Simple Fabrication of a Highly Sensitive Glucose Biosensor Using Enzymes Immobilized in Exfoliated Graphite Nanoplatelets Nafion Membrane

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A novel and highly sensitive electroanalytical sensing nanocomposite material is reported for the development of a glucose biosensor. Exfoliated graphite nanoplatelets (xGnP) were tested to enhance the sensing capability. The xGnP has a diameter of 1 μm and a thickness of 10 nm, on average. The glucose biosensing interface was prepared by casting glucose oxidase and xGnP in a Nafion water–isopropyl-alcohol solution with a high concentrated organic solvent (85 wt%). The resulting biosensors showed rapid response time within 5 s, limits of detection of 10 μM glucose ($S/N = 3$), a linear detection range up to 6 mM, and high sensitivity of 14.17 μA/(mM·cm^2) with an optimum glucose oxidase loading. The biosensors also showed good selectivity and long-term stability. These results indicate that xGnP can be an inexpensive alternative to carbon nanotubes for the fabrication of affordable high-performance biosensors.

Introduction

Nanotechnology offers the potential to increase biosensor sensitivity, response speed and selectivity. A wide variety of nanomaterials have been explored for their application in biosensors due to their unique chemical, physical, and optoelectronic properties.[1] For example, incorporation of carbon nanotubes (CNT) and fullerenes has greatly increased biosensor sensitivity and response speed due to their high chemical stability, high surface area, and unique electronic properties.[2–4] Carbon nanotubes have excellent electrocatalytic activities,[5] and have been shown to promote electron-transfer reactions involving hydrogen peroxide,[6] NADH,[7,8] cytochrome,[9] and ascorbic acid.[10] Unfortunately, the high price of CNT (ranging from 20 to 100s of dollars per gram) often makes them cost-prohibitive for some applications.

Exfoliated graphite nanoplatelets (xGnP) provide a more affordable alternative to CNT. These platelets consist of sp^2 hybridized carbon atoms arranged in a sheet like structure instead of the cylindrical geometry found in carbon nanotubes. Research at Michigan State University (MSU) has led to a process that can successfully produce exfoliated graphite nanoplatelets (xGnP) that are 5–10 nm in thickness and from 100 to 1000 nm in diameter.[11] With an expected cost on the order of $5/pound, these nanoplatelets could be a suitable substitute for carbon nanotubes and fullerenes.

Graphite microsized particles have been used in electrochemical sensors for years. Traditionally, they are used as a carbon paste with a mineral oil binder[12] or composites with Teflon[13] or epoxy.[14,15] The advantage of the carbon electrodes is their surface renewability. However, these biosensors are normally not very sensitive and their response is slow. CNT paste electrodes are more stable than graphite paste electrodes at high mineral oil loadings, and CNT composite electrodes showed much higher sensitivity and much lower overpotential compared to the graphite based electrodes.[16] The improved performance by CNT is attributed to their extremely high surface area. The development of inexpensive nano sized graphene sheets may lead to affordable biosensors with high sensitivity and fast response. In addition, due to the nanoscale thickness of these graphene sheets, versatile technologies such as layer by layer self assembly can be used for biosensor fabrication.

The objective of this work was to demonstrate the potential use of graphite nanoplatelets in the biosensor application.

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Biosensors can be prepared by immobilizing an enzyme that selectively reacts the analyte in the vicinity of an electrochemical device that can measure one of the reaction substrates or products. Glucose oxidase (GOx) was used to develop a glucose biosensor, which is the most widely studied biosensor due to the importance in the monitor of blood glucose for treatment and control of diabetes. GOx catalyzes the oxidation of glucose to gluconolactone:[17]

$$\text{glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2 \qquad (1)$$

The quantification of glucose can be achieved via electrochemical detection of H_2O_2. There are several methods to immobilize enzymes for glucose biosensor application. Nafion encapsulation of enzyme is a common practice to prepare biosensors.[17] Nafion is a sulfonated tetrafluorethylene copolymer that has been widely used as a proton conductor for proton exchange membrane, in fuel cells,[18] and biosensor applications.[19] The main advantages of Nafion in biosensor applications are its biocompatibility, excellent thermal and mechanical stability, mechanical strength, and antifouling properties. Pioneering work by Wang, et al. showed that Nafion was an effective solubilizing agent for carbon nanotubes that yielded CNT-based biosensors exhibiting both the efficient electrocatalytic action of CNT toward hydrogen peroxide and the antifouling/discriminative properties of Nafion films.[6] In this paper, a similar methodology was used to disperse xGnP in Nafion. A simple organic solvent casting method was used to develop a highly sensitive and fast responding glucose biosensor by the combination of xGnP, GOx, and Nafion.

Experimental Procedures

Reagents. Glucose oxidase (Type X-S, Aspergillus niger (EC 1.1.3.4), 179 units/mg), Nafion (5 wt% in lower aliphatic alcohol and water mixture), 2-propanol, glucose, sodium phosphate monobasic, and sodium phosphate dibasic were obtained from Sigma-Aldrich and used as received. Exfoliated graphite nanoplatelets (xGnP) with an average diameter of 1 μm and thickness of 10 nm were processed at the Michigan State University by a microwave process, followed by sonication and milling process.[11] All aqueous solutions in the processes were prepared with deionized (DI) water (>18.1 mΩ) supplied by a Barnstead nanopure Diamond-UV purification unit equipped with a UV source and final 0.2 μm filter.

Preparation of xGnP–Nafion Composite Film. A 0.5 wt% Nafion solution was prepared by diluting the 5 wt% Nafion stock solution with 2-propanol. Various amounts of xGnP were mixed with 1 mL of 0.5 wt% Nafion–isopropyl-alcohol solution by ultrasonication for ~30 mins. Prior to the surface modification, gold electrodes were cleaned in Piranha solution (7 parts by volume concentrated sulfuric acid and 3 parts 30% (v/v) hydrogen peroxide) for 20 s. The electrodes were then rinsed with DI water, and dried under nitrogen. A 60 μL aliquot of xGnP–Nafion solution was drop cast on the gold electrode with a surface area of 1.5 cm^2. The solvent was allowed to evaporate at room temperature.

Preparation of Glucose Biosensor. The enzyme suspension in a water–isopropyl-alcohol solution (85% alcohol) was prepared as follows: glucose oxidase was dissolved in 50 mM phosphate buffer solution (pH 7.4) to a final concentration of 20–60 mg/mL. Then, the casting solution was prepared by adding the xGnP–Nafion–isopropyl-alcohol suspension to the enzyme solution to meet the desired enzyme concentration. The suspension was sonicated for 5 mins using a bath sonicator (longer sonication is not recommended because it may denature the enzyme). From this solution, 60 μL was drop cast onto the gold electrode with an area of 1.5 cm^2. The coating was dried at room temperature for 1 h. Then, the modified electrodes were soaked in 50 mM phosphate buffer (pH 7.4) for 20 mins, washed thoroughly with DI water, and stored at 4 °C dry before use.

Electrochemical Measurement. Cyclic voltammetry and amperometric experiments were performed on an electrochemical analyzer (CH instruments, Austin, TX, model 650 A) connected to a personal computer. All experiments were carried out using a conventional three-electrode system with the gold electrode as the working electrode, a platinum foil as the auxiliary electrode, and a saturated Ag/AgCl electrode as the reference electrode. The cyclic voltammograms (CVs) of the xGnP–Nafion composite film were obtained in 5 mM $K_4Fe(CN)_6$ and 0.1 M KCl solution. In steady-state amperometric experiments, the potential was set at 700 mV versus Ag/AgCl electrode with magnetic stirring. All measurements were carried out in 50 mM phosphate buffer solution with a pH of 7.4.

Particle and Surface Characterization. The dispersion of xGnP in the Nafion solution was characterized using scanning electron microscopy (SEM, JEOL 6300F). A clear xGnP–Nafion suspension was filtered with an alumina membrane which has a pore size of 0.2 μm. The membrane was naturally dried and then a layer of osmium was coated with a pure osmium coater (NEOC-AN, Meiwa Shoji Co., Ltd, Japan) for 20 s for enhanced conductivity before SEM measurements. The morphology of GOx–Nafion and GOx–xGnP–Nafion composite films was characterized using scanning electron microscopy (JEOL 6300F) and atomic force microscopy (AFM, a Nanoscope IV version from Veeco Instruments (Santa Barbara, CA)) in tapping mode.

Results and Discussion

Dispersion of xGnP in Nafion and Choice of Solvent. The process used to produce exfoliated graphite nanoplatelets from natural crystalline graphite by microwave and milling has been previously reported.[11] BET surface area analysis showed a specific surface area of 100 m^2/g.[20] Assuming a hypothetical monolayer of graphite would exhibit a specific surface area close to 2700 m^2/g, and an interlayer spacing of 0.335 nm,[21] the average thickness of graphite nanoplatelets was estimated to be 5–10 nm.[20] The thickness of graphite nanoplatelets is also confirmed with transmission electron microscopy (TEM).[20] A major challenge of using graphite nanoplatelets in biosensor applications is their insolubility in most enzyme compatible solvents due to their large hydrophobic basal plane. Oxidation of graphite is a common way to achieve a homogeneous dispersion of graphite nanoplatelets aqueous solution.[22,23] However, this approach causes a decrease in conductivity. Recently we found that the polyelectro-

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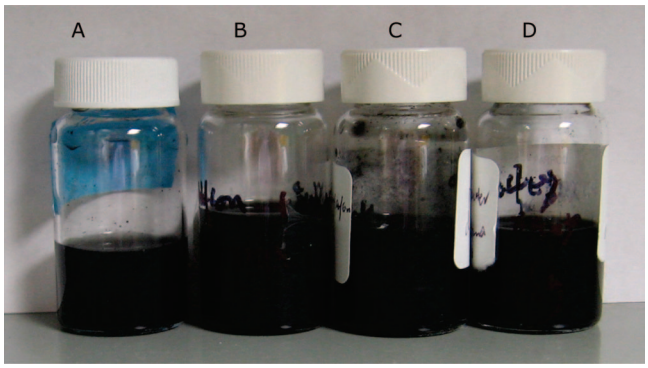

Figure 1. Photograph of vials containing xGnP–Nafion suspension: A 0.1 wt% xGnP in Nafion–water, B 0.1 wt% xGnP in Nafion–isopropyl-alcohol, C 1.0 wt% xGnP in Nafion–water, and D 1.0 wt% xGnP in Nafion–isopropyl-alcohol.

lytes previously used to solubilize carbon nanotubes can also stably disperse graphite nanoplatelets, such as poly(diallydimethylammonium chloride) (PDAC), sulfated poly(styrene) (SPS), and polyethyleneimine (PEI).[24] Since Nafion is a negative charged polyelectrolyte, it can be used to suspend graphite nanoplatelets in water or alcohol. Figure 1 shows a photograph of vials containing xGnP in Nafion water or isopropyl alcohol solution after settling for two weeks. At 0.1 wt% xGnP, both aqueous and isopropyl alcohol solutions show a good dispersion and relative stability. When the xGnP concentration increases to 1 wt%, however, the Nafion–isopropyl-alcohol suspension is more homogeneous than aqueous suspension because isopropyl-alcohol is a better solvent for graphite than water. In all the cases, there is some sedimentation after 24 hours which is largely due to the variation in graphite particle size. The degree of dispersion of xGnP in Nafion–isopropyl-alcohol was further illustrated by SEM. A few drops of xGnP–Nafion suspension were filtered with an alumina membrane. It is assumed that no aggregation would occur during the filtration as this process takes a few seconds. As shown in Figure 2, the extremely thin graphite nanoplatelets observed using SEM were almost translucent on the alumina filter membrane. Thicker graphite particles also exist, which we believe is mostly due to incomplete exfoliation of graphite during the microwave process, although agglomeration of nanoplatelets is also possible. Thus, this xGnP solubilization using Nafion opens their application in modifying electrode and biosensor preparation.

A simple method was developed by Tsai, et al. in which multiwalled carbon nanotubes, Nafion, and glucose oxidase were deposited as a nanobiocomposite film coating on a glassy carbon electrode, yielding a high-performance biosensor.[25] In our study, it was found that the biosensor based on xGnP using same method was not stable on gold surface during the electrochemical measurement. This may be due to the nonuniformity of the composite membrane as Nafion does not dissolve in water.[26] In addition, adhering of carbon on the gold surface may not be as good as on the glassy

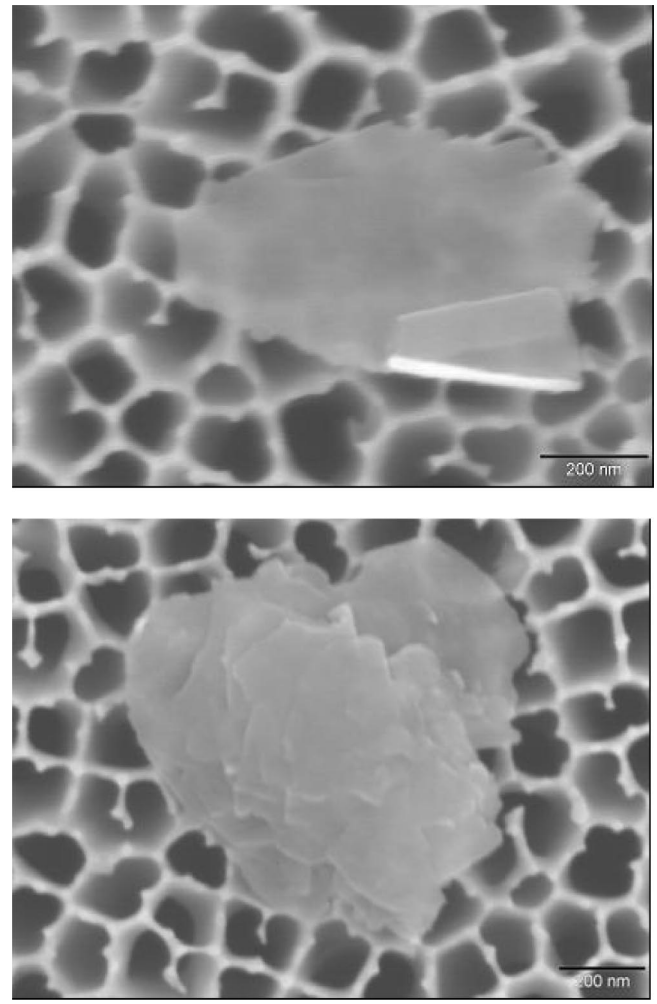

Figure 2. SEM images of xGnP dispersed in Nafion–isopropyl-alcohol solution filtered on the alumina membrane.

carbon surface. Karyakin et al. found that deposition of the enzyme–Nafion layers from water–organic solutions with a high concentration of organic solvent showed better sensitivity and stability. This is because Nafion solution was deposited from a solution where it is truly dissolved.[26] The enzymes in concentrated solvent can retain up to 100% of their initial activity because the enzyme becomes insoluble and exist as colloidal particles which are protected by Nafion. Since xGnP has a better solubility in Nafion–isopropyl-alcohol than water, a water–isopropyl-alcohol Nafion mixture (15 wt% water and 85 wt% isopropyl alcohol) was chosen for subsequent biosensor preparation to maximize the enzyme activity.

Evaluation of xGnP–Nafion Composite Film. Cyclic voltammetry is a useful tool to evaluate performance of xGnP–Nafion composite films as a transducer. Figure 3 shows the cyclic voltammograms at the different concentration of xGnP recorded in 5 mM ferrocyanide (in 0.1 M KCl). The Nafion modified gold electrode shows no redox peaks because Nafion film acts as a barrier to electron transfer. The addition of xGnP increases the peak current, and at 1 wt% xGnP, well defined oxidation and reduction peaks are

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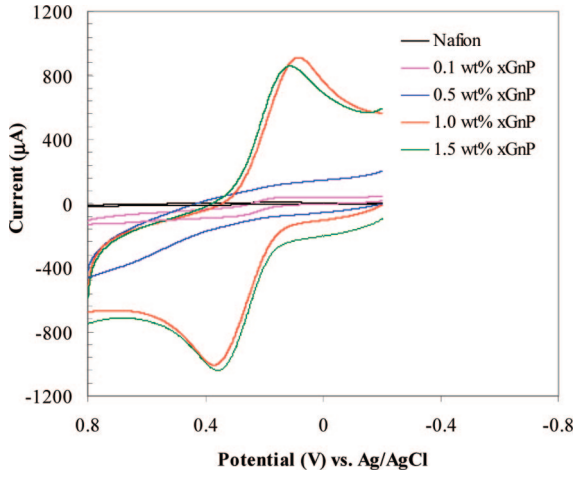

Figure 3. Cyclic voltammetric response of 5 mM $Fe(CN)_6^{4-}$ in 0.1 M KCl for xGnP-Nafion composite at different loading of xGnP (inner to outer): 0 wt%, 0.1 wt%, 0.5 wt%, 1.0 wt%, and 1.5 wt% xGnP, scan rate, 100 mV/s.

shown, although the peak separation is large, indicating slow electrode kinetics.[27] It is possible that nanocomposite film behaves as arrays of small electrodes.[25,28] Based on the Randles–Sevcik equation,[29] the electroactive surface area can be estimated:

$$I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} \gamma^{1/2} C \qquad (2)$$

where n is the number of electrons participating in the redox reaction, A is the area of the electrode, D is the diffusion coefficient of the molecule (equal to $(6.70 \pm 0.02) \times 10^{-6}$ $cm^2 s^{-1}$). C is the concentration of the probe molecule in the solution, and γ is the scan rate. The effective electroactive surface for 1 wt% xGnP–Nafion composite film obtained using eq 2 is $0.90 \pm 0.1 cm^2$. Figure 3 also shows that the increase of xGnP concentration to 1.5 wt% did not enhance redox peak current. This is mostly attributed to the limitation of the ability of Nafion to solubilize xGnP. At a high concentration of xGnP, most nanoplatelets are possibly agglomerated which could not effectively increase the electroactive surface area. Luong, et al. found that the addition of 3-aminopropyltriethoxysilane (APS) in Nafion–ethanol solution enhanced the solubility of CNT due to covalent bonding between amino groups and CNT.[30] Unfortunately, the use of APS as a solubilizing agent for xGnP along with Nafion did not effectively increase the redox peak current (data not shown). Based on these results, 1 wt% xGnP was used for subsequent biosensor experiments. Figure 4 shows the cyclic voltammograms of 1 wt% xGnP–Nafion composite film at different scan rates. Peak-to-peak separation increases with increasing scan rate, suggesting a quasireversible behavior.[31]

Figure 5 shows the catalytic activity of xGnP towards hydrogen peroxide evaluated with cyclic voltametry by

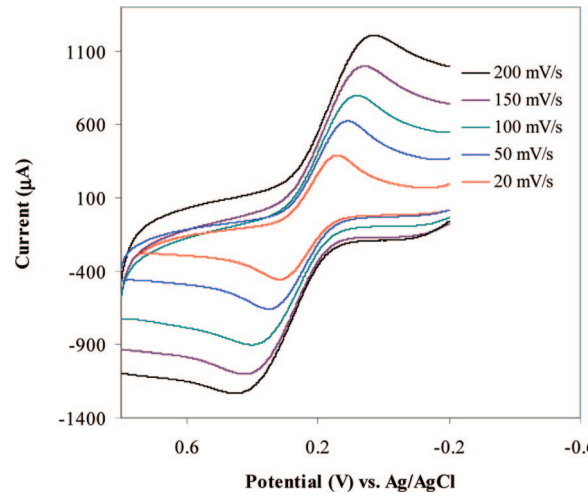

Figure 4. Voltammetric response of 1 wt% xGnP–Nafion composite modified gold electrode to 5 mM ferrocyanide in 0.1 M KCl at different scan rates.

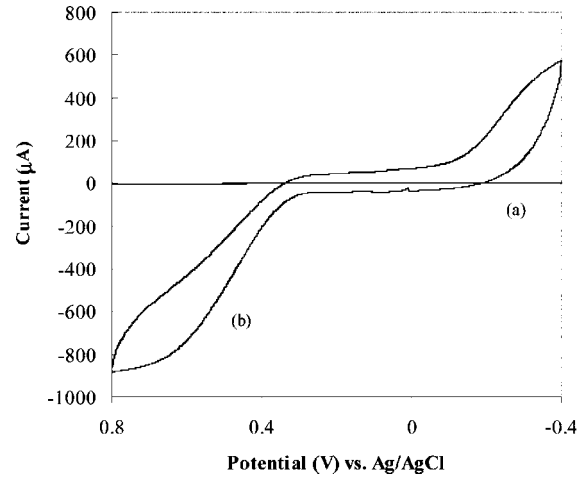

Figure 5. Cyclic voltammograms of 2 mM hydrogen peroxide in 0.05 M phosphate buffer solution (pH 7.4) at Nafion (a) and xGnP–Nafion (b) modified gold electrodes, scan rate, 20 mV/s.

adding 2 mM H_2O_2 in 50 mM phosphate buffer solution. The xGnP–Nafion modified electrode exhibits significant oxidation and reduction currents starting at around 0.3 and −0.15 V, respectively. In contrast, Nafion modified gold electrode shows very weak electrochemical response over this potential range. These results indicate that xGnP catalyzes the oxidation and reduction of the hydrogen peroxide; in this case hydrogen peroxide is not enzymatically generated, which lowers the overpotential of hydrogen peroxide detection. Thus, these graphite nanoplatelets have similar catalytic activity for hydrogen peroxide as carbon nanotubes,[25,32,33] but the same activity is not observed for microsized graphite epoxy composite electrode.[16]

Morphology of Biocomposite Films. Figure 6 shows the SEM images of GOx–Nafion and GOx–xGnP–Nafion composite films. Glucose oxidase is insoluble in water–isopropyl-alcohol mixture with highly concentrated organic solvent (85 wt%); rather it situates inside the colloid particle which is

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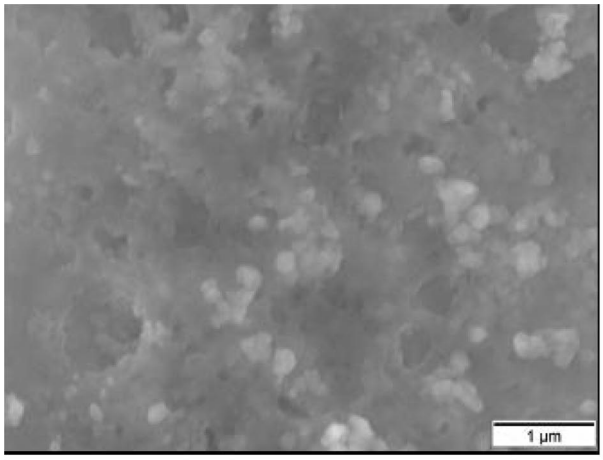
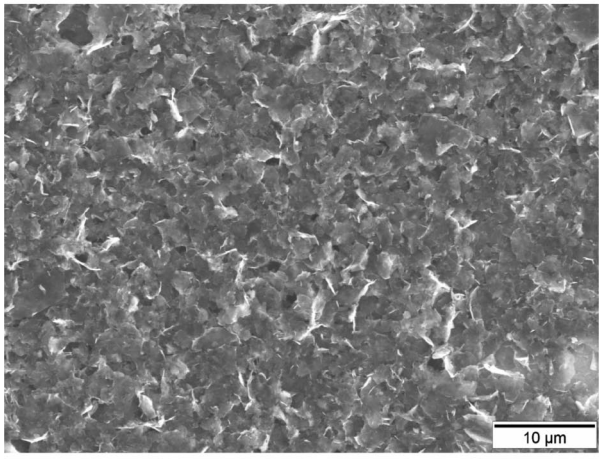


(a)



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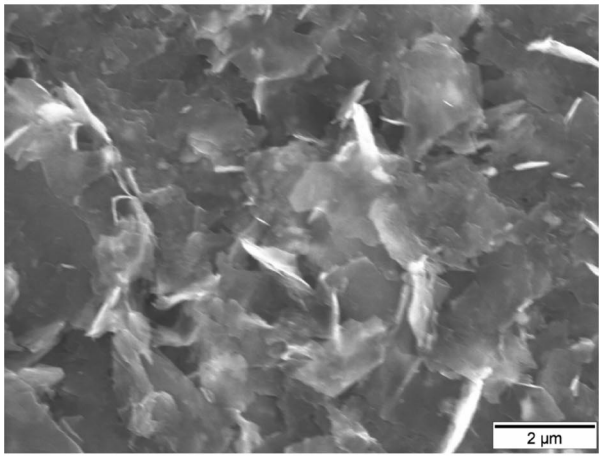

(c)

Figure 6. SEM images of GOx–Nafion film (a) (white dots are GOx nanoparticles) and GOx–xGnP–Nafion composite film (b), low magnification, and (c) high magnification.

protected by Nafion. As shown in Figure 6 (a), GOx forms nanoparticles with size less than 100 nm homogeneously dispersed in Nafion membrane. Figure 6 (b) and (c) show the surface morphology of GOx–xGnP–Nafion composite. At low magnification, it can be seen that xGnPs are packed densely which makes the coating as a conductive film. Most of nanoplatelets are laying flat on the surface although some edges of platelets are curled or sticking out of the surface, which makes a very rough surface. At a high magnification, the roughness of the surface is clearly shown. As shown in Figure 7, the general roughness of the biocomposite film is also confirmed by the AFM amplitude image. This rough surface is generally beneficial in increasing the sensitivity of an amperometric electrode. The GOx nanoparticles are not visible in the xGnP-Nafion composite, possibly they are absorbed on the graphite nanoplatelets or trapped between platelets, which can prevent the leakage of glucose oxidase during the electrochemical measurement.

Amperometric Measurement. Figure 8 shows the typical amperometric response of the GOx–xGnP–Nafion biosensor compared to the GOx–Nafion biosensor for the successive addition of various amounts of glucose at an applied potential of +0.7 V. In both cases, the GOx concentration was 3 mg/mL. The GOx–Nafion biosensor gives much weaker current responses than the GOx–xGnP–Nafion biosensor. The time to achieve 95% steady state current is no more than 5 s. The detection limit of glucose is 10 μM (S/N = 3). Figure 9 shows the calibration curves of GOx–Nafion and GOx–xGnP–Nafion biosensor. The linear detection range of xGnP-based glucose biosensor is 6 mM. The sensitivity of xGnP based glucose biosensor is calculated to be 14.17 ± 1.76 μA/(mM·cm^2) when a glucose oxidase concentration of 3 mg/mL was used. The sensitivity of xGnP-based glucose biosensor cast from 15 wt%/85 wt% water/isopropyl-alcohol is three times of that reported for carbon-nanotube-based biosensor cast from aqueous solution,[25] and this value is superior to most literature values for the glucose biosensors prepared with carbon nanotubes.

Varying Glucose Oxidase Concentration. The influence of glucose oxidase concentration on the biosensor sensitivity was also studied. As shown in Figure 10, the higher the concentration of glucose oxidase, the higher the sensitivity of the biosensor. Unfortunately, the increasing enzyme concentrations resulted in deterioration of the physical properties of the composite and thus in peeling of the composite layer from the gold surface during the electrochemical measurement. The stability of composite film could be improved with the increase of Nafion concentration, but the sensitivity of the resulting biosensor decreased (data not shown).

Selectivity and Storage Stability. The common existing interfering species in the physiological samples of glucose, such as ascorbic acid (AA) and uric acid (UA), can cause bias in the determination of glucose. The common interferences of AA and UA in the blood of human beings have normal concentration ranges of 34–79 μM and 0.18–0.42 mM, respectively.[34] Thus, the interfering effect of 0.1 mM ascorbic acid and 0.2 mM uric acid compared to 4 mM glucose (the average physiological concentration of blood glucose[35]) were evaluated at the potential of +0.7 V. As shown in Figure 11, the interfering signals of AA and UA are significant, representing 56.8% for 0.1 mM ascorbic acid and 125% for 0.2 mM uric acid of the signal from 4 mM glucose, respectively. Although Nafion is a negatively charged polyelectrolyte matrix, which is supposed to reduce the permeability of negatively charged substrates, Wang, et al. reported that the permselective properties of Nafion are

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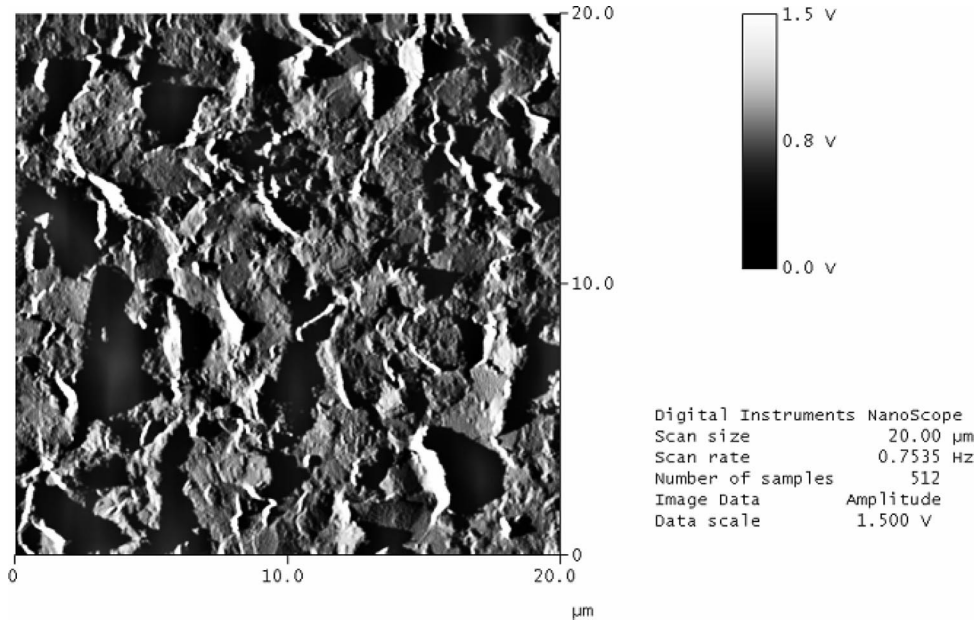

Figure 7. AFM amplitude image (20 × 20 μm) shows an overall roughness of the GOx–xGnP–Nafion composite surface.

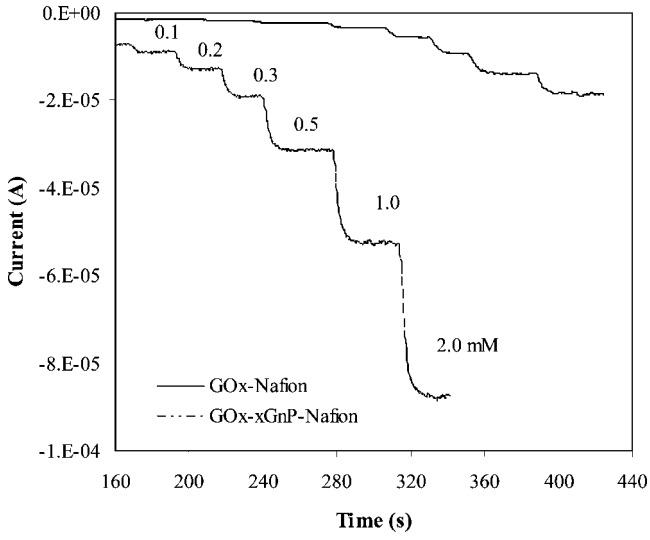

Figure 8. Amperometric response of xGnP–Nafion glucose biosensor (glucose oxidase, 3 mg/mL) at an applied potential of +0.7 V to successive addition of varied amount of glucose in a stirred 0.05 M, PH 7.4, phosphate buffer solution.

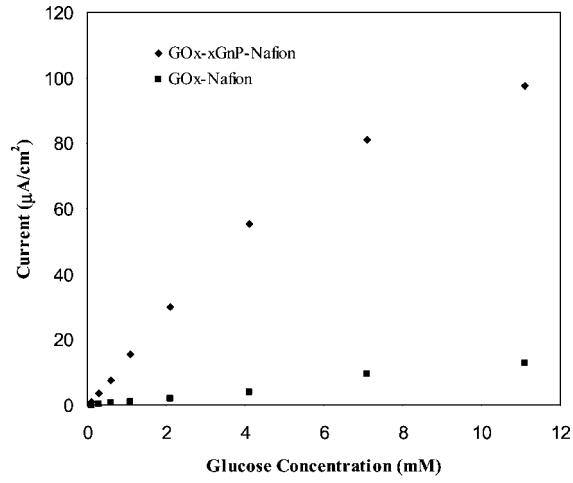

Figure 9. Calibration curves and linear range of Nafion glucose biosensors with and without xGnP at an applied potential of +0.7 V versus Ag/AgCl in 0.05 M PBS solution.

not sufficient to fully eliminate anionic interferences, especially at high potential.[6] We doubled the Nafion concentration in the casting solution and also added an additional layer of Nafion on the composite film, but there was no significant reduction in interference of AA and UA (data not shown). Rather, the sensitivity of biosensor was significantly reduced, because more Nafion in the casting solution could reduce the activity of enzyme[36] and the additional layer of Nafion could block fast electron transfer. Another method to prevent interference by electroactive compounds is to vary detection potential. As shown in Figure 5, the xGnP based biosensor has a wide operating potential range for detecting H_2O_2. For example, at the operating potential of −0.2 V, there is relatively strong current response with the addition of glucose, but the interference of UA and AA is completely avoided (Figure 11). Figure 12 shows the calibration curves of the xGnP–Nafion glucose biosensor operated at +0.7 V and −0.2 V. The sensitivity of the biosensor operated at −0.2 V is half of the sensitivity at +0.7 V, and the linear detection limit can be up to 5 mM. Thus, by varying operating potential, high sensitivity or medium sensitivity without interference can be achieved for the xGnP-based biosensors.

The long term storage stability of these biosensors was also studied. The biosensors were stored dry at 4 °C and their response to 0.5 mM glucose was measured intermittently. The best sensitivity of biosensor was found after a day or two of storage, and the first week was quite stable. After that, the response decreased to 75% of the initial value after one month storage and 50% after two months storage. The decrease in sensitivity is possibly due to the leakage of enzyme during electrochemical measurement. The enzyme may also be degrading with time.

(36) Karyakin, A. A.; Kotel'nikova, E. A.; Lukachova, L. V.; Karyakina, E. E.; Wang, J. *Anal. Chem.* **2002**, *74* (7), 1597.

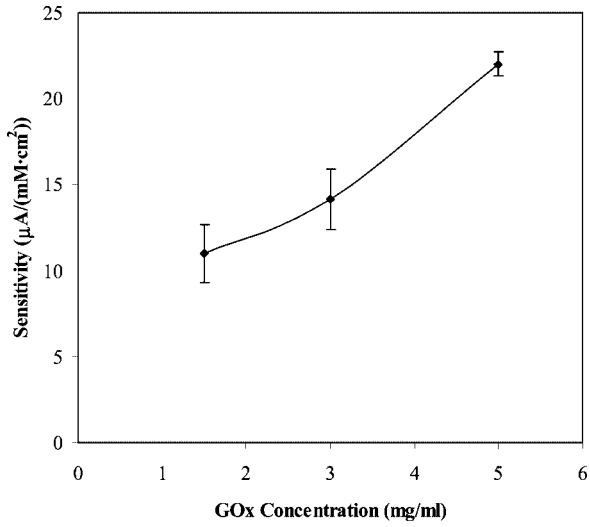

Figure 10. Effect of glucose oxidase loading upon the sensitivity of the biosensors.

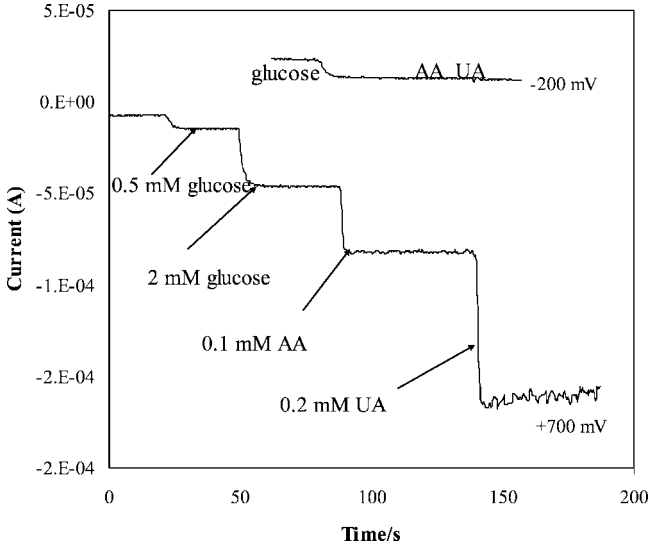

Figure 11. Effects of interfering signals of 0.1 mM ascorbic acid and 0.2 mM uric acid on the performance of GOx–xGnP–Nafion bioelectrode at different potentials.

Conclusion

In this paper, for the first time, we have demonstrated that graphite nanoplatelets can be used as an inexpensive alternative to carbon nanotubes in fabrication of a highly sensitive and fast responding glucose biosensor. A simple water–organic Nafion solution cast with a high content (85 wt%) of organic solvent was used to prepare glucose biosensor. The addition of xGnP greatly enhanced the redox peak current of ferrocyanide solution and also lowered the overpotential for monitoring enzymatically produced hydrogen peroxide. The xGnP based glucose biosensor exhibits excellent sensitivity, resistance to interference, and long-term stability. In fact, the sensitivity of the xGnP-based glucose biosensor cast from high concentrated organic solvent is three times the literature value of a carbon nanotube based biosensor cast from aqueous solution, and this value is superior to the most literature values for the glucose biosensors prepared with carbon nanotubes. Other enzymes can presumably be combined with graphite nanoplatelets to obtain useful biosensors. Thus, this simple, controllable method can be used for the mass production of affordable high sensitive biosensors.

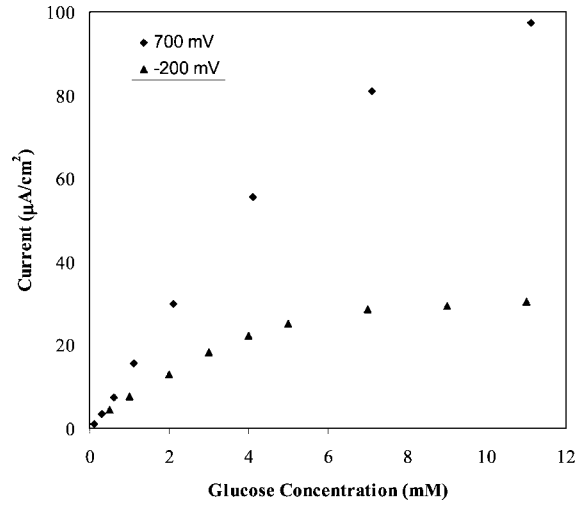

Figure 12. Calibration curves of the xGnP–Nafion glucose biosensor at different operating potential.

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