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Mediatorless amperometric glucose biosensing using 3-aminopropyltriethoxysilane-functionalized graphene

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ABSTRACT

A mediatorless glucose biosensor was developed by the immobilization of glucose oxidase (GOx) to graphene-functionalized glassy carbon electrode (GCE). The surface of GCE was functionalized with graphene by incubating it with graphene dispersed in 3-aminopropyltriethoxysilane (APTES), which acted both as a dispersion agent for graphene and as an amine surface modification agent for GCE and graphene. This was followed by the covalent binding of GOx to graphene-functionalized GCE using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) based crosslinking. Graphene provided signal enhancement by providing greater surface area for GOx binding, while APTES-functionalization led to a higher GOx immobilization density by providing free amino groups for crosslinking. The developed biosensor used a redox potential of −0.45 V (vs. Ag/AgCl) for detecting glucose in the diabetic pathophysiological range 0.5–32 mM. There was no interference from endogenous electroactive substances and drug metabolites. The developed biosensor was further validated for detecting blood glucose in commercial artificial blood glucose linearity standards in the range 1.4–27.9 mM. Therefore, it is ideal for diabetic blood glucose monitoring. The developed bioanalytical procedure for preparation of GOx-bound graphene-functionalized GCEs had high production reproducibility and high storage stability, which is appropriate for the commercial mass production of enzyme-bound electrodes.

1. Introduction

Graphene has been extensively investigated for the development of optoelectronic devices [1], supercapacitors [2] and various types of high performance sensors [3–8] due to its large surface-to-volume ratio [9,10], excellent electrical conductivity, and electron mobility [11]. The large surface area of graphene enhances the surface loading of desired biomolecules such as enzymes and proteins, either through passive adsorption or by covalent crosslinking to the reactive groups of biomolecules. The excellent conductivity and small band gap of graphene are beneficial for the conduction of electrons between biomolecules and the electrode surface [11]. It has been demonstrated that graphene has about two times more effective surface area than carbon nanotubes [12] and is more cost-effective due to its direct synthesis from graphite [13].

Graphene has been widely employed in mediatorless electrochemical glucose biosensors based on the direct electron transfer between oxidoreductase and the electrode surface [14,15]. Glucose oxidase (GOx) undergoes a reversible two-proton and two-electron transfer reaction, at a rapidly heterogeneous electron transfer rate with a rate constant of 2.83 s^{-1} [15], when it is bound to the graphene–chitosan modified glassy carbon electrode (GCE). The direct electrochemistry of GOx has been observed on polyvinylpyrrolidone-protected graphene-ionic liquid [14] and electrochemically reduced graphene oxide [16] modified GCE. The redox peaks of GOx active centers were also observed when GOx was immobilized on graphene–metallic nanoparticles composites with gold [17] or cadmium sulfide (CdS) [18]. However, these graphene-based electrodes employed a lengthy procedure with a long preparation time greater than 24 h [14,16–18] and were unable to detect in the entire diabetic pathophysiological glucose range of 0.5–32 mM [14–19], which render these approaches unsuitable for diabetic glucose monitoring. Moreover, most of the developed

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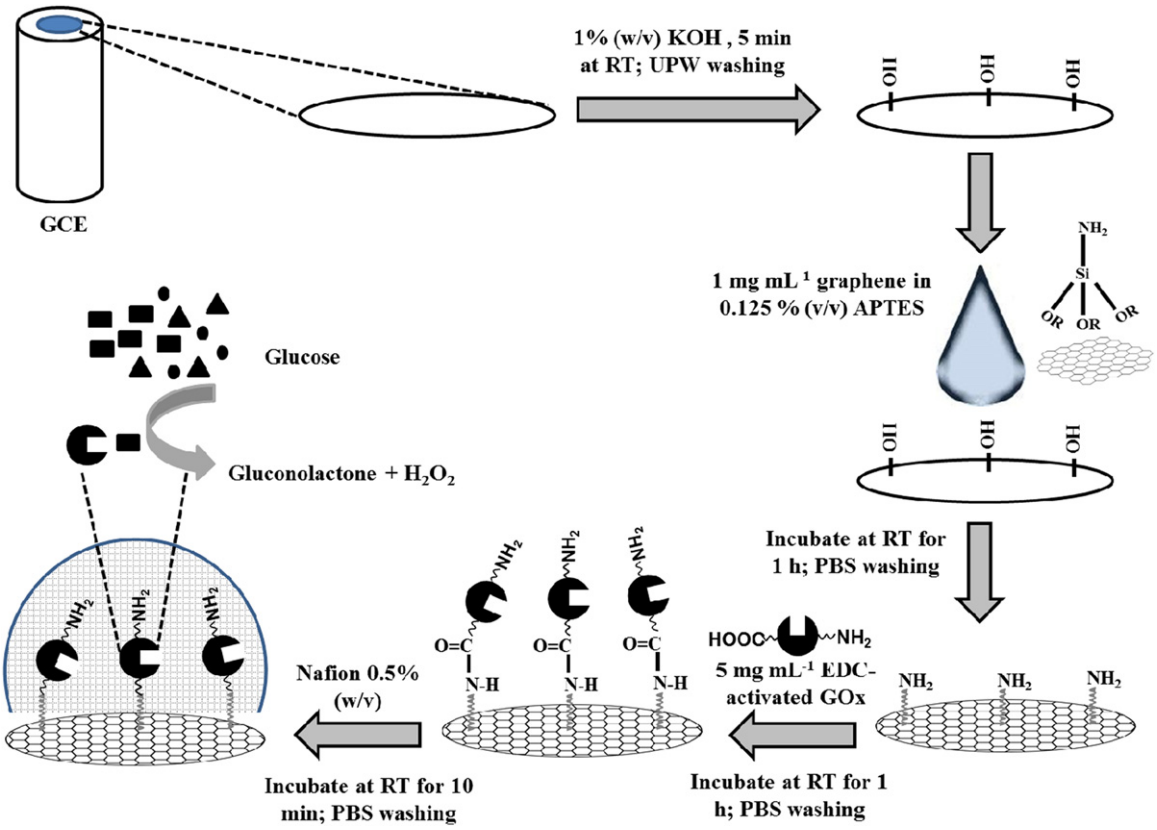

Fig. 1. Schematic of the developed graphene based electrochemical glucose biosensor.

graphene-based electrochemical sensors have employed an organic or inorganic solvent, such as dimethylformamide [20], dichlorobenzene [21], and chitosan [15,22], for the dispersion of graphene. To our knowledge, 3-aminopropyltriethoxysilane (APTES) has not been employed for the dispersion of graphene.

We have developed a simple bioanalytical procedure (Fig. 1) for the rapid preparation of GOx-bound graphene-functionalized GCEs in less than 3 h. It involved the sequential generation of hydroxyl groups on GCE by treatment with 1% KOH; graphene functionalization of GCE by incubating with graphene dispersed in APTES (denoted as graphene–APTES/GCE); GOx binding by 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) based heterobifunctional crosslinking to graphene-functionalized GCE (denoted as GOx/graphene–APTES/GCE); and covering the GOx-bound GCE with 0.5% Nafion (denoted as Nafion/GOx/graphene–APTES/GCE). Nafion acts as a glucose-limiting membrane, which allows the diffusion of glucose molecules but prevents the diffusion of larger contaminating substances and interferences. The developed Nafion/GOx/graphene–APTES/GCE based amperometric glucose biosensor was employed for the detection of commercial as well as artificial blood glucose in the clinically-relevant pathophysiological range in diabetic monitoring. The production reproducibility of the developed bioanalytical procedure was also determined along with the storage stability of prepared Nafion/GOx/graphene–APTES/GCEs.

2. Experimental

2.1. Reagents

Graphene was purchased from Cheap Tubes (USA, diameter 5 μm) and was used as such. GOx (EC 1.1.3.4, Type X-S from *Aspergillus niger*, G7141), D-glucose, 5 wt% Nafion, 70 wt% glutaraldehyde and all the interfering substances, i.e. ascorbic acid, uric acid, acetaminophen, dopamine, creatinine, tetracycline, bilirubin, salicylate, ibuprofen, tolazamide, tolbutamide and ephedrine, were purchased from Sigma-Aldrich, Singapore. BupH phosphate buffered saline (PBS), BupH 2-(N-morpholino)ethanesulfonic acid (MES) buffered saline, EDC and bicinchoninic acid (BCA) protein assay kit were procured from Thermo Scientific, USA. Sugar-Chex Linearity (whole blood glucose linearity standards) was purchased from Streck, Inc. (USA). The dilution of APTES and glutaraldehyde was made in ultrapure water (UPW, 18.2 MΩcm at 25 °C, Direct Q, Millipore) whereas GOx and glucose were made in 50 mM PBS, pH 7.4. The EDC solution was prepared in 100 mM MES, while the dilution of 0.5 wt% Nafion was made in absolute ethanol. The GOx stock solution was prepared by mixing equal volumes of 10 mg mL^{-1} GOx and 5% (w/v) glutaraldehyde, and was stored at 4 °C for at least 1 h prior to use. The Glucose solution was stored at RT overnight, while interfering substances were freshly prepared just before use. All experiments were performed at RT in 50 mM PBS, pH 7.4.

2.2. Electrode preparation

GCEs (3 mm diameter, CH Instruments, Austin, TX, USA) were polished consecutively using 0.3 and 0.05 μm alumina powder, and subsequently cleaned by putting in an ultrasonic bath (Model 2510, Branson) for 20 min. GCEs were then dipped in 1% KOH for 5 min to generate hydroxyl groups on their surface, and then washed extensively with UPW. 1 mg of graphene was mixed with 1 mL of 0.125%, 0.25%, 0.5%, 1%, 2%, 4%, 6%, 8% and 10% APTES and dispersed in ultrasonic bath for 1 h. 4 μL of the resulting graphene–APTES suspension was drop-casted on the GCE surface, incubated at RT for 1 h, and washed extensively with UPW to

form graphene–APTES/GCE. 4 μL of EDC-activated GOx (5 mg mL^{-1}) was drop-casted on each graphene–APTES/GCE, incubated at RT for 1 h, and washed extensively with PBS to form GOx/graphene–APTES/GCE. Finally, 0.5% Nafion was drop-casted, incubated at RT for 10 min, and washed extensively with PBS to form Nafion/GOx/graphene–APTES/GCE.

Fourier transform infrared (FTIR) spectra were collected from pristine and APTES (0.125%)-functionalized graphene samples in KBr pellets over 4000–400 cm^{-1} for 64 scans at a resolution of 4 cm^{-1} using a Bruker Tensor 27 FTIR spectrophotometer. The surface characterization of the graphene–APTES functionalized glassy carbon substrate was done by imaging the surface using a JEOL scanning electron microscope (SEM) at 15 kV (JSM-6010LV, Japan).

2.3. Electrochemical analysis

The electrochemical characterization was performed using a CHI 660A electrochemical workstation with a three electrode system, i.e. developed bioelectrode, Pt wire counter electrode and Ag/AgCl (3 M KCl) reference electrode. Cyclic voltammetry (CV) was performed in both air- and nitrogen-saturated solutions at a scan rate of 100 mV s^{-1}. Amperometric *i–t* curve technique was used to detect glucose in stirred PBS at −0.45 V. All potentials were referred to Ag/AgCl.

2.3.1. Assay curve for glucose and Sugar-Chex blood glucose linearity standards

The detection of varying concentrations of glucose was obtained by injecting varying volumes of 1 M glucose stock solution into stirred PBS to form 2 mL of 0.5, 1, 2, 4, 8, 16 and 32 mM. All the concentrations were detected individually in triplicate. The assay curve of Sugar-Chex blood glucose linearity standards was obtained by injecting 400 μL of Sugar-Chex blood glucose linearity standards, having different glucose concentrations of 1.4, 2.7, 6.8, 12.0, 20.3, and 27.9 mM, into 2.8 mL of stirred PBS. The results obtained were then multiplied by the dilution factor.

2.3.2. Effect of interfering substances

Stocks of bilirubin and uric acid solutions were prepared in 10 mM NaOH; creatinine, acetaminophen, ascorbic acid, dopamine and ephedrine solutions were prepared in 0.1 M PBS; ibuprofen, salicylate and tolbutamide solutions were prepared in absolute ethanol; tetracycline solution was prepared in 3 M HCl; and tolazamide solution was prepared in acetone. The effect of interfering substances was determined by analyzing their effect on the electrochemical detection signal for the 6.8 mM Sugar-Chex blood glucose linearity standard.

2.3.3. Production reproducibility

The production reproducibility was determined from the reproducibility of electrochemical responses for the detection of 4 mM glucose (in triplicate) using 25 GOx-functionalized GCEs prepared using the developed procedure.

2.3.4. Storage stability

The developed Nafion/GOx/graphene–APTES/GCEs were stored in dry state at RT and employed for detecting 4 mM glucose (in triplicate) each week for a period of 5 weeks.

2.3.5. Effect of biofouling

The developed Nafion/GOx/graphene–APTES/GCE was kept immersed in the 1 mM Sugar-Chex blood glucose linearity standard for 7 days. It was employed each day for the detection of the 6.8 mM Sugar-Chex blood glucose linearity standard in triplicate.

2.4. BCA protein assay

The developed Nafion/GOx/graphene–APTES/GCE and the control electrode i.e. Nafion/graphene–APTES/GCE were immersed in 200 μL of BCA working reagent inside microcentrifuge tubes and incubated at 80 °C for 15 min. The colored products formed (180 μL each) were then transferred to the microtiter plate, whose absorbance was read at 562 nm on Tecan Infinite M200 Pro microplate reader. The absorbance of the control electrode, i.e. Nafion/graphene–APTES/GCE, was subtracted from that of the developed Nafion/GOx/graphene–APTES/GCE to determine the total concentration of GOx bound to the electrode.

3. Results and discussion

3.1. Development of graphene-based glucose biosensor

The graphene-based amperometric glucose biosensor was developed using a simple bioanalytical procedure, which enabled the rapid preparation of Nafion/GOx/graphene–APTES/GCEs. The procedure was based on the use of APTES, which acts as a dispersion agent for graphene and as a surface modification agent for GCE and graphene. We have extensively employed APTES as a surface modification agent for inducing free amino groups that were used for the EDC–sulfoNHS based crosslinking of antibodies [23–25] on various bioanalytical platforms such as microtiter plates, surface plasmon resonance chips, microarrays, and optical chips. To our knowledge, the rapid one-step preparation of graphene-functionalized GCE by incubating with graphene dispersed in APTES has not been demonstrated. APTES in the graphene sample binds to the hydroxyl groups on the GCE surface and graphene by its alkoxy groups, while its amino groups on the other end are free for crosslinking to GOx. As shown in supplementary Fig. S1A, the FTIR spectrum of pristine graphene was virtually featureless with the exception of the ~1650 cm^{-1} peak associated with the skeletal vibrations of the graphitic sheet [26,27]. The free primary amine NH_2 bending mode of APTES at 1600 cm^{-1} could not be discernible from the FTIR of the graphene–APTES sample due to the overwhelming signal of graphene at this wavelength. CH_3 asymmetric stretching at ~2970 cm^{-1}, CH_2 asymmetric stretching of APTES at ~2930 cm^{-1} and the NH_2 stretch vibration of APTES at 3350–3380 cm^{-1} [28] were overlapped by the strong peak of pristine graphene (supplementary Fig. S1B). Nevertheless, the FTIR signature of APTES–graphene was distinctly different from that of graphene in the region 700–1000 cm^{-1} (supplementary Fig. S1C), exemplified CH_3 rocking (~740 cm^{-1}) and H–SiO_3 bending (887 cm^{-1}). Such a difference indicated the interaction between graphene and APTES to form a stable complex on the electrode surface. When the graphene dispersed in APTES is provided to GCE, the APTES molecules functionalized on GCE surface and graphene form siloxane bonds (–Si–O–Si–), thereby leading to the binding of graphene with GCE. GOx is then bound covalently to graphene–APTES/GCE by EDC-based crosslinking. GOx is initially incubated with EDC for 15 min, which crosslinks EDC to the carboxyl groups on GOx and forms EDC-activated GOx. Subsequently, the EDC-activated GOx is provided to graphene–APTES/GCE, which leads to the formation of covalent amide bonds between the carboxyl groups on GOx and the free amino groups on the graphene–APTES/GCE. The GOx/graphene–APTES/GCE was then covered with 0.5% Nafion, which acts as a glucose limiting membrane. As a control, the same bioanalytical procedure was

used for the direct crosslinking of GOx to 2% APTES-functionalized GCE without employing graphene (denoted as Nafion/GOx/GCE).

Graphene was well dispersed in APTES (Fig. 2A), whereas it forms aggregates in UPW (Fig. 2B). The SEM images of graphene functionalized glassy carbon substrate clearly showed the flake-like structure of graphene, which was uniformly bound to the glassy carbon (Fig. 2C).

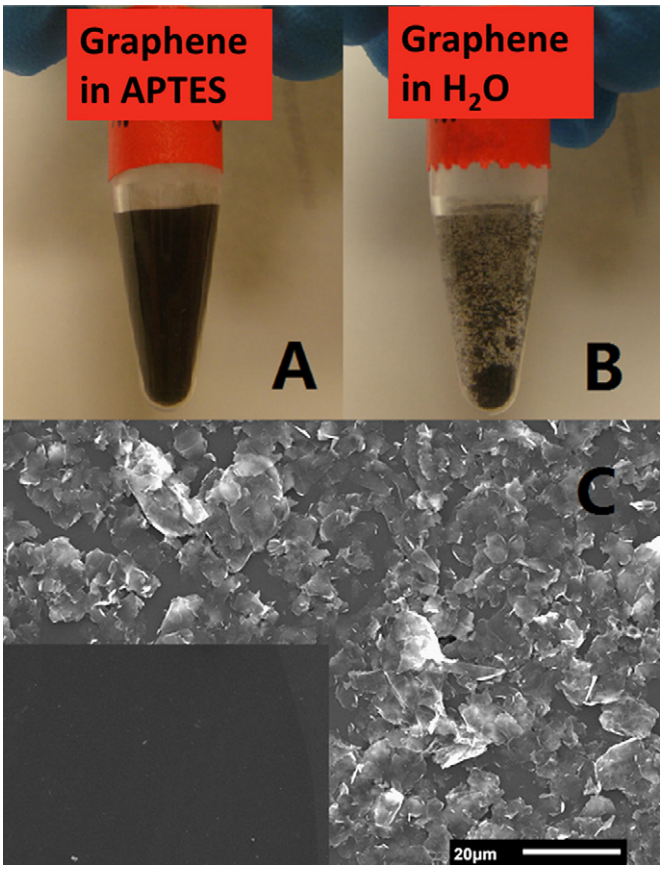

Fig. 2. Graphene dispersed in (A) APTES and (B) water. (C) SEM image of graphene-functionalized glassy carbon substrate (the inlet is the SEM image of blank glassy carbon).

3.2. Detection of commercial and blood glucose

The developed electrode was characterized by CV in the absence and presence of oxygen before it was used for glucose detection. There were no redox peaks of FAD/$FADH_2$ on the Nafion/GOx/graphene–APTES/GCE whether it was employed under N_2-saturated PBS (Fig. S2) or ambient PBS (Fig. 3A). However, the cathodic current (from −0.2 to −0.8 V) decreased with the increase in glucose concentration in the presence of oxygen (Fig. 3A). The decrease in cathodic current was caused by the GOx–$FADH_2$ catalyzed reduction of oxygen [29–31]. The proportionate decrease in cathodic current with the increase in glucose concentration can be employed for glucose detection. The optimum applied potential was found to be −0.45 V (Fig. S3). The amperometric responses of the developed electrode for the detection of 1–32 mM glucose are shown in Fig. 3B.

We have previously demonstrated that 2% APTES is the optimum concentration for surface modification of bioanalytical platforms [23,25] based on polystyrene and gold-coated substrates. Similarly, 2% APTES was also found to be the most appropriate as the surface modification agent of GCE. However, in the present study, where APTES was used both as a surface modification and dispersion agent, 0.125% APTES was found to be the optimum concentration (Fig. 4A). The developed amperometric glucose biosensor had a dynamic range of 0.5–32 mM with a linearity of 1–32 mM that covered the entire diabetic pathophysiological range 1–28 mM. The current response of developed Nafion/GOx/graphene–APTES/GCEs for the detection of glucose was much higher than that of Nafion/GOx/GCE (Fig. 4B), which shows the signal enhancement provided by graphene due to increased surface area that results in higher GOx immobilization density. Moreover, the developed bioanalytical procedure for the preparation of Nafion/GOx/graphene–APTES/GCEs takes the same hands-on time as that of Nafion/GOx/GCE because graphene functionalization of GCE follows the same procedure and time as those of APTES-functionalization of GCE.

The developed glucose biosensor was further employed for the detection of blood glucose using Sugar-Chex blood glucose linearity standards from Streck, USA, which are widely used as the industrial calibration and clinical validation standards for diabetic blood glucose monitoring. Streck blood glucose standards are made from stabilized whole blood [32] having the same composition as that found in humans. The developed biosensor detected the entire set of Sugar-Chex blood glucose linearity

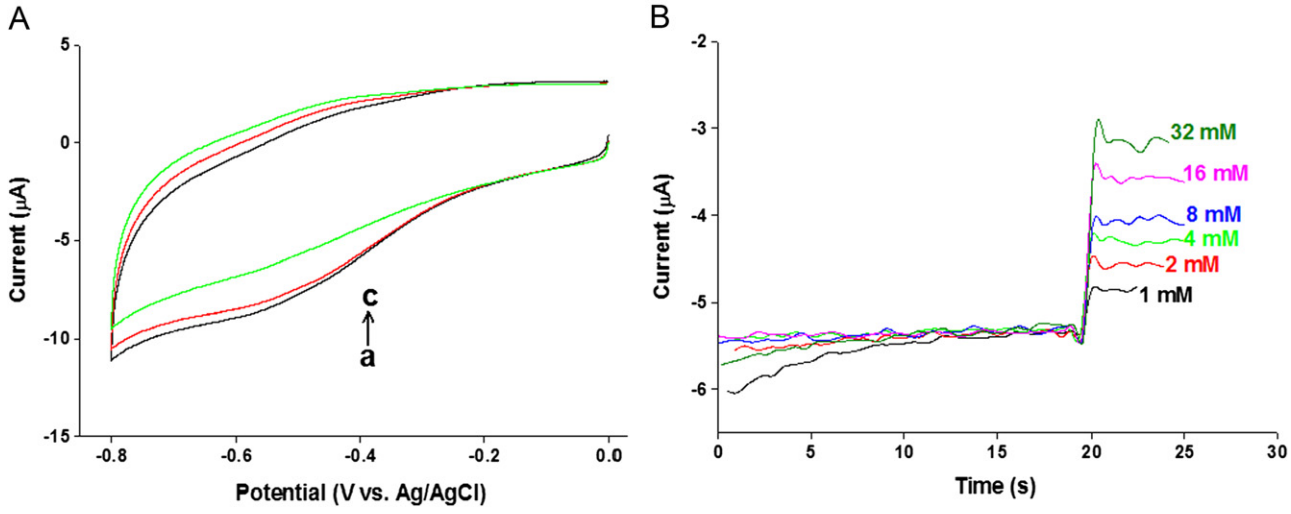

Fig. 3. (A) CVs of Nafion/GOx/graphene–APTES/GCE in (a) PBS, and (b) 1 mM and (c) 4 mM glucose solutions in the presence of oxygen at a scan rate of 100 mV s^{-1}. (B) Amperometric detection of 1–32 mM glucose at −0.45 V in the presence of oxygen.

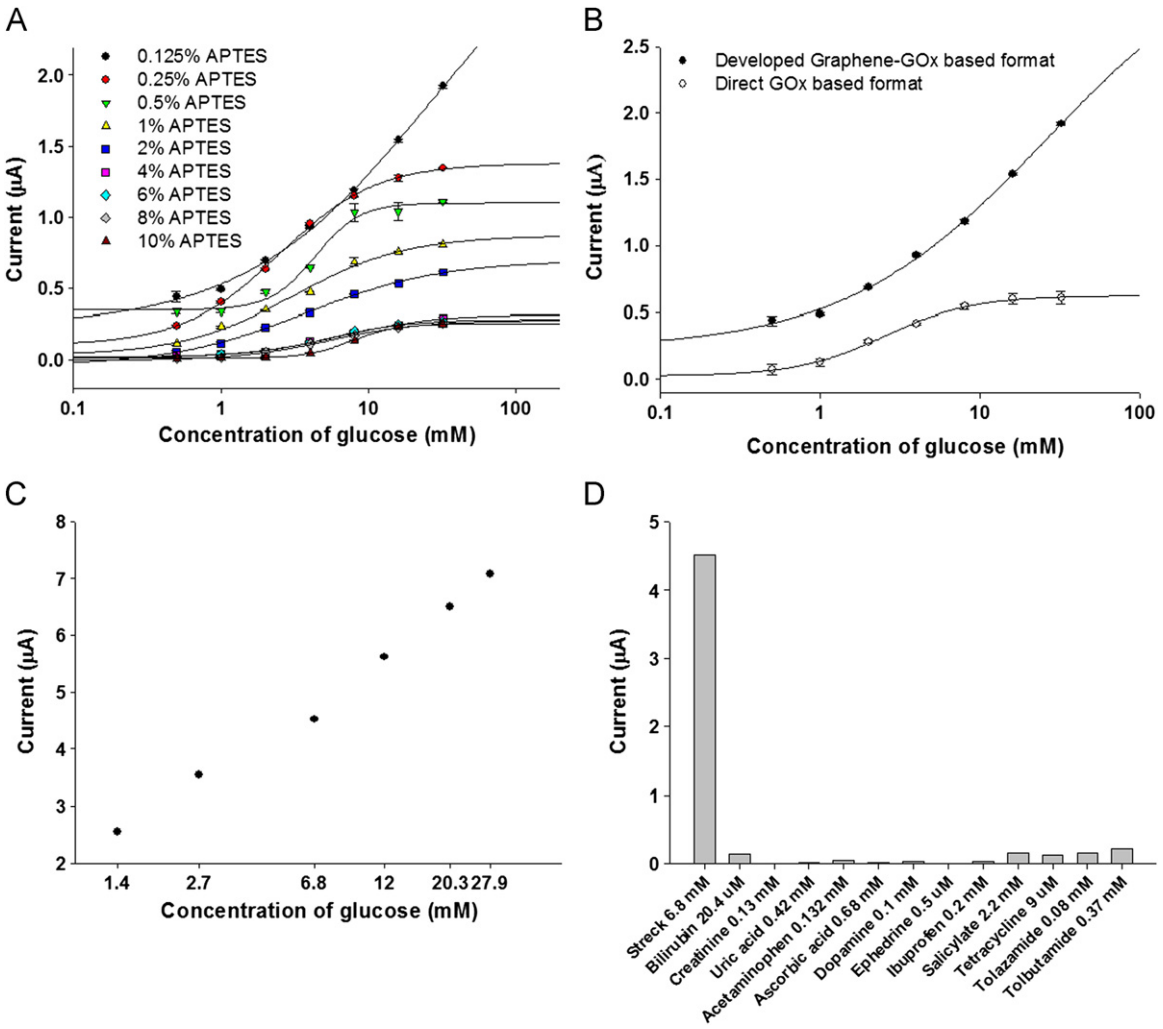

Fig. 4. (A) Effect of APTES concentration on the electrochemical glucose sensing by the developed Nafion/GOx/graphene–APTES/GCE. (B) Comparison of electrochemical glucose sensing by developed Nafion/GOx/graphene–APTES/GCE and Nafion/GOx/GCE. (C) Detection of Sugar-Chex whole blood glucose linearity standards from Streck, USA, by the developed Nafion/GOx/graphene–APTES/GCE. (D) Effect of physiological interferences and drug metabolites on the specific detection of 6.8 mM blood glucose by the developed Nafion/GOx/graphene–APTES/GCE. The error bars represent standard deviation.

standards in the range 1.4–27.9 mM, i.e. the diabetic pathophysiological glucose range (Fig. 4C), which demonstrates its clinical utility for diabetic blood glucose monitoring. However, the current signals for the detection of Sugar-Chex blood glucose linearity standards [32,33] were very different from those of commercial glucose, which may be attributed to different matrices. Therefore, these results warrant the need to determine the analytical biosensing parameters of glucose biosensing approaches using the blood glucose only instead of the commercial glucose that has been used in many published reports. The Sugar-Chex blood glucose linearity standards have not been used in any report. Only a few reports detected varying glucose concentrations in human serum samples [17,34]. The Sugar-Chex blood glucose linearity standards are made from stabilized whole blood of humans. Therefore, they have the same hematocrit, viscosity, and surface tension, which enable them to highly mimic the flow rate and performance of fresh whole blood on commercial glucose test strips. It has been demonstrated that these characteristics varied only slightly over a period of 90 days in comparison to that of fresh whole blood [32]. On the other hand, the commercial glucose solutions are incapable of mimicking the cellular impedance and viscosity of whole blood in order to obtain the pre-optimized sample delivery time on the test strip/electrode surface. They reach the test strip/electrode surface much faster than the whole blood, which adversely affects the kinetic rate of reaction [32]. The developed glucose biosensor was found to have the best dynamic and linear range in comparison to other graphene-based glucose sensing approaches reported so far (Supplementary Table S1).

3.3. *Effect of interfering substances*

The pathophysiological levels of biologically interfering species and drug metabolites [35] did not interfere with the detection of glucose by the developed biosensor as it was mediatorless and employed a lower applied potential of −0.45 V (Fig. 4D). The interference of glucose biosensor is one of the most critical analytical parameters that determine the commercial success of blood glucose monitoring devices [36–40]. Most of the reported graphene–GOx based glucose biosensors have either not tested [14–16] or employed only a few interfering substances [41,42] such as ascorbic acid, uric acid, dopamine and acetaminophen. However, we have critically investigated the effects of various interferences on the detection of glucose by the developed biosensor. The absence of interference from physiological substances and drug metabolites clearly shows the high precision of blood glucose measurements by the developed biosensor for diabetic monitoring.

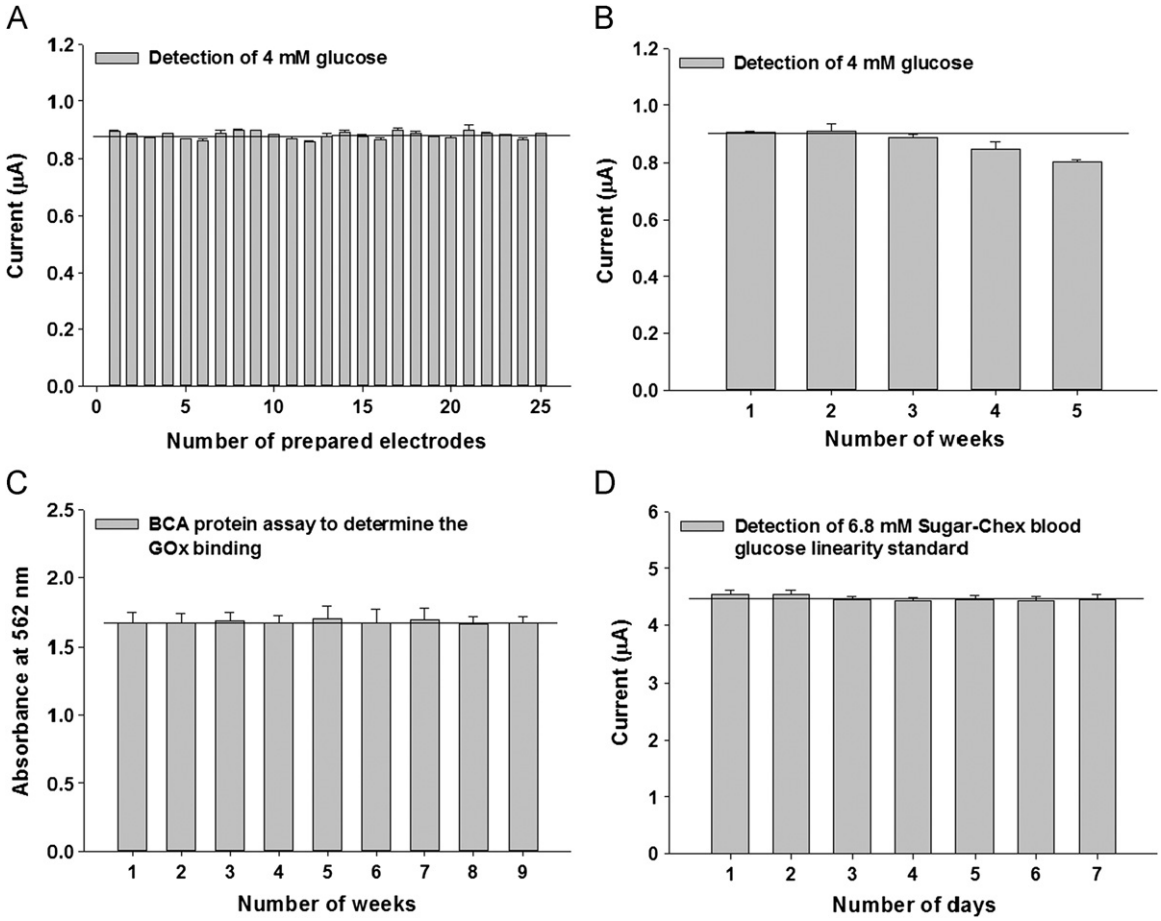

Fig. 5. (A) Determination of reproducibility of developed bioanalytical procedure for the production of 25 Nafion/GOx/graphene–APTES/GCEs based on the detection of 4 mM glucose. (B) Determination of stability of developed Nafion/GOx/graphene–APTES/GCE that was stored at RT in a dry state based on their detection of 4 mM glucose. (C) BCA protein assay for the determination of GOx binding to developed Nafion/GOx/graphene–APTES/GCEs that were used for glucose detection for 9 weeks. (D) Determination of the effect of biofouling by keeping the Nafion/GOx/graphene–APTES/GCE immersed in the 1 mM Sugar-Chex blood glucose linearity standard for 7 days but used intermittently each day for detecting the 6.8 mM Sugar-Chex blood glucose linearity standard in triplicate. The error bars represent standard deviation.

3.4. Analytical performance

The capability of the developed bioanalytical procedure for reproducible mass production of GOx-bound GCE-functionalized GCEs was tested by preparing 25 Nafion/GOx/GNP/GCEs and employing them for the triplicate detection of 4 mM glucose. As shown in Fig. 5A, the developed bioanalytical procedure has excellent production reproducibility, which is ideal for the commercial mass production. It can be easily transduced to screen- or inkjet-printing, which are cheaper techniques for the commercial mass-production of enzyme-bound electrodes [43–46]. The screen-printing will enable the production of graphene–APTES/GCE, GOx/graphene–APTES/GCE and Nafion/GOx/graphene–APTES/GCE in consecutive steps.

The storage stability of the developed Nafion/GOx/graphene–APTES/GCE, stored at RT under ambient conditions, was determined from the current response pertaining to the detection of 8 mM glucose each week for a duration of 5 weeks (Fig. 5B). There was no decrease in the current response of developed Nafion/GOx/graphene–APTES/GCE for the first 3 weeks and only a minor decrease of 12% in the next 2 weeks. The decrease in the functional activity after 3 weeks may be due to the spreading of GOx and its conformational change under ambient conditions [23]. This storage stability is good for demonstrating the developed laboratory prototype of glucose biosensors under ambient conditions. However, more intensive studies are underway to determine the actual shelf-life of developed Nafion/GOx/graphene–APTES/GCEs using the commercial strategies being employed for storage and packing. Various strategies are also being tried to prolong the shelf-life of developed Nafion/GOx/graphene–APTES/GCEs, which will be a key determinant for their commercialization. The total concentration of GOx bound to graphene-functionalized GCE, as determined by BCA protein assay, was found to be consistently uniform for 2 months (Fig. 5C), which confirmed the leach-proof binding of GOx to graphene-functionalized GCE by the developed bioanalytical procedure. Moreover, there was no effect of biofouling when the developed Nafion/GOx/graphene–APTES/GCE was kept immersed in the 1 mM Sugar-Chex blood glucose linearity standard for 7 days and used each day for the detection of 6.8 mM Sugar-Chex blood glucose linearity standard in triplicate (Fig. 5D). Therefore, the developed method will be of tremendous utility for the development of continuous glucose monitoring devices based on its higher analytical performance and stability in comparison to the commercially-available continuous blood glucose monitoring devices, where the enzyme-coated electrode can only be used for only 5 days.

4. Conclusion

A simple bioanalytical procedure was developed for the preparation of Nafion/GOx/graphene–APTES/GCE that was employed for the mediatorless amperometric glucose biosensing in diabetic pathophysiological range of 0.5–32 mM using a redox potential of

−0.45 V (vs. Ag/AgCl). The developed biosensor was validated for detecting blood glucose in Sugar-Chex blood glucose linearity standards (1.4–27.9 mM), and was demonstrated to be free from any potential interference by physiological substances and drug metabolites. There was no decrease in functional activity of the developed Nafion/GOx/graphene–APTES/GCE for 3 weeks, when it was stored in a dry state at RT under ambient conditions. However, the concentration of GOx bound to graphene-functionalized GCEs remained consistently constant for 9 weeks, which shows the leach-proof binding of GOx to GCE by the developed bioanalytical procedure. The developed procedure had excellent production reproducibility for the preparation of Nafion/GOx/graphene–APTES/GCEs and can be easily transduced to screen-printing that will enable the cost-effective mass production of Nafion/GOx/graphene–APTES/GCEs for commercialization. There was no effect of biofouling on the glucose detection of Nafion/GOx/graphene–APTES/GCE that was kept immersed in the 1 mM Sugar-Chex blood glucose linearity standard for 7 days. It has tremendous commercial potential for the development of diabetic blood glucose monitoring devices based on its simplicity and superior analytical performance.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.05.014.

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