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ORIGINAL PAPER

Direct electrochemistry of glucose oxidase and sensing glucose using a screen-printed carbon electrode modified with graphite nanosheets and zinc oxide nanoparticles

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Abstract We have studied the direct electrochemistry of glucose oxidase (GOx) immobilized on electrochemically fabricated graphite nanosheets (GNs) and zinc oxide nanoparticles (ZnO) that were deposited on a screen printed carbon electrode (SPCE). The GNs/ZnO composite was characterized by using scanning electron microscopy and elemental analysis. The GOx immobilized on the modified electrode shows a well-defined redox couple at a formal potential of −0.4 V. The enhanced direct electrochemistry of GOx (compared to electrodes without ZnO or without GNs) indicates a fast electron transfer at this kind of electrode, with a heterogeneous electron transfer rate constant (K_s) of 3.75 s^{-1}. The fast electron transfer is attributed to the high conductivity and large edge plane defects of GNs and good conductivity of ZnO-NPs. The modified electrode displays a linear response to glucose in concentrations from 0.3 to 4.5 mM, and the sensitivity is 30.07 μA mM^{-1} cm^{-2}. The sensor exhibits a high selectivity, good repeatability and reproducibility, and long term stability.

Keywords Electrochemical activation · Graphite nanosheets · ZnO nanoparticles · Glucose oxidase · Direct electrochemistry · Glucose biosensor

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Introduction

The electrochemistry of redox active proteins (enzymes) has received substantial attention owing to their interesting electron transfer properties in biological systems, which has been vastly used for the application in biochemical and biophysical sciences [1]. In particular, the direct electron transfer (DET) of redox active proteins between electrode surface and redox active group has paid much notice due to their structural and biological activity for the construction of biosensor and biofuel cells [2]. Glucose oxidase (GOx) is one of the model redox active flavin enzymes, which has been mostly used for the electrocatalysis of glucose [3, 4]. The DET of GOx at bare electrodes is very difficult due to the complicate structure and FAD redox active site of GOx that is deeply buried in the protein cell [5]. Hence, the modified electrodes are utilized to promote the DET between the FAD active site of GOx and the electrode surface, including carbon nanotubes, graphene, metal nanoparticles, metal oxides, conducting polymers, ionic liquids, etc., [6–8]. The carbon nanotubes and graphene composites have been extensively used for the fabrication of enzyme based biosensors due to their excellent conductivity, fast electron transfer and high surface area [9]. Hence, the fabrication of novel materials has gained wider interest in the advancement of redox electrochemical reaction of GOx on the electrode matrix.

Fascinatingly, the electrochemical activation of carbon nanostructures has become more attractive and known for a long time [10]. The activated carbon nanomaterials like graphite, carbon nanotubes and graphene have shown enhanced electrochemical activity and fast electron transfer towards the several analytes, such as NADH, dopamine, hydrazine, and ascorbic acid [11, 12]. Graphite is an anisotropic carbon

material which has been widely used for the fabrication of graphite oxide, graphene oxide and graphene [13]. The graphite has highly reactive large edge planes with inert basal plane defects, which is more favourable for the activation of graphite [14]. Hitherto, different methods have been employed for the activation of graphite including the chemical activation and electrochemical activation [15, 16]. The electrochemical activation method is simple, green, environmental friendly and cost effective when compared to other methods employed for the activation of graphite [14]. Over the past years, the metal oxides have been widely employed for the immobilization of GOx owing to their large surface activity and rapid electron transfer ability [17]. Among them, zinc oxide (ZnO) is a special class metal oxide having the high isoelectric point (9.5), which is more favorable for the immobilization of various redox active enzymes, such as GOx, horseradish peroxidase, cytochrome c, hemoglobin etc., [17–20]. The integration of activated graphite with ZnO is relatively new and novel platform for the immobilization of GOx. To the best of our knowledge, the activated graphite or its composites have never been used as an immobilization matrix for the redox active proteins and not been reported yet in the literature.

In the present paper, the DET of GOx has been investigated at electrochemically fabricated graphite nanosheets (GNs) and ZnO nanoparticles (ZnO) composite modified screen printed carbon electrode (SPCE) for the first time. The GOx immobilized GNs/ZnO composite has been further used for the construction of glucose sensor. The selectivity, stability and practicability of the biosensor have also been studied in detail.

Experimental

Chemicals

Raw graphite with an average diameter of about >20 μm was obtained from Sigma to Aldrich (http://www.sigmaaldrich.com/taiwan.html). Screen printed carbon electrode was purchased from Zensor R&D Co., Ltd., Taipei, Taiwan (http://www.zensor.com.tw/about/about.htm). Glucose oxidase from Aspergillus Niger was obtained from Sigma Aldrich (http://www.sigmaaldrich.com/taiwan.html). D (+) Glucose was obtained from Wako pure chemical industries (http://www.wako-chem.co.jp/english/labchem/). Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$) was obtained from Aldrich (http://www.sigmaaldrich.com/taiwan.html). The supporting electrolyte was prepared by using 0.05 M Na_2HPO_4 and NaH_2PO_4 solutions in doubly distilled water. All the chemicals used in this work were of analytical grade and all the solutions were prepared using doubly distilled water without any further purification.

Apparatus

A computerized electrochemical workstation CHI 750a was used for the cyclic voltammetry (CV) measurements. Scanning electron microscopy (SEM) was performed using Hitachi S-3,000 H electron microscope. An energy-dispersive X-ray (EDX) spectrum was recorded using HORIBA EMAX X-ACT attached to Hitachi S-3,000 H scanning electron microscope. A conventional three-electrode system was used for the electrochemical experiments, a screen printed carbon electrode (active surface area =0.3 cm^2) was used as a working electrode, a saturated Ag/AgCl as a reference electrode and a platinum electrode as an auxiliary electrode. All measurements were carried out at room temperature in the inert atmosphere.

Fabrication of GNs/ZnO composite and immobilization of GOx

The GNs and electrochemically activated SPCE were fabricated according to our previous method [21]. The fabricated GNs modified SPCE was transferred to an electrochemical cell containing 0.5 M $(ZnNO_3)_2 \cdot 6H_2O$ and 0.1 M KNO_3 solution, followed by applying a constant potential of −0.6 V for 200 s. Finally, ZnO was electrodeposited onto the GNs surface and the fabricated composite was named as GNs/ZnO composite. Similarly, ZnO modified electrode was also prepared by the same method without GNs.

For the immobilization of GOx, about 8 μL of GOx solution (5 mg mL^{-1}) was drop-coated on the GNs/ZnO composite and dried at room temperature. The obtained GNs/ZnO/GOx composite modified SPCE was gently rinsed a few times with doubly distilled water to remove the loosely bound GOx. The GNs/ZnO/GOx composite modified electrode was used for further electrochemical experiments and it was stored in pH 7 solution at 4 °C when not in use.

Results and discussion

Characterization

Figure 1 shows the SEM images of ZnO/SPCE (a), electrochemically fabricated GNs (b), and GNs/ZnO at lower (c) and higher (d) magnifications. The SEM image of GNs clearly reveals that the each graphite sheet edge is etched by electrochemical pre-treatment method, leading to the successful transformation of GNs. The obtained morphology of GNs is quite similar to previously reported chemically activated GNs [16]. The thickness of each GN is observed around 82±7 nm, which is quite lower than that of pristine graphite. As shown in Fig. S1b, the observed surface of the pristine graphite sheets are relatively smooth compared to that of GNs. The results

clearly indicate that GNs have more edge plane defects with higher surface area than pristine graphite (Fig. S1B). The cracked surface of the electrochemically activated SPCE confirms that the electrochemical pre-treatment greatly enhances the surface nature of SPCE (Fig. S1A). It can be seen from Fig. 1C to D that ZnO is uniformly deposited on the surface of the GNs surface without altering the morphology of the GNs. The ZnO is found uniform in size on the GNs. The observed morphology and size of ZnO at SPCE are similar to that of ZnO (80±11) which is electrodeposited onto the GNs (Fig. 1a). The result further confirms that the morphology of the electrodeposited ZnO at SPCE and GNs is identical. We have also investigated the surface morphology of GNs/ZnO composite after the immobilization of GOx. It is evident from the Fig. S1C to D that the dense clusters of GOx protein molecules are covered on the whole surface of the GNs/ZnO composite. The result confirms that the GNs/ZnO composite possesses high affinity towards the immobilization of GOx.

The formation of ZnO on GNs is further confirmed by the elemental analysis. Figure 2a and b show the typical EDX profiles of GNs and GNs/ZnO composite modified SPCE. A strong and sharp elemental peak of carbon appears at 0.35 EV at GNs modified SPCE authenticating the pure carbon form of GNs. That the GNs/ZnO composite modified SPCE shows two new elemental peaks at 0.5 and 1.0 EV is attributed to the presence of oxygen and metallic Zn respectively.

Direct electrochemistry of GOx

In order to evaluate the DET of GOx, CV was employed at the different modified electrodes. Figure 3 shows the CV response of bare SPCE/GOx (a), electrochemically activated SPCE/ZnO/GOx (b), SPCE/GNs/GOx (c), and SPCE/GNs/ZnO/GOx (d) in N_2 saturated pH 7 solution at a scan rate of 50 mV s^{-1}. At bare SPCE/GOx, no significant electrochemical signal of GOx appears owing to the very poor immobilization of GOx on the SPCE surface. A well defined redox peak response is observed at a formal potential of −0.4 V (Standard deviation for five electrodes is about 1.7 %) at GOx immobilized GNs/ZnO composite modified electrode. It is attributed to the presence of reversible electron transfer nature of the redox active center ($FAD/FADH_2$) of GOx [22, 23]. The high conductivity and large edge plane defects of GNs with good electron communication capability of ZnO leads to the fast electron transfer of GOx [17]. Moreover, the excellent properties of the GNs/ZnO composite are favorable for the more enzyme adsorption than GNs and electrochemically activated SPCE/ZnO electrodes. The peak-to-peak separation (ΔE_p) at GNs/ZnO/GOx is calculated as 35 mV, which is smaller than those obtained at SPCE/GNs/GOx/SPCE (48 mV) and activated SPCE/ZnO/GOx (50 mV) modified electrodes. The obtained peak current of the redox couple of GOx is relatively small at electrochemically activated SPCE/ZnO/GOx and GNs/GOx modified electrodes compared to GOx immobilized GNs/ZnO composite. The ΔE_p at GOx immobilized GNs/ZnO composite is smaller than that of previously reported GOx immobilized modified electrodes indicating the fast and reversible direct electron transfer process of GOx [3, 22–26]. In addition, the peak current intensities of the GOx redox couple at GNs/ZnO composite is about 2.5 and 5 folds higher than SPCE/GNs and activated SPCE/ZnO modified electrodes. The surface

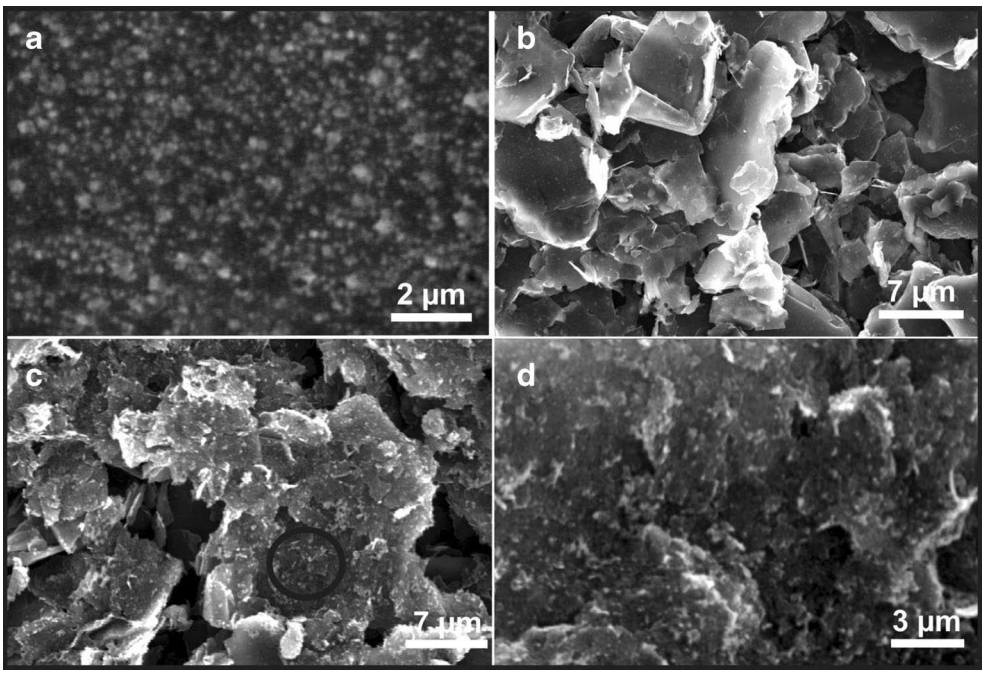

Fig. 1 Typical SEM images of ZnO/SPCE (**a**), fabricated GNs/SPCE (**b**), and GNs/ZnO/SPCE at lower (**c**) and higher magnifications (**d**)

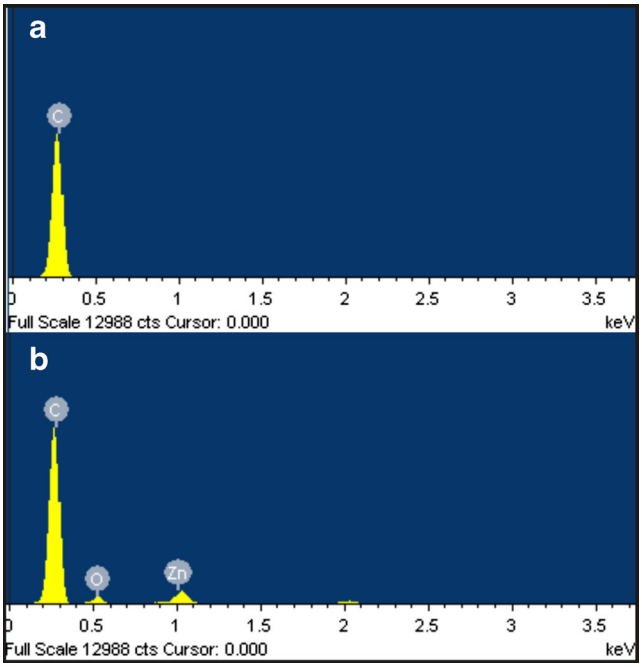

Fig. 2 The EDX spectrum of SPCE/GNs (**a**), and SPCE/GNs/ZnO (**b**)

coverage concentration (Γ) of GOx at GNs/ZnO composite modified electrode is calculated as 8.86×10^{-11} mol cm^{-2} using the Eq. 1.

$$\Gamma = Q/nFA \quad (1)$$

Where a Q = total charge (5.2505×10^{-6} C), the n = number of electrons transferred in the reaction (n=2), F = Faraday

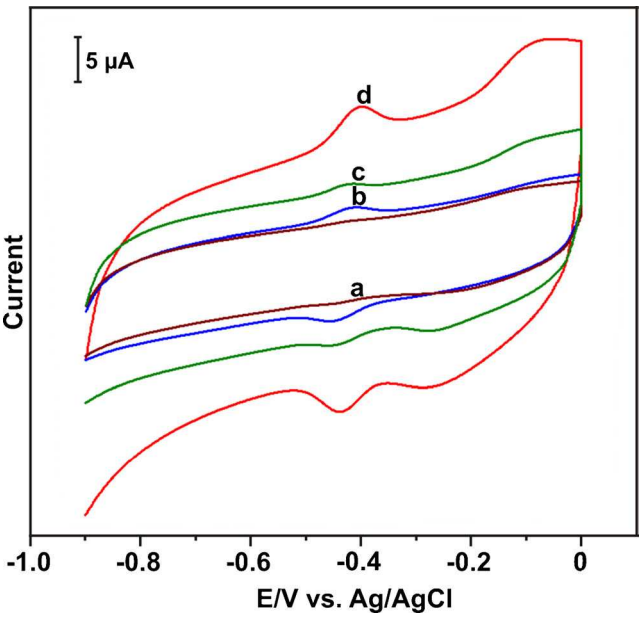

Fig. 3 The CV response of bare SPCE/GOx (**a**), electrochemically activated SPCE/ZnO/GOx (**b**), SPCE/GNs/GOx (**c**), and SPCE/GNs/ZnO/GOx (**d**) in N_2 saturated pH 7 solution at a scan rate of 50 mV s^{-1}

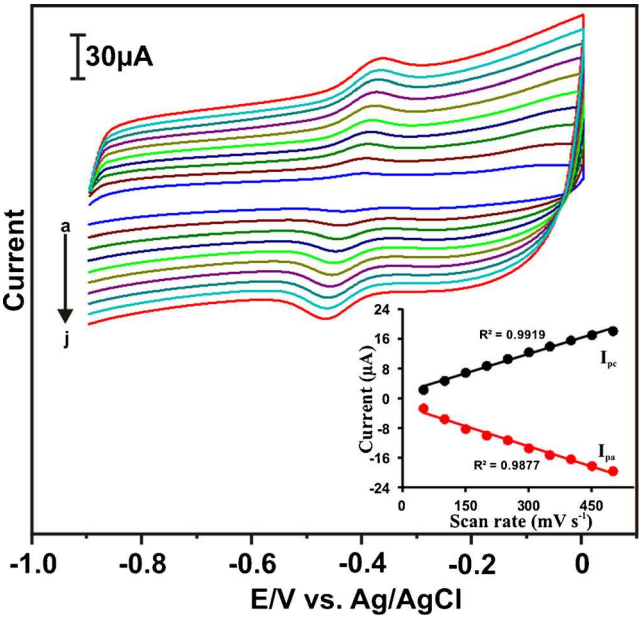

Fig. 4 The cyclic voltammograms obtained at GOx immobilized GNs/ZnO modified electrode in N_2 saturated pH 7 solution at different scan rates (50 to 500 mV s^{-1}). Inset shows the corresponding linear plot for the different scan rates vs. I_{pa} and I_{pc}

constant (96,485.34 C mol^{-1}) and A = active surface area of the electrode (0.307 cm^2). The real active surface area of the GNs/ZnO modified electrode is calculated by CV using equation ($A=n^2F^2I_p.\Gamma v/4RT$) at 0.1 mM $K_4[Fe(CN)_6]$. $3H_2O$ in 0.1 M KCl solution at the scan rate of 50 mV s^{-1}. The obtained surface coverage concentration is much higher than that GOx immobilized at Au nanoparticles/carbon paste (9.8×10^{-12} mol cm^{-2}) [3] and multiwalled carbon nanotubes (2.86×10^{-12} mol cm^{-2}) [27] modified electrodes. The high surface coverage concentration and fast direct electron transfer of GOx are attributed to the large surface area and high electron communication capability of the GNs/ZnO composite.

Effect of different sweeping scan rate and pH

The effect of different scan rate and pH at GOx immobilized GNs/ZnO composite modified electrode was investigated using CV. Figure 4 shows the CVs of GNs/ZnO composite modified electrode in N_2 saturated pH 7 solution at different sweeping scan rates (50 to 500 mV s^{-1}). It can be seen that the anodic (I_{pa}) and cathodic (I_{pc}) peak currents increase linearly with increasing the scan rates from 50 to 500 mV s^{-1}. The cathodic (E_{pc}) and anodic (E_{pa}) peak potentials are slightly shifted with the slight increment of the peak to peak separation (ΔE_p) at higher sweeping rates (>200 mV s^{-1}). Moreover, the I_{pa} and I_{pc} increase linearly with increasing scan rates from 50 to 500 mV s^{-1} (inset), indicating that the redox reaction of GOx at the GNs/ZnO composite electrode is a surface-confined reversible electron transfer process. According to the Lavirons' equation [28], the heterogeneous electron

transfer rate constant (K_s) for GOx immobilized at GNs/ZnO composite modified electrode is calculated as 3.75 s^{-1}. The calculated K_s value is much higher than that of previously reported GOx immobilized multiwalled carbon nanotubes and graphene modified electrodes [24, 29–31], which validates that GNs/ZnO composite facilitates the fast direct electron transfer between the GOx and electrode.

Figure 5 shows the effect of pH on GOx ($FAD/FADH_2$) redox couple at GNs/ZnO composite modified electrode in various pH solutions at the scan rate of 50 mV s^{-1}. It can be seen that a pair of well defined reversible redox peaks of GOx is observed in each pH solution. Both anodic and cathodic peak potentials are shifted to negative and positive direction upon increasing and decreasing the pH and the maximum redox peak current response (I_p) is observed at pH 7 solution. The formal potential (E°) of GOx exhibits a linear dependence over a wide pH range from pH 3 to 11 with a slope value of −61.2 mV/pH (R^2=0.9921, inset of Fig. 5). This slope value is close to the theoretical value of −58.6 mV/pH for equal number of protons (H^+) and electrons (e^-) involving electron transfer process [9]. The result confirms that the direct electrochemical reaction of the redox couple at GOx is involving an equal number of protons (H^+) and electrons (e^-). The direct electrochemistry of GOx at GNs/ZnO composite modified electrode can be expressed by Eq. 2 [22, 32].

$$GOx + FAD + 2\,e^- + 2\,H^+ \leftrightarrow GOx{-}FADH_2 \quad (2)$$

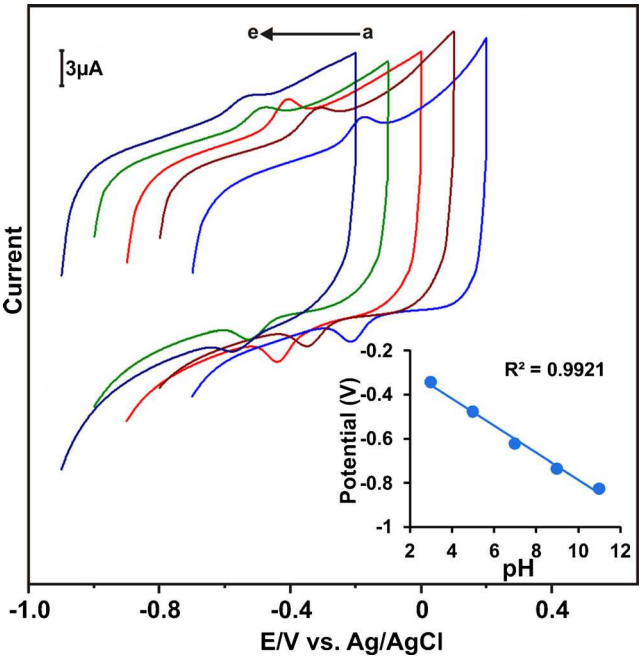

Fig. 5 The CV response of GOx immobilized GNs/ZnO modified electrode in different pH solutions at the scan rate of 50 mV s^{-1}. The corresponding calibration plot for pH vs. E^0 (inset)

Oxidation of glucose and reduction of O_2

In order to evaluate the electrocatalytic activity of the GOx towards glucose, CVs were recorded using GOx immobilized GNs/ZnO composite modified SPCE. Figure 6a shows the cyclic voltammetry response of composite modified electrode in O_2 saturated PBS for different concentrations of glucose (0.3 to 6 mM) at the scan rate of 50 mV s^{-1}. It can be seen that the oxidation peak current (I_{pa}) increases while the reduction peak current (I_{pc}) decreases significantly upon increasing the concentration of glucose form 0.3 mM to 6 mM. This is due to the decrease in the O_2 content of the solution as it is consumed during the oxidation of glucose by the immobilized GOx on GNs [33]. The mechanism of glucose oxidation by GOx can be expressed by Eq. 3 as reported previously [34].

$$GOx{-}(FAD) + Glucose + O_2 \rightarrow GOx{-}(FAD) + Gluconolactone + H_2O_2 \quad (3)$$

The mechanism of oxygen reduction by GOx can be found in electronic supplementary information (ESI). The I_{pc} linearly decreases with the glucose concentration over the linear range from 0.3 to 4.5 mM with a correlation coefficient of 0.9904. The equation of the calibration plots (Fig. 6b) are: I (μA)= 9.0213+43.56 c (mM). From the fitted linear regression equation, the sensitivity is calculated as 30.07 $\mu A mM^{-1} cm^{-2}$. The limit of detection (LOD) is estimated as 0.07 mM (S/N=3). The blood glucose level in healthy humans is ranging from 4 to 6 mM. Thus, the obtained linear range of glucose at GOx immobilized GNs/ZnO composite is more favorable for the determination of glucose levels in blood. Here it is essential to explain the advantage of our biosensor compared to existing glucose biosensors, since carbon nanomaterials, metal nanoparticles and metal oxides and their composites have been broadly used for the construction of glucose biosensor [9, 12, 17]. Hence, the analytical performance (linear range, sensitivity, LOD and stability) of our biosensor has been compared with previously reported similar modified electrodes for the glucose detection and the comparative results are shown in Table 1. It is evident from Table 1 that our biosensor exhibits higher sensitivity, stability along with fast heterogeneous electron transfer constant (K_s) than previously reported GOx immobilized carbon nanotubes and reduced graphene oxide composites modified electrodes. The linear range and LOD of our biosensor is comparable to aforementioned glucose biosensors. Most of the glucose biosensors reported in the literature are based on carbon nanotubes, graphene and their composites modified electrodes. However, these modified electrodes are still expensive and need more time for

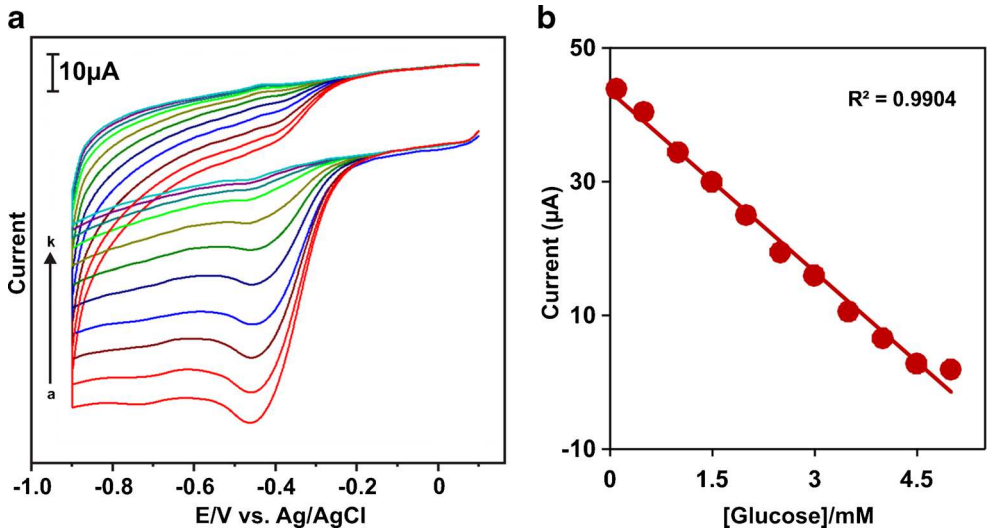

Fig. 6 **a** The cyclic voltammetric responses at GOx immobilized GNs/ZnO modified electrode in the presence of different concentration of glucose in oxygen saturated pH 7 solution.**b** Linear dependence of [glucose] vs. I_{pc}

fabrication when compared to GNs/ZnO modified electrode. Graphite is cheaper and less toxic material compared to carbon nanotubes and graphene. Moreover the fabricated biosensor is disposable one and can be prepared in less than an hour. These advantages of our biosensor will gain much further attention and can be used for real time sensing of glucose in near future.

Selectivity and practicability of the biosensor

The selectivity of the biosensor was investigated by using cyclic voltammetry. The response of the biosensor to 2 mM glucose was checked in the presence of potential interfering biologically active species such as dopamine (DA), ascorbic acid (AA) and uric acid (UA). The biosensor response to 2 mM glucose in the presence of 3 mM DA and AA and 4 mM UA at the GNs/ZnO composite modified electrode was analyzed. Table S1 is evident that the potentially interfering electro active species show less effect (<5 %) on the determination of glucose. These results indicate that the fabricated biosensor has good selectivity even in the presence of excess concentrations of interfering species.

To demonstrate the practical use of the fabricated biosensor, the glucose detection in human serum and urine samples were performed. The experimental conditions are same as mentioned above in the glucose electrocatalysis section. Human serum and urine sample were collected from healthy men and used without any further pretreatment except dilution

Table 1 Comparison of the analytical performance of the GOx immobilized GNs/ZnO composite modified electrode with other GOx modified electrodes reported previously

Modified electrode	K_s (s^{-1})	Linear range (mM)	Sensitivity (μA mM^{-1} cm^{-2})	LOD (μM)	Stability (%)	Reference
CPE/Au/GOx	5.3	up to 0.28	8.4	10	96 (10 days)	[3]
Graphene/GOx/Chi	2.83	up to 12	37.93	20	NA	[24]
GCNT/GOx/GAD	1.08	up to 20.09	2.47	NA	96.3 (12 days)	[29]
CNT/Au/PDDA/GOx	1.01	up to 5.2	0.25	NA	97 (6 days)	[30]
ERGO-MWCNTs/GOx	3.02	up to 6.5	7.95	4.7	90 (30 days)	[31]
RGO/Chi/GOx	NA	up to 2	NA	40	80 (30 days)	[35]
MWCNT/ZnO/GOx	1.96	up to 1.29	NA	2.22	85.1 (27 days)	[36]
RGO/ZnO/GOx	7.55	up to 6.6	13.7	200	80 (10 days)	[37]
RGO/ZnO/GOx	NA	up to 27.2	18.97	20	NA	[38]
Nf/Graphene/GOD	1.96	up to 14	21.9	40	NA	[39]
MWCNT/ZnO/GOx	1.66	up to 27.2	4.18	20	91.3 (15 days)	[40]
GNs/ZnO/GOx	3.75	up to 4.5	30.07	70	89.7 (47 days)	Present study

LOD limit of detection, *CPE* carbon paste electrode, *Au* gold nanoparticles, *GOx* glucose oxidase, *Chi* chitosen, *NA* not available, *GCNT* gelatin-multiwall carbon nanotube, *GAD* glutaraldehyde, *CNT* carbon nanotube, *PDDA* poly (diallydimethylammonium chloride), *ERGO* electrochemically reduced graphene oxide, *MWCNTs* multiwalled carbon nanotubes, *RGO* reduced graphene oxide, *ZnO* zinc oxide, *Nf* nafion

step. The glucose level in a serum sample was pre-determined as 4.62 mM by Tecan Sunrise plate reader (http://www.tecan.com/platform/apps/product/index.asp?MenuID=1260&ID=682&Menu=1&Item=21.2.10.4). The glucose concentration is calculated as 4.5 mM by using fabricated biosensor with a RSD of 1.9 % for two replicate measurements, indicating the good practicality of the biosensor. To evaluate the practicality of the biosensor in Human urine samples, the standard addition method was used for the determination of glucose. The recovery results of glucose in urine samples are summarized in Table S2. The good recovery results of glucose in urine samples (~98.3 %) authenticate the good practicality of the biosensor.

The storage stability of the GOx immobilized GNs/ZnO modified electrode was examined periodically by CV. Only 10.3 % of the response current is lost after 47 days indicating the high enzymatic activity in the biosensor. The stability of the GOx at GNs/ZnO composite before and after overnight drying at room temperature was also checked by using CV. Only slight loss (0.8 %) of the redox peak current and no notable shift in the peak potential of GOx ($FAD/FADH_2$) are observed, suggesting that GOx is highly stable at the GNs/ZnO composite modified electrode. The relative standard deviation (RSD) for 7 successive determinations is 4.4 % for 2 mM glucose determination. These results further indicate that the GOx immobilized GNs/ZnO composite modified electrode has good repeatability and reproducibility along with excellent stability.

Conclusions

In summary, the direct electrochemistry of GOx has been successfully investigated at electrochemically fabricated graphite nanosheets (GNs) and ZnO nanoparticles (ZnO) composite modified screen printed carbon electrode (SPCE) for the first time. Surface morphological studies indicate that GNs have more edge plane defects than pristine graphite. The peak current intensities of the GOx redox couple at GN/ZnO composite is about 2.5 and 5 folds higher than GNs/SPCE and activated SPCE/ZnO modified electrodes. The fast and enhanced electron transfer of GOx is attributed to the high conductivity and large edge plane defects of GNs and good electron communication of ZnO. The fabricated biosensor shows a lower detection limit of 70 μM with the high sensitivity of 30.07 $\mu A m M^{-1} cm^{-2}$ for glucose determination. The fabricated GNs/ZnO composite can bring a novel platform in prospective for the immobilization of other similar redox active proteins like horseradish peroxidase, cytochrome c and hemoglobin.

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