



Achieving direct electrochemistry of glucose oxidase by one step electrochemical reduction of graphene oxide and its use in glucose sensing



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ABSTRACT

In this paper, the direct electrochemistry of glucose oxidase (GOD) was accomplished at a glassy carbon electrode modified with electrochemically reduced graphene oxide/sodium dodecyl sulfate (GCE/ERGO/SDS). A pair of reversible peaks is exhibited on GCE/ERGO/SDS/GOD by cyclic voltammetry. The peak-to-peak potential separation of immobilized GOD is 28 mV in 0.1 M phosphate buffer solution (pH 7.0) with a scan rate of 50 mV/s. The average surface coverage is $2.62 \times 10^{-10} \text{ mol cm}^{-2}$. The resulting biosensor exhibited a good response to glucose with linear range from 1 to 8 mM ($R^2 = 0.9878$), good reproducibility and detection limit of 40.8 μM . The results from the biosensor were similar ($\pm 5\%$) to those obtained from the clinical analyzer.

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1. Introduction

A new report has found that there are now nearly 350 million people on Earth who suffer from diabetes [1]. Diabetes (diabetes mellitus) is classed as a metabolism disorder. A person with diabetes has a condition in which the quantity of glucose in the blood is too elevated. This is because the body does not produce enough insulin. This results in too much glucose building up in the blood. This excess blood glucose eventually passes out of the body in urine. Therefore, for the treatment and control of diabetes, the amount of blood glucose has to be monitored. Conventional techniques such as UV fluorescence [2], chemiluminescence [3,4] and titrimetry [5] have been reported in literatures. However, the conventional methods are generally time-consuming and difficult for an automated detection. Besides, most of these methods show limitations such as lack of sensitivity and susceptibility to interference by other substances in analyte samples. To overcome all these shortcomings, the electrochemical biosensor based on the direct electrochemistry between an electrode and the immobilized enzyme/protein is especially promising because of its simplicity, high sensitivity and selectivity [6–8]. Therefore, many methods are attempted to promote the direct electrochemistry of proteins. Among them, modifying electrode surfaces with proper surfactants and graphene has been demonstrated to be quite effective [9,10]. Surfactants are a type of

amphiphilic molecules with a polar head at one end and a long hydrophobic tail at the other. They can spontaneously adsorb on the interfaces of two phases with different polarities or associate into micelles in solutions. Because of the enhancement effect and the ability to change the properties of electrode/solution interface, surfactants have been widely used in electroanalytical chemistry [11,12]. SDS can increase the immobilization glucose oxidase on ERGO due to its excellent film forming ability. The single-chain surfactant, SDS, can form a stable monolayer containing negative charges on the electrode surface and the positively charged GOD molecules can be adsorbed inside the porous surface of SDS/ERGO/GCE and provide an effective electron-tunneling pathway between GOD and the surface electrode. The biocompatible characteristics of SDS would also provide a favorable microenvironment to keep the activities of the immobilized proteins [13]. Furthermore, it is simple and inexpensive to prepare modified electrode based on surfactants and to immobilize proteins. Also, graphene is currently making an impact in the field of electrochemistry, particularly its use as a sensor substrate [14–17]. The discovery of graphene by Andre Geim and Konstantin Novoselov in 2004 has opened a new chapter in science. Graphene has shown great application potential in various fields, such as ultracapacitors [18], batteries [19], fuel cells [20,21] and bioscience/biotechnologies [22–25]. In fact, most of the graphenes that were synthesized contain other elements, such as oxygen, so it is more reasonable to call the graphene “reduced graphene oxide” (rGO). This composite can be synthesized by various methods [26–40]. Among the current methods of generating reduced graphene oxide, hydrazine

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and sodium borohydride are used for the chemical reduction of exfoliated graphene oxide (GO) [41,42]. But, these reducing agents are unstable and dangerously toxic. Several groups investigated the synthesis of rGO with the eco-friendly methods [43–50]. Electrochemical methods are one promising green strategy for graphene synthesis, and several research works have been reported [51,52]. To the best of our knowledge, the use of ERGO/SDS nanocomposite film for immobilization of GOD and its application as a glucose biosensor based on the direct electron transfer have not yet been reported. The proposed glucose biosensor exhibited excellent electrochemical characteristics and high analytical performance in terms of sensitivity, stability, selectivity, linear range and limit of detection. Herein, SDS increases the immobilization glucose oxidase on ERGO due to its excellent film forming ability.

We expect that the ERGO/SDS will be a suitable nanocomposite for the fabrication of biosensors.

2. Experimental

2.1. Reagents and chemicals

All chemicals were of analytical reagent grade and used without further purification. Double distilled water was used throughout. Glucose oxidase (GOD, EC 1.1.3.4, type VII from *Aspergillus niger*, 221 U mg⁻¹), D-(+)-glucose (97%) and sodium dodecyl sulfate (SDS) were obtained from Sigma (St. Louis, MO, USA). 2 mg of GOD was dissolved in 100 μ L of phosphate buffer solution (0.1 M, pH = 8.0) to prepare GOD working solution. A stock solution of glucose (0.5 M) was prepared with doubly distilled water and stored at 4 °C when not in use. The glucose stock solution was allowed to mutarotate at room temperature for at least 24 h before use.

2.2. Apparatus

Cyclic voltammetry studies were performed using an Autolab potentiostat-galvanostat model PGSTAT30 (Eco Chemie, Utrecht, The Netherlands) with a conventional three electrode set-up, in which a GCE/ERGO/SDS/GOD, an Ag|AgCl|KCl_{sat} and a platinum rod served as the working, reference and auxiliary electrodes, respectively. The working potential was applied in the standard way using the potentiostat and the output signal was acquired by Autolab Nova software. Atomic force microscope (AFM) was taken on Dualscope DS 95-200. All measurements were performed at room temperature. Transmission electron microscopy (TEM) measurements were made on a HITACHI H-8100 EM with an accelerating voltage of 200 kV. X-ray diffraction (XRD) spectra were obtained using a D8ADVANCE (Bruker, Germany) with Cu K α (1.5406 Å) radiation. Fourier Transform Infra-Red (FTIR) analysis was carried out using a Bruker (Germany) vector 22 spectrometer. Raman scattering was performed on an Almega Thermo Nicolet Dispersive Raman spectrometer using the second harmonic (532 nm) of a Nd:YLF laser source. Brunauer–Emmett–Teller (BET) analysis was carried out using a Belsorp, BELMAX (Japan) at 77 K. The ultrasonication and filtration processes were carried out using an ultrasonic cleaner (Elma – E30H, Powerful cleaning with 37 kHz cavitation) and a vacuum pump (Precision 3/4 HP Vacuum Pump Model D 150), respectively.

2.3. Synthesis of graphene oxide

A graphene oxide was obtained through natural graphite oxidation based on Hummer's method [53]. Briefly, 5.0 g of graphite powder was dispersed in a 120 mL concentrated H₂SO₄ kept at 0 °C under stirring. Then, 15.0 g of KMnO₄ was added gradually to the mixture kept in an ice bath to ensure that the temperature remained around 35 °C. After that, the temperature was raised to 0 °C and the mixture was stirred for 30 min and then diluted gradually with 225 mL deionized water. The mixture was re-diluted with 700 mL deionized water and treated with 3% hydrogen peroxide. The color of mixture changed to

yellow-brown during the dropwise addition of H₂O₂. The mixture was filtered and washed with HCl solution (5%) and then repeatedly washed with water until neutral pH was obtained for filtrate by a vacuum pump. This solution was centrifuged at 3000 rpm for 10 min and then the filtrate was re-dispersed in water and centrifuged for several times. Finally, the dark brown GO powder was obtained through drying at 50 °C in a vacuum oven for a day. The graphite oxide was then exfoliated by ultrasonication into the ultrasonic cleaner. For this purpose, GO powder dispersed in a known volume of water was subjected to ultrasonication for 60 min to give a stable suspension of GO and then centrifuged at 3000 rpm for 30 min to remove any aggregates that remained in the transparent light brown exfoliated GO suspension. The prepared graphene oxide (GO) and electrochemically reduced graphene oxide (ERGO) were characterized by FTIR, XRD, Raman and BET (see Supporting information).

2.4. Preparation of GCE/ERGO/SDS/GOD

The surface of a GCE (i.d. = 3.05 mm, Metrohm, Herisau, Switzerland) was polished successively with 0.3, 0.1 and 0.05 μ m alumina slurry (Struers, Copenhagen, Denmark) and then cleaned in ethanol and water, respectively under ultra-sonication. Then, 3 μ L of the GO solution was cast on the surface of GCE and allowed to dry at ambient temperature. Then, 5 μ L of the SDS solution was cast on the surface of GCE/GO and allowed to dry at ambient temperature again. Finally, the electrode was immersed into the GOD solution (3 mg/100 μ L in pH 5.5 PBS) for 1 h. The electrode was also thoroughly rinsed with water to remove the unadsorbed GOD molecules and was dried in air. Then, the electrochemical reduction of GO was carried out by the Potential Step method: The potential of working electrode was kept constant at -0.85 V with respect to Ag|AgCl|KCl_{sat} for 30 min in a N₂-saturated phosphate buffer solution (0.1 M, pH = 7). The GCE/ERGO/SDS/GOD was stored at 4 °C in a phosphate buffer (0.1 M, pH 7.0) when not in use. AFM was used to characterize and compare the surface morphologies of GO and GO/SDS/GOD films on a GCE, as shown in Fig. S5. The difference of the morphologies reflects that strong interactions take place between GOD and GO/SDS, which have influence on the structure of the composite film.

3. Results and discussion

3.1. Characterization

TEM images of the prepared RGONs are shown in Fig. 1. It can be seen a transparent and flake-like sheet of GO.

Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) have been used to characterize the interface properties of surface-modified electrodes. EIS has been used to characterize the interface properties of surface-modified electrodes [54–56]. The typical impedance spectrum (presented in the form of the Nyquist plot) includes a semicircle portion at higher frequencies corresponding to the electron-transfer-limited process and a linear part at lower frequency range representing the diffusion limited process. The semicircle diameter in the impedance spectrum equals the electron-transfer resistance, R_{et} . This resistance controls the electron-transfer kinetics of the redox probe at the electrode interface.

Fig. 2A shows the EIS of the bare GCE (curve a), GCE/GO/SDS (curve b), GCE/ERGO/SDS (curve c) and GCE/ERGO/SDS/GOD (curve d), respectively. The resistance controls the electron transfer kinetics of the redox probe at the electrode. The R_{et} of bare GCE (curve a) was 377 Ω . After the GO/SDS was added to the bare GCE (curve b), the value of R_{et} increased to 8839 Ω ; this is because GO composite film can decrease the rate of electron transfer. The diameter of the semicircle decreased after the formation of ERGO/SDS to 163.9 Ω (curve c), indicating that the ERGO/SDS composite film may provide higher electron conduction pathways than GO/SDS. However, when GOD was immobilized on GCE/ERGO/SDS, the

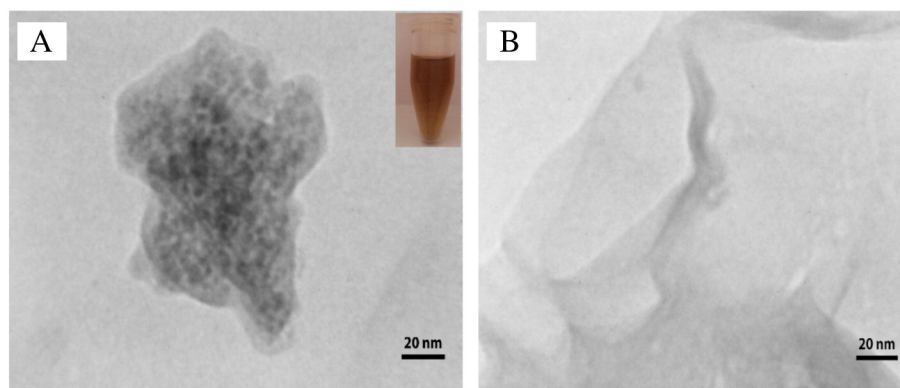


Fig. 1. The TEM images of GO with different magnifications. Photograph showing the change in color of exfoliated GO solution (0.2 mg mL^{-1}). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

diameter of the semicircle increased from 163.9Ω to 5250.2Ω (curve d), indicating that the immobilized GOD led to a great increase in resistance. The inset of Fig. 2.A is the equivalent circuit of the EIS.

CV is also an efficient method for studying the interface properties of electrodes (Fig. 2.B). As indicated, the peak current intensity on GCE/ERGO/SDS (curve c) was higher than GCE/GO/SDS (curve b), indicating that ERGO can effectively improve the rate of electron transfer. Due to

the mass transfer limitation of probe to surface of GCE/ERGO/SDS/GOD (curve d), the peak current intensity decreased dramatically compared with GCE/ERGO/SDS (curve c). It can be seen that the peak to peak separation for the redox probe couple on GCE/ERGO/SDS electrode was less than that observed for GCE electrode (curve a). The peak current intensity on GCE/ERGO/SDS electrode was also higher than that observed on GCE (curve a). The results were consistent with the EIS results.

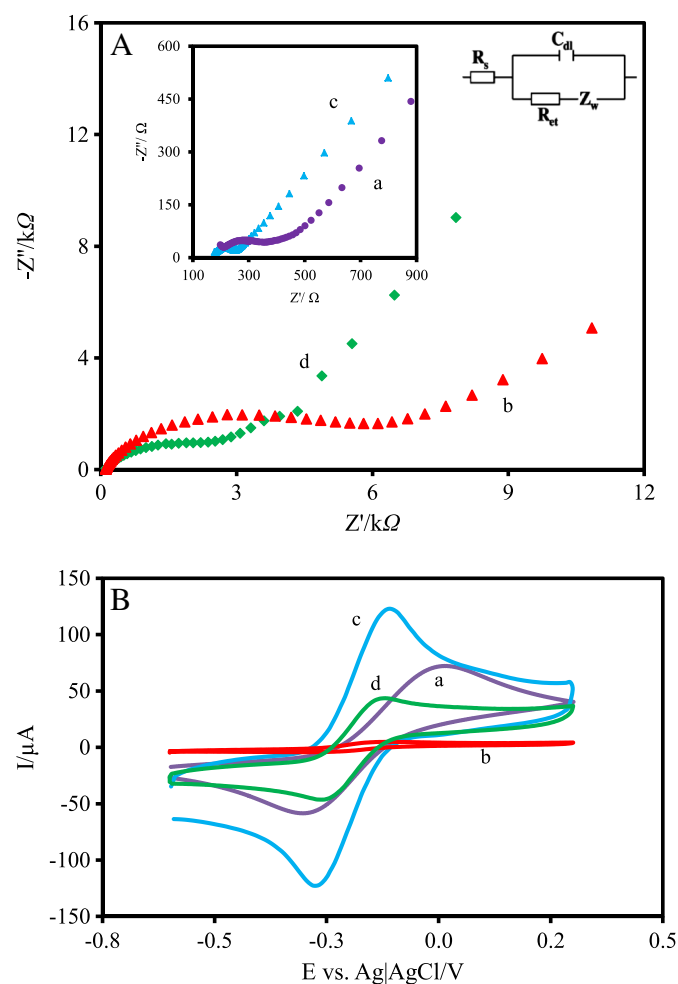


Fig. 2. EIS (A) and CVs (B) of GCE (curve a), GCE/GO/SDS (curve b), GCE/ERGO/SDS (curve c) and GCE/ERGO/SDS/GOD (curve d) in 0.10 M KCl solutions with $5.0 \text{ mM } [\text{Ru}(\text{NH}_3)_6]^{3+/2+}$. The bias potential for EIS is -0.2 V . The inset is the equivalent electric circuit compatible with the Nyquist diagrams. R_s : solution resistance, R_{et} : electron transfer resistance, C_{dl} : double layer capacitance, Z_w : Warburg impedance.

3.2. Direct electrochemistry of the GOD on the GCE/ERGO/SDS electrode

Fig. 3 shows cyclic voltammograms (CVs) of a GCE/SDS/GOD (a), GCE/ERGO/SDS (b) and GCE/ERGO/SDS/GOD (c) in a N_2 -saturated phosphate buffer solution (PBS) (0.1 M , $\text{pH} = 7.0$) at a scan rate of 50 mV/s . No redox peaks were observed at GCE/SDS/GOD (Fig. 3.a), indicating that GCE was not a suitable medium to observe GOD direct electron transfer. But, a couple of well-defined and reversible redox peaks were observed for GCE/ERGO/SDS/GOD (Fig. 3.c). The result indicates that the active redox center of GOD, flavin adenine dinucleotide (FAD), which is deeply embedded in the protective protein shell of GOD is able to communicate electron with electrode, directly. The anodic and cathodic peak potentials were observed at about -382 and -410 mV , respectively. The formal potential (E^0) was about -396 mV (versus $\text{Ag}|\text{AgCl}|\text{KCl}_{\text{sat}}$). The peak-to-peak potential separation (ΔE_p) and the ratio of cathodic to

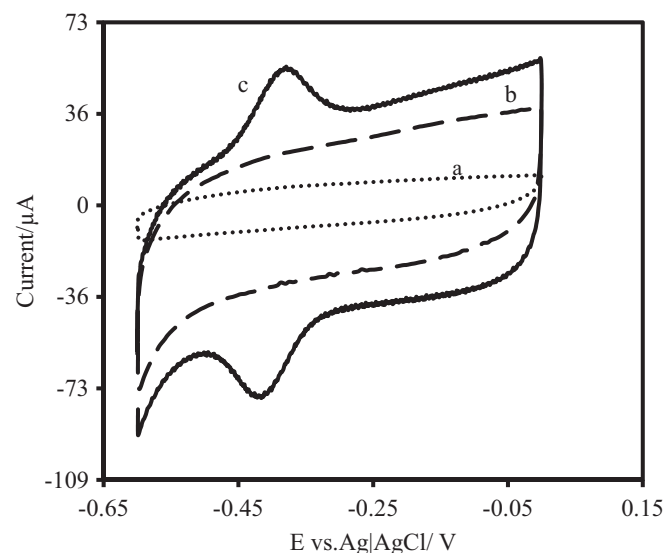


Fig. 3. CVs of the GCE/SDS/GOD, (a) the GCE/ERGO/SDS, (b) and the GCE/ERGO/SDS/GOD (c) in a N_2 -saturated phosphate buffer solution (0.1 M , $\text{pH} = 7.0$) at a scan rate of 50 mV s^{-1} .

anodic current intensities were about 28 mV and 1 mV, respectively. Based on the obtained result, it can be seen that due to adsorption of GOD inside the porous surface of GCE/ERGO/SDS, the electron transfer distance between the redox site (FAD) within the GOD and the electrode surface decreased dramatically.

Fig. 4 shows the cyclic voltammograms of GCE/ERGO/SDS/GOD electrode in PBS (0.1 M, and pH = 7.0) at different scan rates. Both the cathodic and anodic peak currents are linearly proportional to the scan rate in the range from 100 to 650 mV/s, as shown in the inset of Fig. 4, which indicates that the electrode reaction corresponds to a surface-controlled quasi-reversible process. The reduction peak current was nearly equal to the oxidation peak current at the same scan rate. The oxidation peak potential shifted to a more positive value and the reduction peak potential to a more negative value with an increasing scan rate, while the ΔE_p increased gradually. According to the Laviron's formula for a surface controlled electrochemical system ($\Delta E_p < 200$ mV), $k_s = mnFv/RT$ where the apparent heterogeneous electron transfer rate constant (k_s) was estimated to be 4.1 s^{-1} , suggesting that direct electron transfer of adsorbed GOD onto GCE/ERGO/SDS had good reversibility. This k_s is higher than that reported previously for GOD on MWCNT paper (1.7 s^{-1}) [57], on boron-doped MWCNTs (1.56 s^{-1}) [58], on MWCNT-SDS (1.53 s^{-1}) [13], on SWCNT-chitosan (3.0 s^{-1}) [59] and on CNT-poly(diallyldimethylammonium chloride) (PDDA) modified electrode (2.76 s^{-1}) [60]. The surface average concentration of electroactive GOD ($\Gamma \text{ mol/cm}^2$) on the film can be calculated from the charge integration of the cathodic peak in the cyclic voltammogram according to the formula, $Q = nFA\Gamma$, where Q is the charge consumed in C, A is the electrode area (cm^2), F is the Faraday constant, and n is the number of electrons transferred. The electroactive GOD concentration on ERGO/SDS nanocomposite is estimated to be $3.42 \times 10^{-10} \text{ mol/cm}^2$ ($n = 2$). This value is two orders of magnitude higher than that the theoretical value ($2.86 \times 10^{-12} \text{ mol cm}^{-2}$) for the monolayer of GOD on the bare electrode surface [8], suggesting that the nanostructured ERGO/SDS provides a large surface area and a higher capability of composite ERGO/SDS matrix for enzyme immobilization.

3.3. Effect of pH

Previous reports have shown that the solution pH is essential to the electrochemical behaviors of proteins [61,62]. Cyclic voltammetric measurements of the present film modified electrode also show a strong dependence on solution pH. Fig. 5 shows that the anodic and cathodic

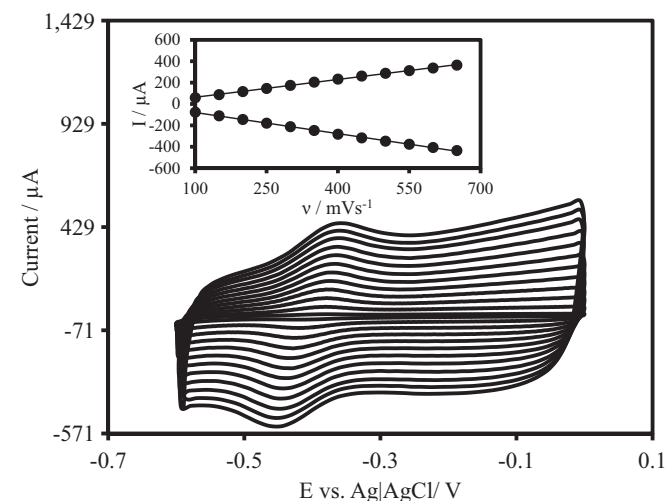


Fig. 4. CVs of the GCE/ERGO/SDS/GOD in a N_2 -saturated phosphate buffer solution (0.1 M, pH = 7.0) at various scan rates from 100 to 650 mV s^{-1} (100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600 and 650 from inner to outer). Inset: plot of the peak current (I_p) vs. scan rate (v).

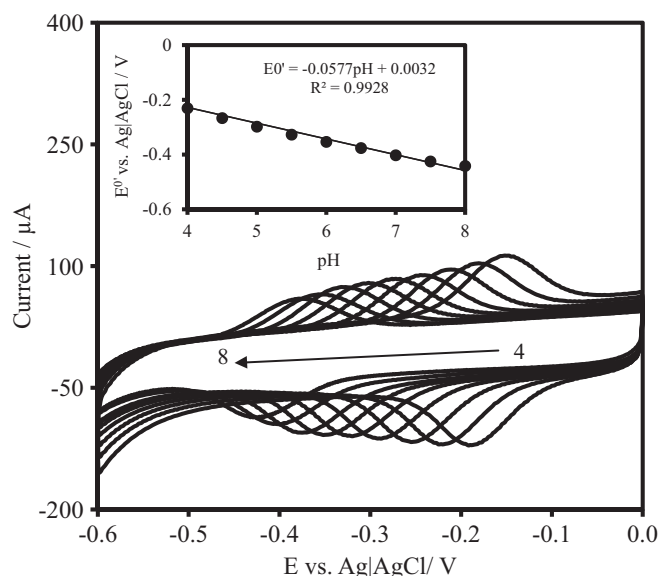


Fig. 5. CVs of the GCE/ERGO/SDS/GOD in a N_2 -saturated phosphate buffer solution (0.1 M, pH = 7.0) at different pH values from 4.0 to 8.0 at a scan rate of 100 mV s^{-1} . Inset: plot of the formal potential ($E^{0'}$) vs. pH.

peak potentials of GOD shift to a negative direction and the formal potential ($E^{0'}$) versus pH gives a straight line with the slope of 57.7 mV pH^{-1} (inset of Fig. 5), which is close to the theoretical value (59.0 mV pH^{-1}) for a two-proton coupled with two-electron redox reaction process shown in Eq. (1) [8].



3.4. The determination of glucose

Fig. 6 shows the CVs of GCE/ERGO/SDS/GOD in a solution containing different concentrations of glucose under the condition of oxygen saturation at a scan rate of 20 mV/s . It can be seen from this figure that the baseline of the reduction decreased with the increase in glucose concentration

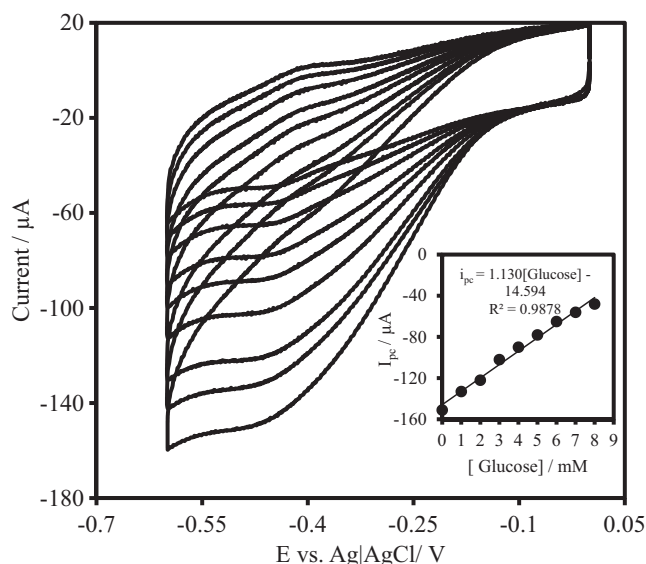


Fig. 6. CVs of the GCE/ERGO/SDS/GOD in an O_2 -saturated phosphate buffer solution (0.1 M, pH = 7.0) containing different amounts of glucose (0, 1, 2, 3, 4, 5, 6, 7 and 8 mM from outer to inner) at a scan rate of 20 mV s^{-1} . The inset is the calibration corresponding to CV responses.

Table 1

Comparison of the analytical performance of the proposed glucose biosensor with other graphene-based glucose biosensors.

Biosensors	Linear range (mM)	LOD (μM)	k_s (s^{-1})	Stability %	R.S.D. %	References
GCE/MGF/GOD	1–12	250	4.8	96 (for 1 week)	6.7	[63]
GCE/rGO–AuNPs/GOD	2–18	20	–	95.4	5.4	[64]
GCE/chit/rGO/Au _{nano} /GOD	2–14	180	–	96 (for 1 week)	4.7	[65]
GCE/rGO–Au _{nano} /GOD	0.2–20	17	–	94.8	6.8	[66]
GCE/G/GOD/chit	0.08–12	20	2.83	95	5.3	[67]
GCE/GQD/GOD	0.005–1.27	1.73	1.12	95	5	[68]
GCE/poly(ViBulm ⁺ Br [−])-rGO GOD	1–20	267	–	97%	3.4	[69]
GCE/rGO–Au _{nano} /GOD	1–8	10	5.35	93.6	3.5	[70]
GCE/ERGO/SDS/GOD	1–8	40.8	4.1	94	3.2	This work

MGF: mesocellular graphene foam, rGO: reduced graphene oxide, GQD: graphene quantum dots, chit: chitosan, poly(ViBulm⁺Br[−])-rGO: poly(1-vinyl-3-butylimidazolium bromide)-reduced graphene oxide, SGN: sulfonated graphene nanosheet, GO: graphene oxide.

indicating the oxygen consumption. It was found that the oxygen consumption is linearly increased with the increase in glucose concentration ranging from 1 to 8 mM with a correlation coefficient (R^2) of 0.9878 and a high sensitivity of about $1.13 \mu\text{A mM}^{-1} \text{cm}^{-2}$ (inset of Fig. 6). Therefore, this GCE/ERGO/SDS/GOD can served as a glucose sensor. The detection limit of the biosensor was estimated to be $40.8 \mu\text{M}$ at a signal-to-noise ratio of 3.

With a series of 7 experiments, the relative standard deviation (R.S.D.) of 3.2% was achieved. These results indicated that the immobilized GOD possesses high enzymatic activity, and the ERGO/SDS film provides favorable microenvironment for GOD to perform DET at the modified electrode. The further measurement indicated that after 100 cycles at a scan rate of 100 mV/s the anodic peak current of GOD is only decreased by 6.0%, illustrating that GOD immobilized on ERGO/SDS possesses the excellent stability. To evaluate the selectivity of the proposed biosensor, the effect of the presence of two possible interfering substances, uric acid 2 mM (UA) and ascorbic acid 2 mM (AA), on the cyclic voltammetry response of the proposed biosensor towards 0.4 mM glucose was investigated. A 7.1% error criterion was adopted. This was probably due to the smaller size of ascorbic acid, which could diffuse through the porous film to the electrode surface and be oxidized. Thus, the response was not interfered by co-oxidizable substances such as AA and UA. The proposed glucose biosensor was employed for the determination of glucose in normal human serum to examine its applicability for real sample analysis. A 400 μL of normal human serum was mixed with 3.6 mL O_2 -saturated PB solution (0.1 M and pH = 7.0) and then analyzed using CV method. The concentration of glucose in normal human serum was determined to be 5.61 mM. The recovery of the analysis was about 102%, considering the value determined by a local hospital (5.50 mM). The comparison of the analytical performance of the proposed glucose biosensor with other graphene-based glucose biosensors is summarized in Table 1. It can be seen that the analytical performance of GCE/ERGO/SDS/GOD is comparable with other graphene-based glucose biosensors.

4. Conclusions

We successfully investigated the direct electrochemistry of GOD at GCE/ERGO/SDS electrode. The apparent heterogeneous electron transfer rate constant (k_s) of GOD at the GCE/ERGO/SDS electrode surface is estimated to be 4.1 s^{-1} . The dependence of the formal potential on solution pH indicated that the direct electron transfer reaction of GOD was a two-proton coupled with a two-electron redox reaction process. The proposed biosensor can catalyze the reduction of dissolved oxygen, and glucose determination was achieved based on the decrease of peak currents due to the reduction of dissolved oxygen. The proposed composite glucose biosensor can be used for the determination of glucose. The constructed biosensor shows a good reproducibility and stability. Finally, we have successfully fabricated the electrochemical glucose biosensor based on such ERGO/SDS nanocomposites.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.msec.2014.09.002>.

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