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Determination of Glucose using a Biosensor Based on Glucose Oxidase Immobilized on a Molybdenite-Decorated Glassy Carbon Electrode

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We report the use of a molybdenite (MLN) and glucose oxidase (GOD)-modified glassy carbon electrode (GCE)-based glucose biosensor with the aid of hydroquinone (HQ) as a mediator. It was fabricated through covalent bonding of GOD and MLN using glutaraldehyde (GA) as a covalent reagent. The biosensor exhibited a fast and linear response toward glucose from 1.0 to 135 mM by using a cyclic voltammogram (CV) measurement, with a detection limit of 100 μM. The repeatability, reproducibility and applicability were also investigated in this research. The results show that MLN is a good platform for GOD immobilization.

Keywords: molybdenite, glucose biosensor, covalent bonding, second-generation

1. INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar levels over a prolonged period and an endogenous inability to control glycemic excursions [1]. Diabetes causes many complications if left untreated, such as diabetic ketoacidosis, chronic kidney failure, and eye damage. An analysis of glucose has attracted immense research interest since the first report on glucose enzyme biosensors by Clark and Lyons [2]. Glucose biosensors occupy approximately 85% of the current world market for biosensors because of the large scale of diabetes in developed countries [3]. The rapid and accurate detection of glucose concentration is of practical importance for the prevention and treatment of diabetes mellitus.

Glucose oxidase (GOD, EC 1.1.3.4) catalyzes the oxidation of β-*D*-glucose to δ-gluconolactone by using molecular oxygen as an electron acceptor, which then substantially and spontaneously hydrolyzes to gluconic acid and hydrogen peroxide [4]. GOD has been widely used as a biorecognition enzyme in glucose biosensors for the quantitative detection of *D*-glucose in clinical tests, food analysis and fermentation product analysis. Electrochemical biosensors have great potential in glucose concentration detection due to their high sensitivity, excellent selectivity, simple operation and good compatibility with miniaturization technologies [5]. Numerous materials have been applied to the construction of sensors and biosensors, such as noble metal nanomaterials [6], graphene [7, 8], carbon nanotubes [9, 10], organic polymers [11], and molybdenum disulfide (MoS_2) [12-14]. The research interest in two-dimensional (2D) materials has increased since graphene, along with its outstanding properties, was discovered in 2004 [15]. MoS_2, as an analogue of graphene in structure [16], has aroused extreme interest in the field of electrochemical catalysis [17], sensing [18], and supercapacitors [19]. MoS_2 has excellent electrochemical properties, especially fast heterogeneous electron transfer capabilities and tunable band gaps [20].

To avoid a complicated synthesis procedure, we directly use natural molybdenite (MLN) as a support matrix to prepare a GOD-based biosensor. The main component of MLN is 2D-layered MoS_2. In this study, we aim to explore the potential of natural molybdenite toward the development of a second-generation electrochemical glucose biosensor without doping or decoration with carbon materials and noble metal nanoparticles. In addition, this glucose biosensor may be applicable to the detection of real samples. The methodology will be useful not only for studying the interfacial properties of molybdenite and glucose oxidase but also for providing a wide application platform for molybdenite.

2. EXPERIMENTAL

2.1 Reagents

Glucose oxidase (GOD, EC 1.1.3.4 from *Aspergillus niger*; ＞100 units mg^{-1}) was purchased from Sigma-Aldrich Co., China, and used as received. Glucose, glutaraldehyde (GA), catechol (CC), dopamine (DA), ferrocenemethanol (FcMeOH), hydroquinone (HQ), K_2HPO_4 and KH_2PO_4 were obtained from Sinopharm Chemical Reagent Co., Ltd (China). A 0.1 M phosphate buffer (prepared by using K_2HPO_4 and KH_2PO_4) was used to prepare the electrolyte. Molybdenite (MLN) was obtained from Yichun Luming Mining Co. Ltd. (Yichun, China). All reagents were used without further purification.

2.2 Apparatus

An advanced mineral identification and characterization system (AMICS, AMICS-Mining/SIGMA 500, ZEISS/Bruker) was used to evaluate the morphology of the modified surface. Atomic force morphology (AFM) images were recorded by using a Being Nano-instruments CSPM-5500 (Ben Yuan Ltd., Beijing, China). Electrochemical measurements were performed using an electrochemical analyzer (CHI 660E, ALS Co. Ltd.). Cyclic voltammetry of GOD/GA/MLN/GCE was

measured in a glass cell using a modified GC electrode as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl electrode (3 M KCl) as the reference electrode. Electrochemical impedance spectroscopy (EIS) was performed by using deoxygenated 0.1 M phosphate buffer (pH 5.5) containing a 5 mM $[Fe(CN)_6]^{4-/3-}$ redox reaction. All measurements were performed in air at room temperature.

2.3 Preparation of the GOD/GA/MLN/GCE biosensor

A glassy carbon electrode (GCE) with a diameter of 3 mm was successively polished to a mirror shine surface with 1.0 and 0.05 μm alumina powder. After being rinsed with deionized water, it was sonicated in ethanol and deionized water for 5 min, respectively. The GCE was then dried in a nitrogen airflow. First, 30 mg/mL of MLN suspension (10 μL) was dropped on the cleaned GCE surface and dried for 1 h. Second, a GA (5%) aqueous solution was dropped on the MLN/GCE surface for 30 min. Finally, GOD (1.0 mg/mL) was cast on the GA/MLN/GCE surface. The prepared GOD/GA/MLN/GCE was washed with PBS (pH 5.5) three times.

3. RESULTS AND DISCUSSION

3.1 Characterization of the modified electrode surface

A biomolecule-functionalized support matrix is of great importance for substrate recognition in biosensor technology. The selection of materials is important for the development of electrochemical biosensors.

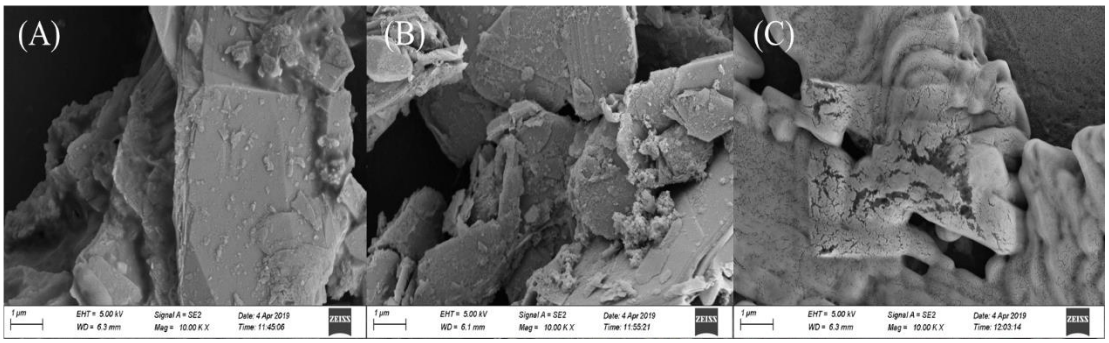

Figure 1. AMICS images of (A) MLN/GCE; (B) GA/MLN/GCE; and (C) GOD/GA/MLN/GCE.

The general morphologies and microstructures of modified GCE surfaces were evaluated by AMICS. As shown in Fig. 1A, the MLN reveals the typical layered structure and large-sized nanosheets. After GA immobilization, the cluster-shaped appearance on the GA/MLN/GCE surface (Fig. 1B) indicated the successful immobilization of GA on the MLN-modified GCE surface. Fig. 1C shows the modification of GOD on the GA/MLN/GCE surface. It is obvious that the film-like macromolecule layer

was covered on the electrode surface, which is evidence of the GOD modification.

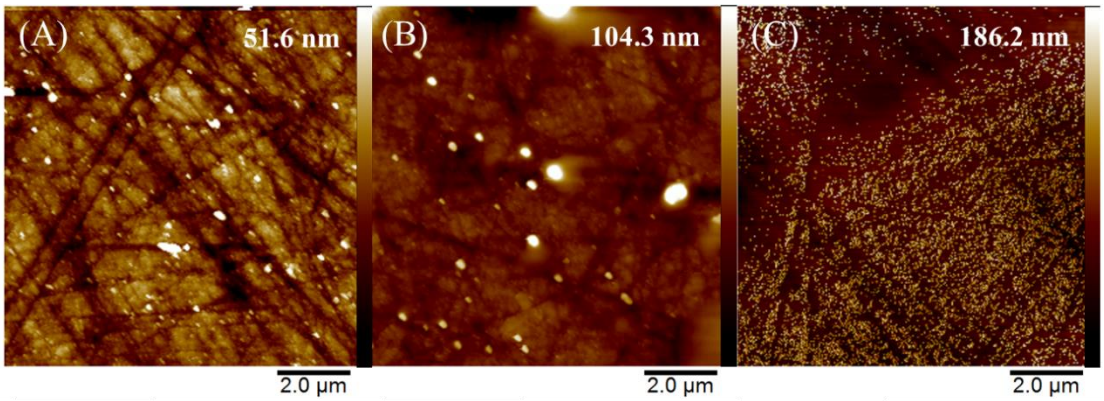

Figure 2. AFM images of (A) MLN/GCE; (B) GA/MLN/GCE; and (C) GOD/GA/MLN/GCE.

In addition, AFM images with the height profiles of the modified GCE were evaluated. Fig. 2A is the MLN-modified GCE, which shows a sliced and striped surface. After GA modification, the surface was covered with GA, while the striped surface was still observed (Fig. 2B). A granular surface was observed when GOD was immobilized on the GA/MLN/GCE surface (Fig. 2C). From these results, the chemical covalent bonding is shown to be effective for the modification of GOD onto the MLN matrix. Moreover, the heights of MLN/GCE, GA/MLN/GCE and GOD/GA/MLN/GCE are 51.6 nm, 104.3 nm and 186.2 nm, respectively, which is evidence of a layer-by-layer self-assembly of GOD.

3.2 Electrochemical studies of the modified electrode surface

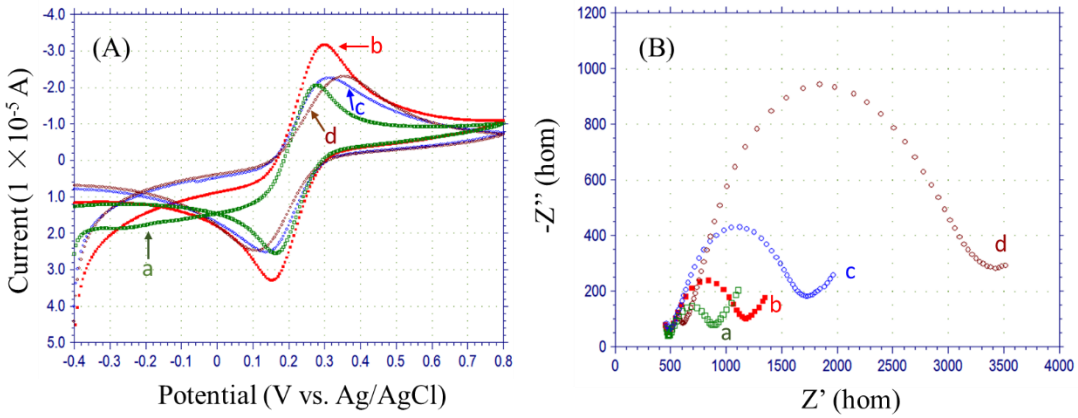

Figure 3. (A) CVs of the bare GCE (a), GOD/GA/MLN/GCE (b), GA/MLN/GCE (c) and MLN/GCE (d) in a deoxygenated 0.1 M PBS (pH 5.5) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. The potential scan rate was 5 mV/s. The starting potential was -0.4 V vs. Ag/AgCl. (B) Nyquist plots of the electrochemical impedance spectroscopy data of the bare GCE (a), GOD/GA/MLN/GCE (b), GA/MLN/GCE (c) and MLN/GCE (d) in deoxygenated 0.1 M PBS (pH 5.5) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. The amplitude was 0.005 V with a frequency range from 0.01 Hz to 10 KHz.

The electrocatalytic activity and electron transfer properties of the modified biosensors are

largely affected by the structures and conformations of the immobilized enzyme. To obtain the electrochemical properties of the modified electrodes, we measured cyclic voltammograms (CV) of 5 mM $[Fe(CN)_6]^{3-/4-}$ on the bare GCE (a) GOD/GA/MLN/GCE (b), GA/MLN/GCE (c) and MLN/GCE (d) by using 0.1 M PBS (pH 5.5) at a scan rate of 100 mV/s (Fig. 3A). The cathodic and anodic peaks are observed at 154.0 and 299.8 mV for GOD/GA/MLN/GCE, respectively. As a result, the peak-to-peak separation (ΔE) is 145.8 mV. The peak-to-peak separations are 174 and 244 mV for GA/MLN/GCE and MLN/GCE, respectively. This result indicates that the electron transfer rate of GOD/GA/MLN/GCE is faster than those of GA/MLN/GCE and MLN/GCE [21].

The electrochemical behaviors of the modified electrodes were also examined by EIS. It is a useful tool to evaluate the interfacial properties of enzyme-modified electrodes [22]. Fig. 3B shows the Nyquist plots of the EIS for the bare GCE (a) GOD/GA/MLN/GCE (b), GA/MLN/GCE (c) and MLN/GCE (d) obtained in 0.1 M phosphate buffer solution (pH 5.5) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. The value of the electron transfer resistance (R_{ct}) can be obtained from the semicircle diameter, which varies with the presence of different substances on the electrode surface. As shown, the R_{ct} of the bare GCE, GOD/GA/MLN/GCE, GA/MLN/GCE and MLN/GCE are 450 Ω, 750 Ω, 1300 Ω and 2800 Ω, respectively. Obviously, the R_{ct} of GOD/GA/MLN/GCE is dramatically decreased compared with MLN/GCE and GA/MLN/GCE, thus exhibiting the fastest electron transfer rate among these modified electrodes. This interpretation is consistent with the results of the CVs for each electrode (Fig. 3A).

3.3 Optimization of the experimental variables

3.3.1 Effect of immobilization procedures toward the oxidation of glucose

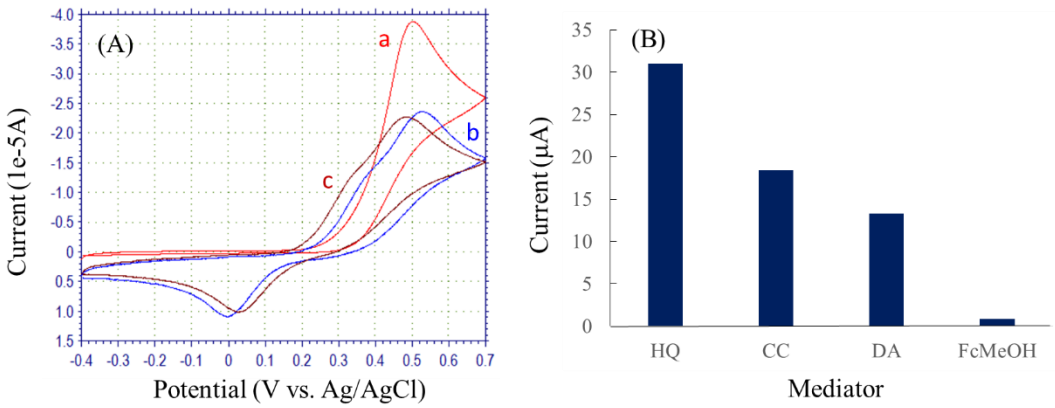

Figure 4. (A) Comparison of the preparation procedures on CVs of the GOD-modified GCE. The construction methods: (a) GOD/GA/MLN/GCE, (b) simultaneous adsorption of the GOD and GA on MLN/GCE, and (c) a step-by-step fabrication method (first the MLN was immobilized on the GCE, then GOD was dropped on the MLN/GCE, finally GA was cast on the GOD/MLN/GCE surface). The CV measurement conditions were the same as in Fig. 3. (B) Comparisons of the different mediators in the electrolyte. ([glucose] = 20 mM, PBS (0.1 M, pH 5.5))

The effect of immobilization steps was studied as shown in Fig. 4A. The bioelectrocatalytic

activity observed in the presence of 20 mM glucose of GOD/GA/MLN/GCE (curve a) was much larger than the case of simultaneous immobilization of GOD and GA on MLN/GCE (curve b) and the step-by-step modification of GA/GOD/MLN/GCE (curve c). This means that the step-by-step immobilization of GOD/GA/MLN/GCE is effective for achieving the best performance.

The GOD/GA/MLN/GCE biosensor exhibits sufficient bioelectrocatalytic activity with the use of HQ as an electron acceptor according to the following scheme (equations 1 to 3) [23].

GOD (FAD) + *D*-glucose → GOD ($FADH_2$) + D-glucono-δ-lactone (1)

GOD ($FADH_2$) + *p*-quinone → GOD (FAD) + hydroquinone (HQ) (2)

Hydroquinone (HQ) → *p*-quinone + $2H^+$ + $2e^-$ (3)

3.3.2 Effect of mediators on the oxidation of glucose

To obtain the best biosensor performance, the mediators were optimized and are shown in Fig. 4B. Among the four mediators, HQ shows the best results. The detection mechanism of glucose in this second-generation biosensor begins with the oxidation of glucose by the flavin redox center (FAD) of GOD (equation 1). HQ works as a mediator instead of O_2 as the cofactor to regenerate GOD (FAD).

3.3.3 Effect of MLN concentration on the oxidation of glucose

The bioelectrocatalytic anodic peak currents toward 5 mM glucose were used to optimize the construction and application of the GOD/GA/MLN/GCE biosensor.

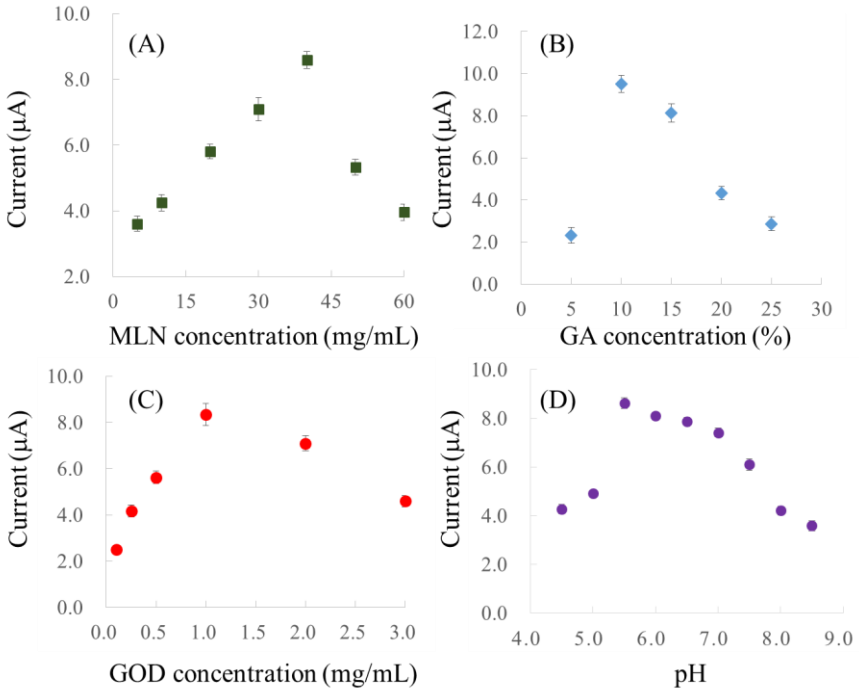

Figure 5. Effect of MLN concentration (A), GA concentration (B), GOD concentration (C) and electrolyte pH (D) on the CV responses of glucose (5 mM) in 0.1 M PBS (pH 5.5).

The effect of MLN concentration was optimized by using the CV data in this research (Fig. 5A). The peak current was selected at a potential of 0.6 V vs. Ag/AgCl. The response toward glucose increases in the concentration range from 5 to 40 mM and reaches a maximum value of 40 mg/mL. Thus, as the optimum MLN concentration, we chose an MLN concentration of 40 mg/mL for the following experiments.

3.3.4 Effect of GA concentration on the oxidation of glucose

We checked the effect of the GA concentration, which is shown in Fig. 5B. The response increases as the concentration of GA increases from 5 to 10% (v/v%) and then decreases when the GA concentration is increased to 25%. A high concentration of GA is not preferable for the enzyme because of its toxicity to the enzyme. Hence, 10% GA was selected for the following experiments.

3.3.5 Effect of GOD concentration on the oxidation of glucose

The effect of the GOD concentration on the CV response to glucose was investigated at a potential of 0.6 V vs. Ag/AgCl. As shown in Fig. 5C, the response increases with increasing GOD concentration up to 1.0 mg/mL. When the concentration is greater than 1.0 mg/mL, the current decreases with increasing GOD concentration. A high concentration of GOD will hinder the electron transfer between the active site of GOD and the substrates [24]. Therefore, 1.0 mg/mL of GOD was chosen for all subsequent experiments.

3.3.6 Effect of electrolyte pH on the oxidation of glucose

The effect of the electrolyte pH on the CV response to glucose was also investigated over a pH range of 4.5 to 8.5 using 0.1 M PBS (Fig. 5D); the maximum current response was obtained at a pH of 5.5. The optimum pH is in good accordance with the GOD isoelectric point. Thus, an electrolyte pH of 5.5 was selected for the following experiments.

3.4 Bioelectrocatalytic activity of GOD/GA/MLN/GCE for the oxidation of glucose.

The cyclic voltammograms (CVs) of GOD/GA/MLN/GCE observed in the absence and presence of *D*-glucose in 0.1 M phosphate solution (pH 5.5) containing 5 mM HQ are shown in Fig. 6.

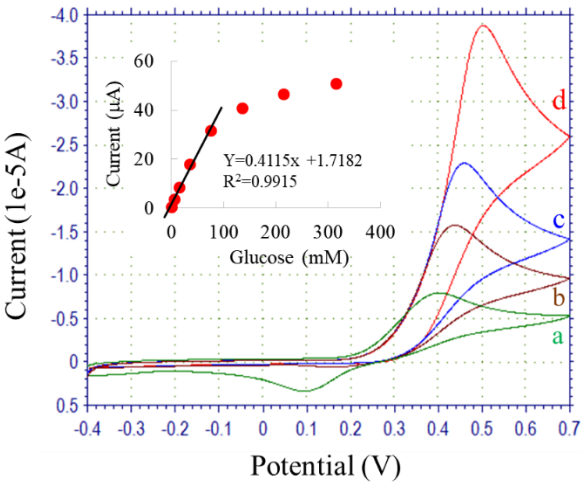

Figure 6. CVs of GOD/GA/MLN/GCE in a 0.1 M phosphate solution (pH 5.5) containing 5 mM HQ in the absence (a) and the presence of 5 mM (b), 15 mM (c), and 35 mM (d) *D*-glucose. The inset graph shows the correlation between the glucose concentrations in the electrolyte and the oxidation peak currents. The potential scan rate was 5 mV/s with a starting potential of -0.4 V.

Table 1. Comparison of MoS_2-based electrochemical glucose biosensors

Electrode platform	Transduction method	Linear range	Detection limit (μM)	Ref.
GOD/rMoS_2/Chitosan/APTES/ GCE/	voltammetry	3-20 μM	---	25
GOD/AuNPs-MoS_2/Au	chronoamperometry	0.25-13.2 mM	0.042	26
GOD/gold/MoS_2/Gold nanofilm	amperometric	10-500 nM	0.01	27
Nafion/AuNPs@MoS_2-GOD/GCE	voltammetry	10-300 μM	2.8	28
Ni/MoS_2	amperometric	0-4 mM	0.31	29
MoS_2/graphene aerogel	voltammetry	2-20 mM	290	30
Cu_2O/MoS_2	amperometric	0.01-4 mM	1.0	31
GOD/GA/MLN/GCE	voltammetry	1.0-135 mM	100	This work

rMoS_2, reduced MoS_2; CTS, chitosan; APTES, 3-aminopropyltriethoxysilane; AuNPs, Au nanoparticles; Ni, Ni nanoparticles; Cu_2O, Cu_2O nanoparticles

A well-defined HQ/*p*-quinone redox couple is observed in the absence of glucose (curve a) and greatly changes in the presence of glucose. The reduction current nearly disappears, and the oxidation current significantly increases (from b to d). This oxidation current is a typical catalytic current for the second generation of glucose biosensors. The mediated current increases with increasing glucose concentration from 1 to 100 mM. The results show that the prepared GOD/GA/MLN/GCE biosensor

exhibits a linear range from 1.0 to 135 mM toward the determination of glucose. In addition, when we added N_2 to the PBS electrolyte (data not shown), the anodic current signals increase more significantly in the presence of glucose. This phenomenon indicates a possible competition with oxygen to regenerate the oxidized form of the redox center of GOD [12]. Despite better performance in N_2 saturation, the GOD/GA/MLN/GCE biosensor also exhibits good characteristics in an air-saturated electrolyte solution. It shows a good linear range and great practicality.

Table 1 summarizes the characteristics of reported GOD-based glucose biosensors compared with our as-prepared biosensor. The performance of the GOD/GA/MLN/GCE biosensor is excellent due to the wide linear range, and good repeatability and reproducibility. The result is comparable in consideration of directly using raw molybdenite.

3.5 Operational stability, preparation reproducibility and application of GOD/GA/MLN/GCE biosensor.

The operational stability and the reproducibility of the GOD/GA/MLN/GCE are important parameters to be evaluated to assess practical usage. To check the operational stability of GOD/GA/MLN/GCE, the biocatalytic activity using HQ as an electron transfer mediator was measured repeatedly. After measuring 5 mM glucose, the GOD/GA/MLN/GCE was washed with a phosphate buffer, the electrolyte was replaced with fresh buffer, and then 5 mM glucose was measured again in the HQ-mediated electrolyte. No apparent degradation is observed after 10 consecutive measurements, which implies good operational stability for the prepared biosensor.

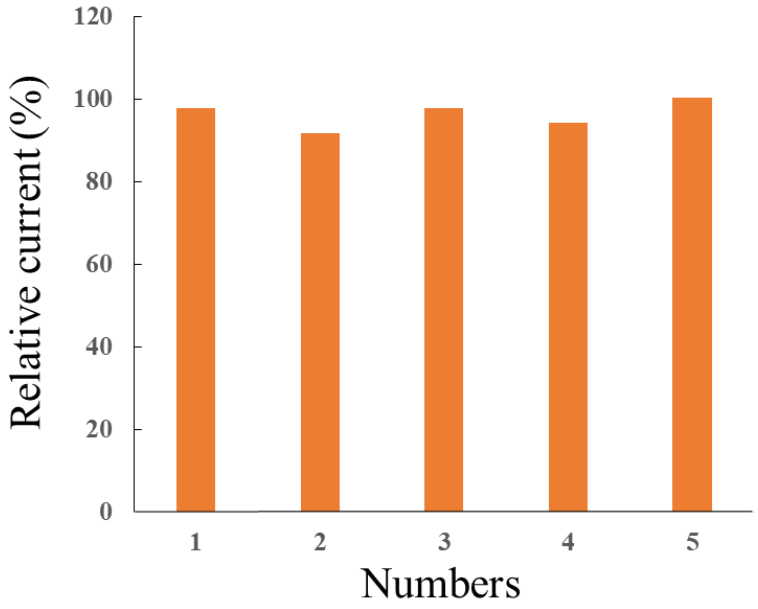

Figure 7. Reproducibility of five independent GOD/GA/MLN/GCE biosensors toward 20 mM glucose in 0.1 M PBS (pH 5.5).

Table 2. Analytical results for the glucose biosensor and the spectrophotometric method for the determination of glucose concentrations in beverages

Samples	Glucose concentration (mM)	
	Biosensor[a]	Spectrophotometric[b]
Beverage 1	6.80	6.95
Beverage 2	8.95	9.19
Beverage 3	18.29	18.88

[a] For the biosensor analysis, the beverage samples were diluted 100-1000 times. An aliquot of a suitably diluted ample (150 □L) was added to an electrolyte solution (15 mL).
[b] For the spectrophotometric method, the beverage samples were diluted 10 times.

The reproducibility of GOD/GA/MLN/GCE was examined to detect 5 mM glucose by using 5 independently prepared biosensors under the same conditions (Fig. 7). The relative standard deviation (R.S.D.) was 2.2%, indicating good reproducibility of this preparation method, especially considering the use of natural minerals.

To evaluate the practical usage for the analysis of real samples, we measured glucose in beverages by using the as-prepared GOD/GA/MLN/GCE-based biosensor. The analytical results are compared in Table 2. The results obtained by the biosensor agree with those of the spectrophotometric method. This suggests that the biosensor can be used to detect and measure the concentration of glucose in real samples with reasonably good performance.

4. CONCLUSIONS

In this study, a natural layered sulfide mineral was developed for the first time to detect glucose with an electrochemical signal enhancement. MLN provided a good platform for the immobilization of glucose oxidase. The GOD/GA/MLN/GCE biosensor displayed a current response that was linearly related to glucose concentration in a range from 1.0 mM to 135 mM. In future work, the prepared GOD/GA/MLN/GCE system will be useful not only for the study of bio-interfaces between a sulfide mineral and enzyme but also as an extended application for sulfide minerals.

DECLARATION OF INTEREST
There was no interest to declare.

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