.1–1.1 mM for GOD immobilized on nitrogen-doped graphene modified electrode [28] and 0.08–12 mM for glucose oxidase–graphene–chitosan modified electrode [24].

The stability of the biosensor was investigated. The response current of the modified electrode changed by 5.4% of its initial response after storing in the refrigerator at 4 °C for two weeks. The relative standard deviation (RSD) of the current response to 4 mM glucose was 3.6% for 8 successive measurements, which proved that the biosensor also had good reproducibility.

3.5. Interference and sample analysis

As mentioned above, some reductive biomolecules, such as AA and UA, may coexist with glucose in blood serum, which may interfere with the determination of glucose based on the oxidation method. In this work, we detected glucose based on the concentration of oxygen and the potential was selected at more negative than −0.4 V. However, the signal of AA began after −0.1 V and the signal of UA was even more positive. In this situation, the interference of these coexisting molecules did not occur (Fig. S4).

To verify the reliability of the sensor, the IL-graphene–GOD modified electrode was applied for the determination of glucose in real blood serum samples. The results were shown in Table 1. Here the electrolyte solution used for the CV experiment was a mixing solution containing the same volume of blood serum and PBS. Beside, 0.5 mM standard glucose was added to estimate the recovery rate, and our sensor showed a good recovery (≥94%). The results obtained from the proposed sensor corresponded well with the widely accepted glucose meter “ACCU-CHEK Active” from Roche, with whom we have been doing a program to develop the glucose test trips.

4. Conclusions

We constructed a novel graphene and liquid composite, which dispersed well in water. The composite showed high electrocatalytic activity towards the reduction of O₂ and H₂O₂. Based on the perfect biocompatibility of both graphene and liquid, graphene and glucose oxidase biocomposite bridged by liquid unit was prepared for further research. The as-prepared bionanocomposite achieved direct electron transfer of glucose oxidase, and exhibited excellent performance as a glucose biosensor. Here, GOD was connected to the graphene material based on forceful ionic interaction instead of the usual method of weak physical adsorption. Ionic liquid here acted as not only a structure to bridge graphene and glucose oxidase, but also a promoter to electron transfer. The good selectivity, wide linear range and acceptable stability made this modified electrode a novel glucose sensor. Besides, graphene material in our experiment had the potential to overmatch carbon nanotubes because it can be obtained more easily. The modified electrode can be easily fabricated and used as an amperometric biosensor for the routine analysis of glucose in real blood serum samples. Further and applied investigation is on the way.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2011.11.023.

References


Biographies

Yuanyuan Jiang was born in 1986 in Shanxi Province, China. She received her B.S. degree in Jilin University in 2009. Then she moved to Changchun Institute of Applied Chemistry as a Ph.D. student, majoring in analytical chemistry and material electrochemistry. Her scientific interests focus on carbon and metal nanomaterials for electrochemical, analytical, and biosensing application.

Qixian Zhang studied in Jilin University (B.S.M.S., 1996–2003). Then he devoted himself to Changchun Institute of Applied Chemistry as research assistant in State Key Laboratory of Electroanalytical Chemistry with Professor Li Niu. He has done many significant jobs for the development of this lab, and given helpful guidance to many of the students. His interests focus on ionic liquid synthesis and application, material electrochemistry.

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Li Niu received his Ph.D. degree in Changchun Institute of Applied Chemistry (CIAC) under the guidance of Professor Shaojun Dong in 1998. After postdoctoral work at Abo Akademi University (Finland, with Ari Ivaska) he joined the State Key Laboratory of Electroanalytical Chemistry of CIAC as a professor of Chemistry. Now he is the master of the Engineering Laboratory for Modern Analytical Techniques, c/o State Key Laboratory of Electroanalytical Chemistry. He is the Member of Chinese Chemical Society and Member of International Society of Electrochemistry. He has published over 100 papers, and his research interests concentrate in carbon and metal nanoparticles based materials for electrochemical, analytical, and biosensing application, ionic liquid synthesis and application, spectroelectrochemistry, electrochemical instrumentalization.