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**PAPER**

# Rapid and simple preparation of a reagentless glucose electrochemical biosensor†

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A rapid and simple procedure was developed for the preparation of a highly stable and leach-proof glucose oxidase (GOx)-bound glassy carbon electrode (GCE). Crosslinked GOx *via* glutaraldehyde was drop-cast on a KOH-pretreated GCE followed by drop-casting of 3-aminopropyltriethoxysilane (APTES) to form a stable bioactive layer. At −0.45 V, the biosensor exhibited a wide dynamic detection range of 0.5–48 mM for commercial glucose and 1.3–28.2 mM for Sugar-Chex blood glucose linearity standards. Several endogenous electroactive substances and drug metabolites commonly found in blood were tested and provoked no signal response. To our knowledge, the developed procedure is the most rapid method for preparing a glucose biosensor. The biosensor suffered no biofouling after 7 days of immersion in Sugar-Chex blood glucose. With excellent production reproducibility, GOx-bound electrodes stored dry at room temperature retained their initial activity after several weeks.



## Introduction

Continuous glucose monitoring systems (CGMS) play an important role in diabetes management due to a better glycemic control by continuous monitoring of fluctuations in blood glucose levels, which is otherwise not available by point-of-care glucose meters.[1] Real-time CGM can provide useful insights for diabetic healthcare professionals for the effective management of diabetes[1,2] to circumvent harmful complications. Besides their high cost, CGMS have not been overwhelmingly accepted by diabetic patients.[3] In brief, the high cost of CGMS can be attributed to the efforts required to improve sensing stability,[4] accuracy, reliability and other bioanalytical parameters.

In most commercial CGMS such as Guardian REAL-Time (Medtronic MiniMed, Sylmar, CA), DexCom™ STS™-7 (DexCom, San Diego, CA), and FreeStyle Navigator® (Abbott Diabetes Care, Alameda, CA), flavin-containing glucose oxidase is extensively used (eqn (1)). Molecular oxygen, $O_2$, acts as a natural electron acceptor to oxidize the reduced form of GOx or $FADH_2$-GOx back to FAD-GOx (eqn (2)). The concentration of $H_2O_2$, a by-product of the enzymatic reaction, is measured and equated to the glucose concentration.[2,5] Alternatively, if a mediator is used to facilitate the electron transfer between the active center of GOx and the electrode, the GOx-$FADH_2$ can be oxidized electrochemically through the electrode reaction[2,5–7] (eqn (3) and (4)). Although mediator based chemistry has been used in commercial blood glucose monitoring devices,[2,5,6,8] the search for an ideal mediator has not been fruitful. The mediator must be insoluble, non-toxic and chemically stable in both reduced and oxidized forms.[9] It must be firmly retained on the electrode for repeated use[7] and should not interact with endogenous electroactive substances and drug metabolites commonly found in blood.[9]

$$\text{Glucose} + \text{FAD-GOx} \rightarrow \delta\text{-Gluconolactone} + \text{FADH}_2\text{-GOx} \quad (1)$$

$$\text{FADH}_2\text{-GOx} + \text{O}_2 \rightarrow \text{FAD-GOx} + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{FADH}_2\text{-GOx} + 2\text{M}_{ox} \rightarrow \text{FAD-GOx} + 2\text{M}_{red} + 2\text{H}^+ + 2\text{e}^- \quad (3)$$

$$2\text{M}_{red} \rightarrow 2\text{M}_{ox} + 2\text{e}^- \quad (4)$$

For the past few decades, various efforts have focused on the immobilization of GOx on a solid support with a minimal effect on its functional performance. Of interest is the use of chitin- and chitosan-based materials,[10] conducting polymers[11,12] (*e.g.* polyaniline and polypyrrole), sol–gel encapsulation[13,14] and other methods such as enzyme wiring.[2,5,7] However, such methods employ time-consuming and complex chemical procedures, resulting in an increased manufacturing cost for the preparation

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of GOx-bound electrodes. Therefore, a highly convenient, leach-proof and reproducible procedure is needed for mass production of highly stable and interference- and biofouling-resistant GOx-bound electrodes.

We describe here a simple and rapid procedure for the development of reagentless GOx-bound electrodes, applicable for the detection of glucose in the diabetic pathophysiological range. The GOx-bound GCE was employed for the detection of commercial and blood glucose. The reproducibility of the procedure, storage stability, biofouling and the effect of physiological interferences were also investigated. The developed procedure will be of tremendous significance for the rapid preparation of leach-proof enzyme-bound electrodes for the detection of other analytes.

## Experimental

### Chemicals

98% APTES, glucose oxidase (GOx, EC 1.1.3.4, Type X-S from Aspergillus niger, G7141), D-glucose, 70% glutaraldehyde, 5% Nafion, and the interfering substances were purchased from Sigma-Aldrich, Singapore. BupH phosphate buffered saline (PBS) was procured from Thermo Fisher Scientific, USA. Sugar-Chex whole blood glucose linearity standards were purchased from Streck Inc (Omaha, NE). The dilutions of APTES and glutaraldehyde were made in ultrapure water (18.2 MΩ cm@25 °C, Direct Q, Millipore); the dilutions of GOx and glucose were made in 50 mM PBS; and, the dilution of 0.5 wt % Nafion was made in absolute ethanol. Crosslinked GOx was prepared by mixing equal volumes of 20 mg $mL^{-1}$ GOx and 5% (w/v) glutaraldehyde solution followed by storage at 4 °C for at least 1 h prior to use.

### 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. 2 μL of 10 mg $mL^{-1}$ crosslinked GOx by glutaraldehyde was drop-cast on GCE followed by the immediate drop-casting of 2 μL of 4% (w/v) APTES to form an APTES–GOx mixture on GCE. The APTES–GOx/GCE was dried at room temperature (RT) for 1 h, washed extensively with 50 mM PBS and drop-casted with 3 μL of 0.5% Nafion to form a Nafion/APTES–GOx/GCE. The developed procedure is illustrated in Fig. 1.

### Apparatus

Electrochemical measurements (cyclic voltammetry and amperometric *i–t* curve) were performed using a CHI 660A electrochemical workstation with a three-electrode system: working electrode, Pt wire counter electrode and Ag/AgCl (3 M KCl) reference electrode. All potentials were measured and reported *vs.* Ag/AgCl. All experiments were performed in 50 mM PBS under ambient conditions. The absorbance measurements for the BCA protein assay based determination of GOx binding to the electrode were performed on a Tecan Infinite M200 Pro microplate reader.

### Detection of glucose

For the detection of commercial glucose, varying volumes of 1 M glucose stock solution were injected into the stirred PBS to make a final concentration of 0.5, 1, 2, 4, 8, 16, 32 or 48 mM in a 2 mL solution. All the concentrations were detected individually in triplicate. For the detection of blood glucose, 400 μL of Sugar-Chex blood glucose linearity standards with varying glucose concentrations, *i.e.* 1.3, 2.8, 6.6, 11.8, 20.3 and 28.2 mM, was injected into 2.8 mL of stirred PBS and detected individually.

### Effect of interference

Ascorbic acid, dopamine, creatinine and acetaminophen were dissolved in 50 mM PBS. Uric acid and bilirubin were prepared in 10 mM NaOH; tetracycline was prepared in 1 M HCl; ibuprofen, salicylate and tolbutamide were dissolved in absolute ethanol, and tolazamide was dissolved in acetone. The stock solutions of all interfering substances were prepared freshly just before testing. The effect of interference was determined by the measurement of the response signal for 6.6 mM Streck blood glucose in the presence of such interferents.

### Production reproducibility

Production reproducibility was determined from the response signals obtained for 8 mM glucose (in triplicate) using 25 different GOx-bound GCEs.

### Effect of biofouling

The current signal obtained for 8 mM glucose was recorded using the freshly prepared electrode. The electrode was then stored overnight at room temperature in the 0.825 mM Sugar-Chex blood glucose linearity standard and then used again for the detection of 8 mM glucose the next day. This procedure was repeated for five consecutive days.

### Storage stability

The stability of Nafion/APTES–GOx/GCEs was assessed for four different storage conditions that are most widely used in biomedical diagnostics. Each of the four electrodes was stored under a different storage condition: in 50 mM PBS at 4 °C, in dry state at 4 °C, in 50 mM PBS at room temperature, and in dry state at room temperature. They were used for detecting 8 mM glucose ten times each day for about 2 months.

### Bicinchoninic acid (BCA) protein assay

The Nafion/APTES–GOx/GCE and the control electrode *i.e.* Nafion/APTES/GCE were immersed in 200 μL of BCA working reagent inside the microcentrifuge tubes and incubated at 80 °C for 15 min. The colored products formed (180 μL each) were then transferred to the 96-well microtiter plate, whose absorbance was read at 562 nm by Tecan Infinite M200 Pro microplate reader. The absorbance of control electrode was subtracted from that of

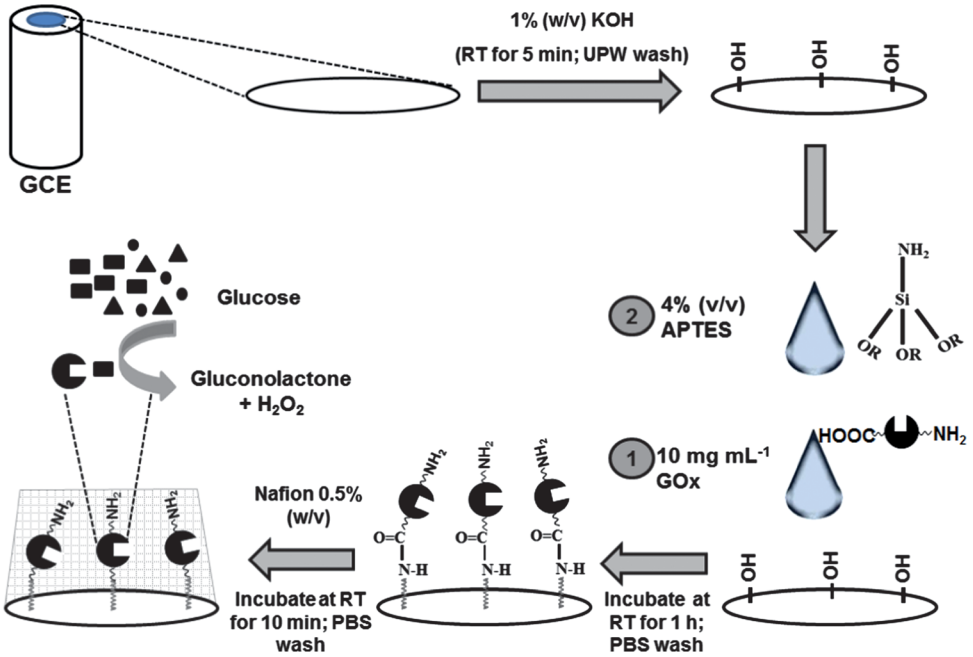


**Fig. 1** Schematic diagram for the preparation of glucose oxidase-bound glassy carbon electrodes.

developed Nafion/APTES–GOx/GCE to determine the total concentration of GOx bound to the electrode.

## Results and discussion

APTES has been used as a surface modification agent for immobilization of biomolecules[15,16] on various bioanalytical platforms. The APTES-functionalized bioanalytical platforms have several potential advantages such as long-term stability, high immobilization density, less biofouling and high reproducibility. APTES has been used commercially for the development of highly stable continuous glucose monitoring systems.[17–19] Recently, we developed a novel multisubstrate-compatible APTES based immobilization strategy[20–24] and employed it widely for various bioanalytical applications in industrial and clinical settings. However, the developed simplified procedure reported in this manuscript, where APTES has been used for GCE surface modification and GOx binding, has not been reported. As shown in Fig. 1, GOx was drop-cast on the KOH-treated GCE surface, followed by immediate drop-casting of APTES. In the micro-environment of the droplet, hydroxyl groups were generated on the KOH-treated GCE. Therefore, GOx was covalently bound to the GCE surface in a leach-proof manner. Moreover, as no artificial mediator is used, the electrochemical glucose sensing by the developed simplified procedure is based on eqn (1) and (2). To our knowledge, the developed procedure is the most rapid method to date for the preparation of a glucose biosensor in comparison to the procedures used in the published reports (Table S1†).

As shown in Fig. 2A, in the presence of oxygen, although the redox peaks of GOx were not observed, the CVs of the glucose sensor show the maximum cathodic current in the absence of glucose (in the potential window of −0.3 to −0.5 V). Additionally, the cathodic current decreased with increasing glucose concentration. The reduction wave actually corresponds to the $FADH_2$-

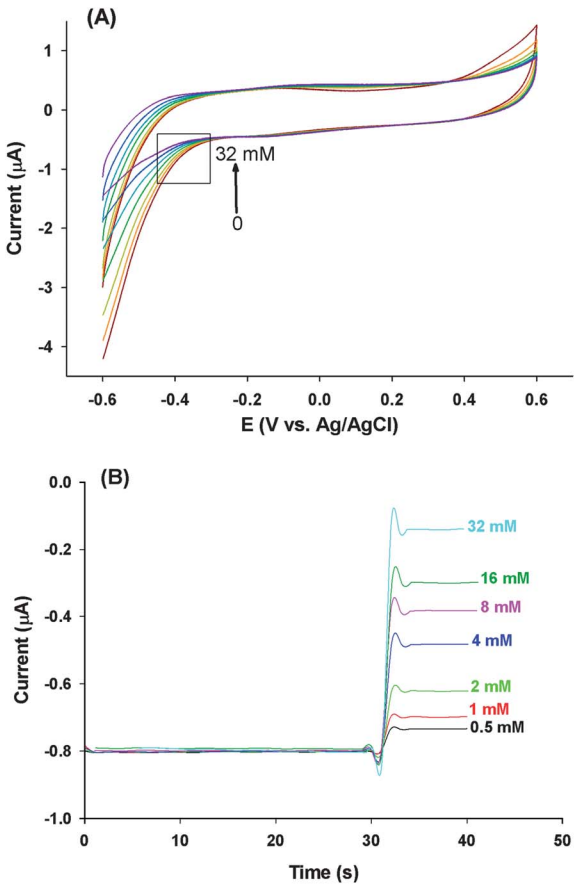


**Fig. 2** (A) The cyclic voltammograms and (B) amperometric response of the developed glucose biosensor for varying glucose concentrations (0.5–32 mM) in 50 mM PBS in the presence of oxygen. The scan rate in (A) was 100 mV s$^{-1}$, while the applied potential in (B) was −0.45 V.

 

GOx catalysed reduction of $O_2$. As the oxygen was consumed by the reaction between glucose and GOx (eqn (2)), the concentration of oxygen near the electrode surface was reduced, resulting in the decline in reduction current with an increase in glucose concentration. Our result is in good accordance with the published results.[25,26] The detection of higher levels of glucose will be restricted due to the limited concentration of $O_2$ dissolved in PBS. Therefore, a thin layer of 0.5% Nafion was employed as a glucose-limiting membrane, to prevent the enzyme from reacting with the excessive glucose molecules. Nafion also acted as an anti-interference membrane to prevent the diffusion of electroactive interferents and contaminating substances. The proportionate decrease of reduction current with the increase in glucose concentration was employed to determine the glucose levels using the *i–t* curve technique in the presence of oxygen. As shown in Fig. 2B, the cathodic current decreases with the injection of glucose in the range of 0.5–32 mM. The use of Nafion in the Nafion/APTES–GOx/GCE enabled the successful determination of glucose concentration in the entire pathophysiological range.

The assay curve obtained from the *i–t* curves (Fig. 3A) demonstrates that the developed electrochemical glucose biosensor can detect commercial glucose in the dynamic range of 0.5–48 mM at an applied potential of −0.45 V. The glucose sensing results obtained by the developed procedure were better than those obtained using various glucose sensing (including non-enzymatic[27,28] and enzymatic[29,30]) approaches. The developed biosensor was further evaluated for the detection of blood glucose in Sugar-Chex whole blood glucose linearity standards, which are industrial calibration standards for blood glucose monitoring as employed by most glucose meter manufacturers (Fig. 3B). These blood glucose standards have excellent open-vial stability of critical parameters for a period of over 90 days.[31] The biosensor detected the entire range of Sugar-Chex blood glucose linearity standards *i.e.* 1.3–28.2 mM, which covered the entire pathophysiological range in diabetics as detected by most commercial glucose meters. However, the current signal for the detection of 8 mM Sugar-Chex blood glucose was about 2.2-fold higher than that for the detection of 8 mM commercial glucose. The difference between the current signals of blood glucose and commercial glucose may be attributed to the matrix effect. As Sugar-Chex blood glucose linearity standards highly mimic the flow rate, hematocrit, viscosity, and surface tension of fresh human whole blood, they provide more reliable readings in comparison to commercial glucose.[31,32]

The effect of common interfering substances such as bilirubin, creatinine and uric acid, and medications such as

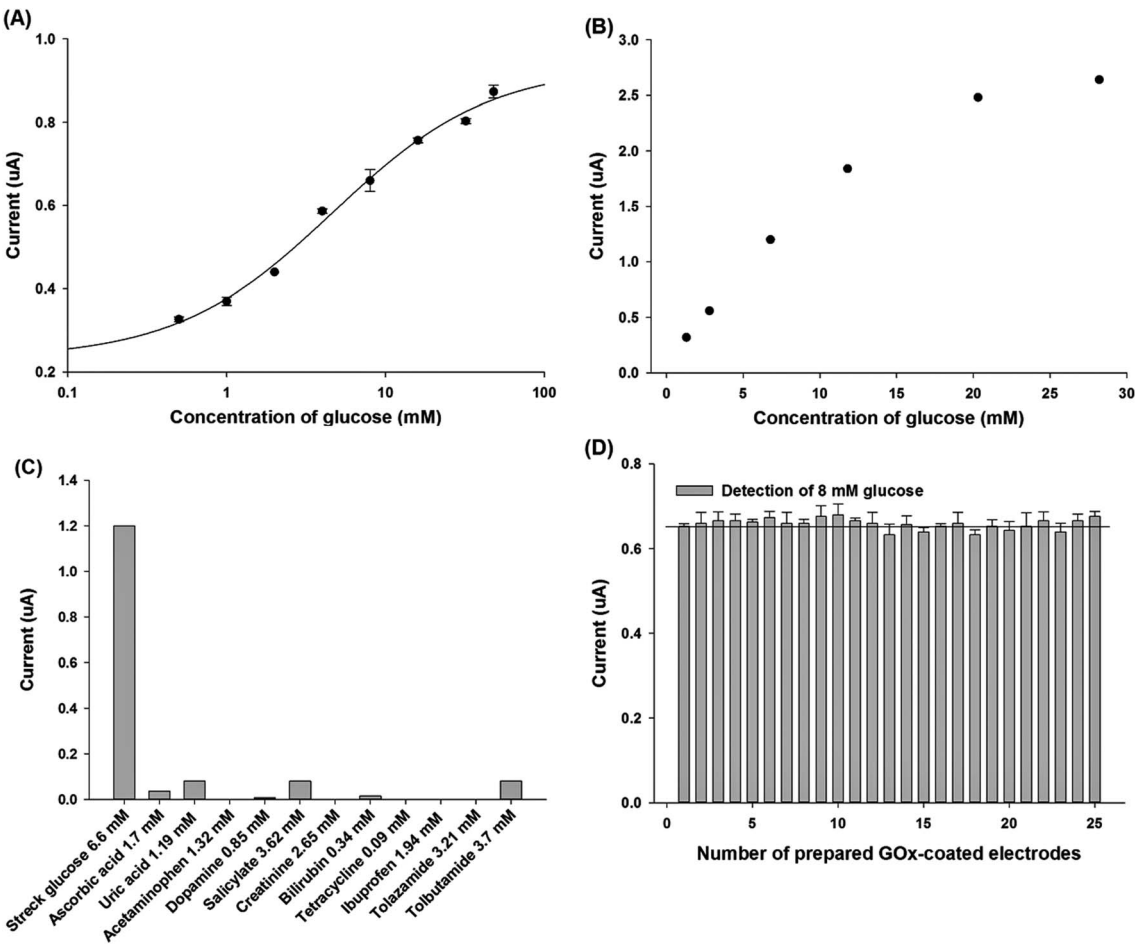


**Fig. 3** (A) Detection of commercial glucose, (B) detection of Sugar-Chex blood glucose linearity standards, and (C) the effect of interfering substances on the detection of blood glucose by the glucose biosensor. (D) Production reproducibility of the developed simplified procedure for the preparation of Nafion/APTES–GOx/GCEs.

acetaminophen, ibuprofen and tetracycline on the detection of blood glucose was studied (Fig. 3C). The testing concentrations of these interfering substances were 10–40-fold higher than their physiological or therapeutic levels.[33] About 8% false current signal was observed by the injection of uric acid, salicylate and tolbutamide, while less than 4% extra signal was observed for ascorbic acid. Dopamine and bilirubin provoked only negligible signal response. However, there was no detectable interference by these substances at their physiological or therapeutic levels (data not shown). These results were not totally unexpected as most electroactive interfering substances are not detected at −0.45 V. The developed mediatorless approach eliminated any plausible interaction between the mediator and electroactive interferents. Finally, the negatively charged Nafion membrane also circumvented the diffusion of electroactive negatively charged substances to the sensing area of the biosensor. Therefore, our strategy holds tremendous potential for highly precise blood glucose monitoring in diabetics with remarkable selectivity, which is one of the most critical factors for the development of commercial glucose meters.[5,34–38]

The production reproducibility (Fig. 3D) and the anti-biofouling behavior (Fig. 4A) of the developed glucose sensing procedure were also investigated. The current signals corresponding to the detection of 8 mM glucose by 25 different GOx-bound GCEs were highly reproducible, as the percentage coefficient of variance was only 3%. The anti-biofouling performance of the developed GOx-bound GCEs was excellent as the current signal for the detection of 8 mM glucose by a particular electrode was stable for five days. Therefore, the developed GOx-bound GCEs have the same anti-biofouling performance as found in commercially available CGMS, where the GOx-bound electrodes are used continuously for five days.

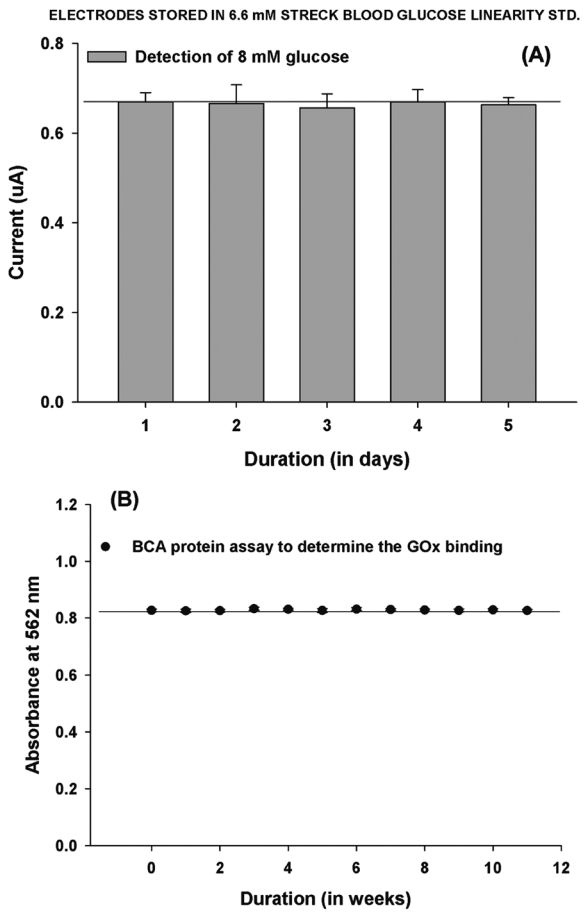


**Fig. 4** (A) Effect of biofouling on the electrochemical sensing of glucose by the developed Nafion/APTES–GOx/GCEs. (B) BCA protein assay for the determination of GOx bound to the developed Nafion/APTES–GOx/GCEs for 12 weeks.

The total concentration of GOx bound to GCE, as determined by BCA protein assay,[39] was found to be consistently uniform for about three months (Fig. 4B), which confirmed the leach-proof binding of GOx to GCE by the developed bioanalytical procedure. The stability of the developed GOx-bound GCEs was also studied under four different storage conditions that are employed routinely in biomedical diagnostics (Table S2†). Our results clearly demonstrated that the stability of the developed GOx-bound GCE was highly affected by the storage conditions. The biosensor was most stable when stored at RT in the dry state, where there was no decrease in the sensing signal for about four weeks. The storage at 4 °C in the dry state was not good as the response signal kept decreasing constantly with time and reached 50% of its initial value after about five weeks. On the other hand, the stability of the developed GOx-bound GCEs stored in PBS at RT or 4 °C was quite similar but not better than that stored at RT in the dry state. Therefore, there were changes in the conformation of GOx under different storage conditions, which affect its functional activity. The performance of our approach was comparable to the GOx-entrapped poly(vinyl alcohol) (PVA) modified platinum electrode,[40] which could also detect up to 37 mM glucose at −0.6 V *vs.* saturated calomel electrode (SCE) and maintains 87% of the initial sensing signal after 35 days when stored in the dry state at room temperature. However, our strategy employs the most simple and rapid procedure for preparing a GOx-bound electrode in comparison to 48 h for the PVA membrane.[40] Therefore, the developed procedure has immense potential for the simple and rapid preparation of enzyme-bound electrodes for the electrochemical detection of other analytes in biomedical diagnostics, environmental monitoring and industrial settings. Moreover, this procedure can be easily extended to other electrode materials such as screen printed carbon, graphene, and carbon nanotubes.

## Conclusions

We developed the most rapid and simple procedure for the preparation of leach-proof and highly stable GOx-bound GCEs, which detected glucose in the dynamic range of 0.5–48 mM at the applied potential of −0.45 V. The developed procedure is highly reproducible, selective and amenable to mass production. The glucose sensing signal was not affected by physiological substances and drug metabolites. There was no biofouling, when the developed GOx-bound GCE was kept for 7 days in Sugar-Chex blood glucose linearity standard.

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