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Research Article

Glucose biosensor based on nanohybrid material of gold nanoparticles and glucose oxidase on a bioplatform

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A simple and relatively cheap glucose biosensor based on a combination of gold nanoparticles (Au NPs) and glucose oxidase (GO_x) immobilized on a bioplatform eggshell membrane was established. Scanning electron microscopy showed successful immobilization of Au NPs/GO_x on the eggshell membrane. The effects of pH, phosphate buffer concentration, and temperature on the glucose biosensor were studied in detail. The biosensor shows a linear response at a glucose concentration range of 5–525 μM. The detection limit of the biosensor is 2.5 μM (S/N = 3). The biosensor exhibits good repeatability with RSD = 3.6% (n = 6), good operational stability with over 300 measurements and long-term storage stability with a shelf life of at least 6 months. The response time is less than 60 s. The glucose level in commercial food samples has been successfully determined. The proposed work shows potential to develop cost-effective biosensors for biotechnological, biomedical and industrial use.

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1 Introduction

Glucose is an important component of food samples. Quantitative determination of glucose is very important in the food industry. The development of glucose biosensors is growing rapidly [1–4]. However, the determination may be affected by other compounds coexisting with glucose or the sample may need to go through complicated pretreatment procedures. Since enzymes possess the specific property of substrate recognition, enzyme-based biosensors have been the subject of extensive studies due to their excellent selectivity [5–10]. Sensitivity and response time are important parameters to be considered for the development of an enzyme-based biosensor. To improve the sensitivity and shorten the response time of glucose biosensors, covalent attachment of glucose oxidase (GO_x) onto metal nanoparticle (NP) surfaces [11], physical adsorption of GO_x into metal NP [12, 13] and graphitic nanocages [14] surfaces have been studied and reported. Nanostructure materials exhibit interesting properties such as large surface-to-volume ratio, high surface-reaction activity, high catalytic efficiency, and strong adsorption ability [15, 16] that render them potential materials for the fabrication of biosensors. The large surface area of NP provides a better matrix for the immobilization of enzymes, leading to increased enzyme load per unit mass of particles. The multipoint combination of nanomaterial surfaces and enzyme molecules can

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Abbreviations: EDS, energy dispersive X-ray spectrum; **NP**, nanoparticle; **SEM**, scanning electron micrograph

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reduce enzyme protein unfolding due to the steric effect, resulting in the enhanced sensitivity of the enzyme [17]. Since gold nanoparticles (Au NPs) can provide a biocompatible environment for enzymes [18, 19], many researches focus on assembling GO_x with Au NPs [20–25].

The stability of enzymes is also crucial for the fabrication of biosensors. A number of techniques have been employed to immobilize GO_x in different solid substrates containing Au NPs in order to improve enzymatic stability. For instance, GO_x was immobilized in composite immobilization membrane composed of Au NPs and polyvinyl butyral by a sol-gel method [26], in a multilayer Au NPs/multiwalled carbon nanotubes membrane by electrostatic assembly [27], and in polyamidoamine dendrimers membrane containing Au NPs and poly(vinylsulfonate) [28]. At present, the demand for non-toxic and environmentally friendly ("green chemistry") materials continues to grow [29, 30]. Eggshell membrane is a light pink double-layered membrane inside the eggshell composed of highly cross-linked proteins similar to keratin, collagen, and elastion. Because of its excellent gas and water permeability and good biological compatibility with the enzyme, eggshell membrane has been identified as an ideal bioplatform for enzyme immobilization [31–33]. Moreover, eggshell membrane is a cheap and easily available biomaterial that has been successfully used to grow Au NPs and exploited for its application in biosensing [34].

In this study, we further extend and improve our previous work [35] by initially binding GO_x to Au NPs using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/*N*-hydroxysuccinimide coupling. Though glutaraldehyde may affect the activity of enzyme, some studies reported [32, 36, 37] that the effect could be negligible. In addition, the enzyme immobilized by physical adsorption is prone to aggregation [38], which would affect the activity of GO_x. The membrane encapsulation method is prone to enzyme leakage. The fabrication of enzyme layer-by-layer assembly is time-consuming and laborious. Therefore, we attempted to use the glutaraldehyde cross-linking method to attach our Au NPs-GO_x onto an eggshell membrane to produce a more stable biosensing interface. The resulting biosensor in this work shows better sensitivity of detection to glucose, better stability and longer shelf life than that of our previous work [35]. Compared to other biosensors with complicated, bulky or expensive experimental set-up, a dissolved oxygen (O_2) electrode acting as a transducer is a good choice for biosensor development since it is well developed, relatively cheap, stable and reliable. Herein, the sensing mechanism of the biosensor is based on the enzymatic reaction of GO_x on glucose accompanied by consumption of dissolved O_2:

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{GO_x} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

The decrease in O_2 level was conveniently monitored by an O_2 electrode and related to the glucose concentration. The effects of pH, buffer concentration and temperature on the biosensor response were investigated in detail. The biosensor has been successfully applied to determine the glucose level in some commercial food samples.

2 Materials and methods

2.1 Reagents

Glucose oxidase (EC 1.1.3.4. Type II-S: from *Aspergillus niger*) with a specific activity of 360 U/mg solid and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was obtained from Sigma (St. Louis, MO). β-D-Glucose was from Acros Organics (Geel, Belgium). A 50% (w/w) glutaraldehyde solution in water was purchased from Beijing Chemical Reagent Corporation (Beijing, China). *N*-Acetyl-L-cysteine (>99%) and *N*-hydroxysuccinimide (NHS) were obtained from International Laboratory (San Bruno, CA). The buffer solutions for preparing glucose standards were 50 mM monosodium dihydrogen phosphate-disodium hydrogen phosphate solutions at pH 7.0. All other reagents were of analytical-reagent grade and used without further purification. All solutions were prepared with deionized (DI) water. Fresh eggs were bought from a local market.

2.2 Immobilization of Au NPs/GO_x on eggshell membrane

N-acetyl-L-cysteine–protected Au NPs were synthesized according to our previous solution-based method [39]. An eggshell membrane was carefully peeled from an eggshell after the albumen and yolk had been removed. It was cleansed with copious amounts of DI water. The membrane was placed in a clean glass Petri dish and cut into pieces with a diameter of about 15 mm. An aliquot of 30 μL mixtures (1:1 v/v) of Au NPs (5000 ppm) and EDC (75 mM)/NHS (75 mM) was dropped on an eggshell membrane and followed with 100 μL of 0.8% (w/v) GO_x solution in PBS (50 mM) at pH 7.0. After about 1 h, 10 μL of 50% (w/w) glutaraldehyde solution was added to the Au NPs/GO_x solution on the eggshell membrane and left for another hour. The Au NPs/GO_x immobilized eggshell membrane was

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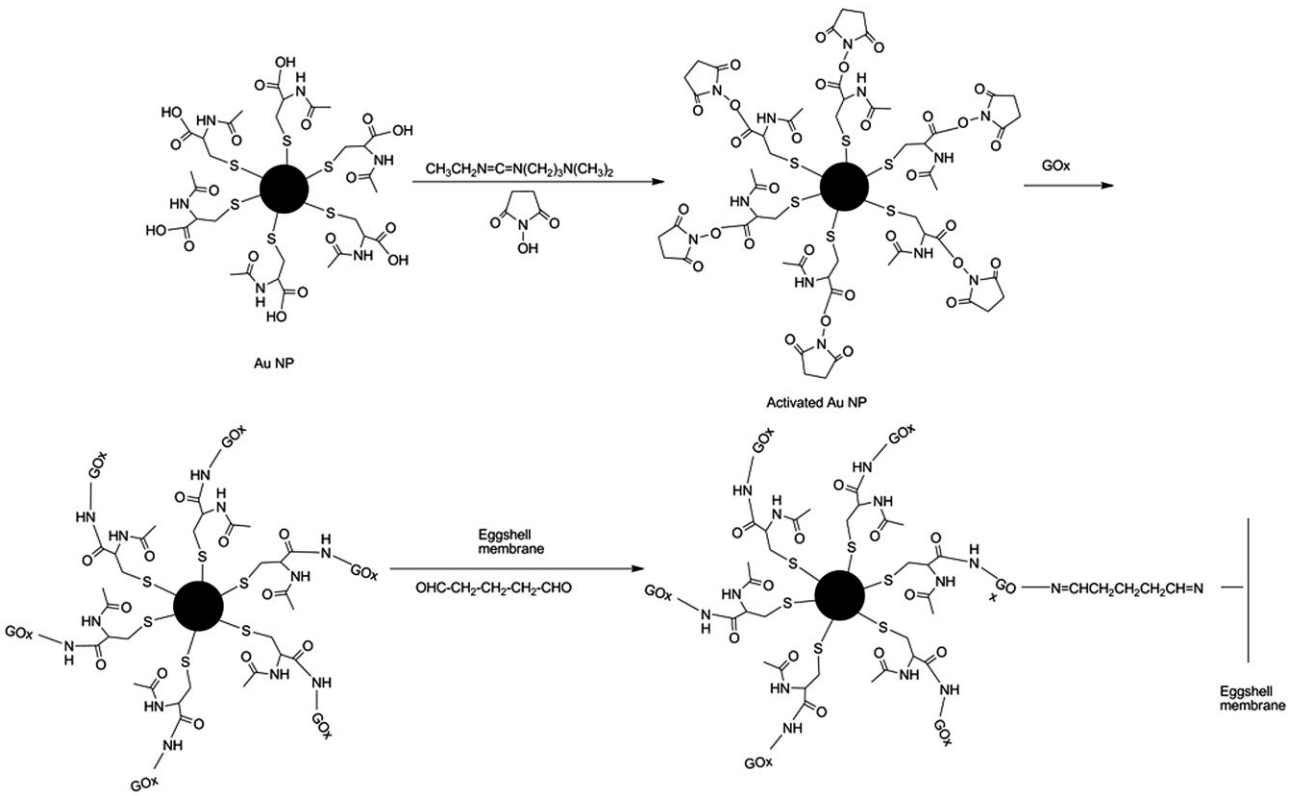

Figure 1. Au NPs activation via EDC/NHS coupling and immobilization of Au NPs/GO_x on eggshell membrane. The top equations show that the Au NP was activated by EDC/NHS and then linked to GO_x. The bottom equation displays that the Au NPs/GO_x was immobilized on eggshell membrane by using glutaraldehyde as a cross-linker.

then washed with a pH 7.0 PBS (50 mM) for 5 min. A schematic of Au NPs/GO_x eggshell membrane immobilization process is illustrated in Fig. 1. The Au NPs/GO_x immobilized eggshell membrane was stored in a pH 7.0 PBS (50 mM) at 4°C until further use.

2.3 Scanning electron micrograph of eggshell membrane

The scanning electron micrograph (SEM) and energy dispersive X-ray spectrum (EDS) of the eggshell membrane immobilized with Au NPs/GO_x were captured by a LEO 1530 field emission scanning electron microscope equipped with an energy dispersive X-ray spectrometer (Berlin, Germany).

2.4 Construction of gold nanoparticles/glucose oxidase-based biosensor

An Au NPs/GO_x immobilized eggshell membrane was positioned on the surface of a Pasco CI-6542 O_2 sensor (Roseville, CA) and kept in position by an *O*-ring. The electrode was immersed into a stirred 10 mL of 50 mM PBS solution at pH 7.0. Various volumes of a glucose standard (50 mM) or samples were injected into the PBS with a syringe. The sample solutions were under constant stirring by a 90-1 thermostat magnetic stirrer (Shanghai Yarong Biochemical Apparatus Corporation, Shanghai, China). The dissolved O_2 signal was captured and processed by a datalogger consisting of a Pasco CI-6760 Science Workshop 500 interface, serial cables, a power supply, and control software. The data were logged in a computer for real-time display and processing. When the biosensor was not in use, the Au NPs/GO_x immobilized eggshell membrane was stored in a pH 7.0 PBS (50 mM) solution at 4°C.

2.5 Food sample preparation

All the food samples were purchased from a local market and kept at 4°C before analysis. The liquid samples were determined without any pretreatment. The solid samples were dissolved by PBS solutions (50 mM, pH 7.0) into known concentration solutions before determination.

3 Results and discussion

3.1 Response behavior of glucose biosensor

GO_x catalyzes the selective oxidization of D-glucose into D-gluconic acid with a concomitant consumption of dissolved O_2 as shown in Eq. (1). The depletion of the O_2 level can be picked up by the O_2 sensor. The analytical signal of the glucose biosensor is the decrease in dissolved O_2 content upon exposure to glucose. Figure 2 shows a typical response curve of the glucose biosensor when exposed to successive step changes of glucose concentration. A good linear relationship between glucose concentration and decrease in oxygen level was obtained. The calibration curve is depicted in the inset of Fig. 2.

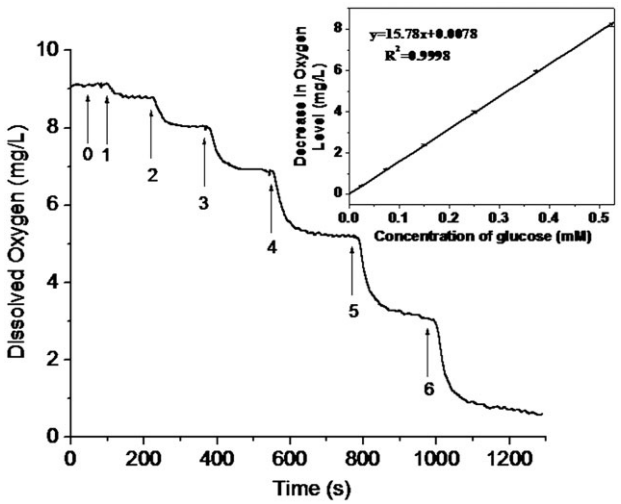

Figure 2. Typical response curve of the glucose biosensor at pH 7.0 PBS (50 mM) after each successive addition of various volumes of glucose standard (50 mM): (0) 0.0, (1) 5.0, (2) 10, (3) 15, (4) 20, (5) 25, and (6) 30 µL. The inset displays the linear calibration curve for glucose from 0.025 to 0.525 mM. Error bars are 95% confidence intervals calculated from three replicate measurements.

3.2 Scanning electron micrograph of Au NPs/GO_x eggshell membrane

The microstructure of various eggshell membranes including a fresh one and membranes with glutaraldehyde, GO_x and Au NPs/GO_x was studied. An eggshell membrane has highly cross-linked protein fibers (diameter ca. 1–5 µm) and cavities, which make it gas- and water-permeable. When the eggshell membrane was treated with glutaraldehyde, GO_x and Au NPs/GO_x, the protein fibers retained their integrity and cross-linked structure, which is more or less the same as the fresh one. Both the membranes immobilized with GO_x (Fig. 3A) and Au NPs/GO_x (Fig. 3B) have some specks or clusters of enzymes on their protein fibers, which can be clearly observed in the SEM images. In contrast to the GO_x-immobilized membrane, the Au NPs/GO_x-immobilized membrane has larger lumps of granular Au NPs/GO_x on its protein fibers. These lumps were confirmed by EDS

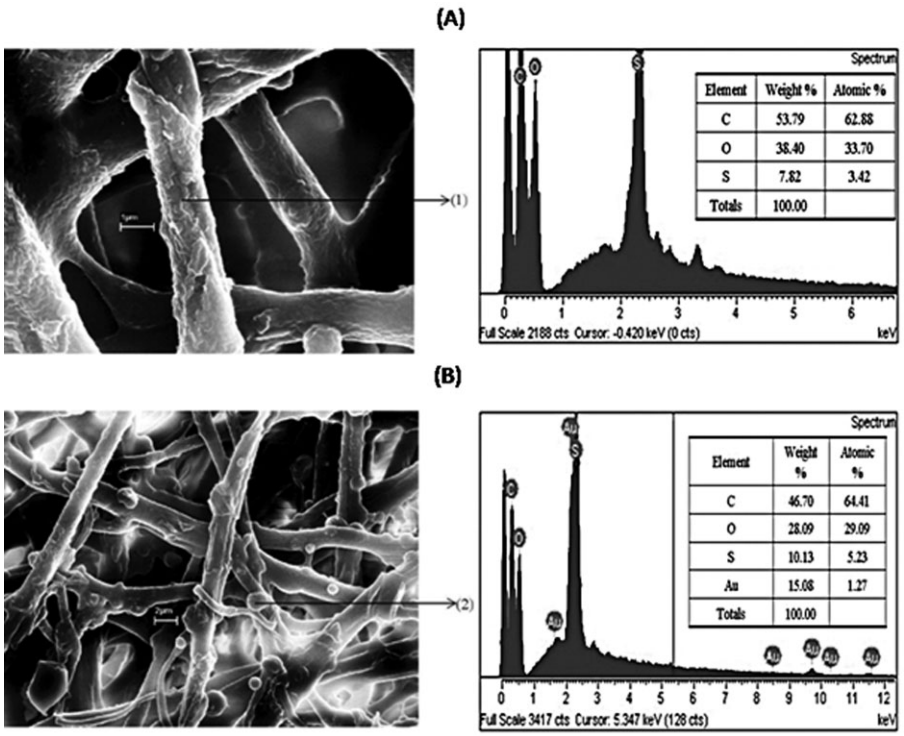

Figure 3. Scanning electron micrographs and energy dispersive X-ray spectra of eggshell membrane immobilized with GO_x (A), and eggshell membrane immobilized with Au NPs/GO_x (B). (1) Protein fiber immobilized with GO_x, and (2) protein fiber immobilized with Au NPs/GO_x.

(Fig. 3B) to have high contents of Au compared with GO_x-immobilized membrane (Fig. 3A). The Au NPs with GO_x increased to about 500 nm after immobilization on the eggshell membrane which is much larger than that of the as-synthesized Au NPs (about 2 nm) [39]. The change in size demonstrates that GO_x were connected and cross-linked to the Au NP surfaces. The SEM supports that Au NPs/GO_x were successfully immobilized onto the eggshell membrane.

3.3 Comparison of GO_x- with Au NPs/GO_x-immobilized eggshell membranes

When the Au NP/GO_x-immobilized eggshell membrane was tested with a series of glucose solutions, it was found that its sensitivity was three times that of a GO_x-immobilized eggshell membrane without Au NPs. The apparent Michaelis-Menten constants (K_m) for the immobilized GO_x eggshell membrane with and without Au NPs were determined to be 0.45 and 1.04 mM, respectively, by the Lineweaver-Burk's plot. The K_m value for an enzymatic reaction determines the affinity of the enzyme for the substrate. The smaller K_m value indicates higher affinity of enzyme for substrate. The K_m value for the covalently immobilized GO_x with Au NPs is 2.31–fold lower than that of GO_x without Au NPs. Our results are consistent with reports that Au NPs are capable of increasing the activity of GO_x [40].

The response time of a biosensor is an important analytical feature. It is defined as the time taken to obtain a 95% steady-state signal (t_{95}) when a biosensor is exposed to a known concentration of standard solution. The experimental results showed that the response time of the biosensor with Au NPs (58 s) was shorter than that of the biosensor without Au NPs (120 s) because Au NPs can speed up the enzymatic reaction. In addition, Au NPs can adsorb enzyme and thus prevent enzyme leakage. The excellent electrical conductivity of Au NPs can also accelerate the response of the O_2 sensor.

3.4 Effect of the amount of Au NPs

The amount of Au NPs on the eggshell membrane would affect the sensitivity of the biosensor. Eggshell membranes immobilized with various amounts (30–150 μL) of activated Au NPs (5000 ppm) and 100 μL of 0.8% (w/v) GO_x were used to fabricate the biosensors. The dissolved O_2 contents were measured in 0.075 mM glucose standard at pH 7.0 PBS (50 mM). It was found that the sensitivity of the biosensors increased with the increase of Au NPs loading on the eggshell membrane. However, not much increase in sensitivity was observed when the amount of Au NPs is above 30 μL. As such, 30 μL activated Au NPs solution was chosen for all experiments throughout our studies.

3.5 Effect of pH

Although the optimal pH for GO_x is in the range of 6.5–7.5 [41, 42], enzymatic activity majorly depends on the immobilization method used and the microenvironment around the enzyme. The pH effect on the response of the biosensor was investigated by subjecting it to 0.075 mM glucose standard in PBS solutions (50 mM) at various pH (4.0–9.0). The biosensor response could be maintained above 90% throughout the whole pH range investigated, showing that pH changes apparently do not affect the dynamic working range of the biosensor. It is anticipated that the biosensor can function satisfactorily over a very broad working range from pH 4.0 to 9.0. In addition, the response times (t_{95}) at various pH were studied. The t_{95} is shortest at pH 7.0, indicating that the rate of catalytic reactions is fastest at this pH. The increase in t_{95} at low or high pH may be attributed to the decrease in enzymatic activity as a result of denaturation of the enzyme at extreme pH conditions [43].

3.6 Effect of buffer concentration

The ionic strength of a buffer solution can affect the enzymatic rate of the enzyme [32], which can influence the sensitivity and response time of a biosensor. The effect of buffer concentration on the response of the biosensor was investigated by subjecting it to 0.075 mM glucose standard in various buffer concentrations of 0–300 mM at pH 7.0. It has been reported that the response of a glucose biosensor greatly depends on the buffer concentration since the buffer species act as carriers in transporting protons out of the enzyme membrane [44]. The response increased with the increase in buffer concentration until it reached 50 mM and was relatively constant at above 50 mM. In addition, the response time increased with the increase in PBS concentration. Therefore, 50 mM was chosen as the optimal PBS concentration for our biosensor to achieve faster response time and relatively higher sensitivity.

3.7 Effect of temperature

The effect of temperature on the glucose biosensor was investigated over the range 4–55°C. The consumption of dissolved O_2 increased with the increase in temperature when the biosensor was

exposed to a 0.075 mM glucose solution (50 mM PBS, pH 7.0). It is well-known that the activity of an immobilized enzyme is governed by the kinetics of the enzymatic reaction [36]. The enzyme can acquire higher activities at higher temperatures and subsequently consume O_2 at a faster rate in the enzymatic reaction of glucose. The biosensor showed that the response time decreases from 4 to 55°C since O_2 is consumed at a faster rate under higher temperatures. On the other hand, higher working temperatures have a counter balance effect on the biosensor. Temperature also affects the solubility of dissolved O_2, which has lower solubility in water at higher temperature. The concentration of dissolved O_2 in water is 9.1 mg/L at 20°C and 1 atmospheric pressure. Increasing the temperature will decrease the concentration of O_2, resulting in a narrower working range for the biosensor. Higher temperature would result in denaturation of enzyme, shortening the lifetime of the biosensor. For practical reasons, room temperature is recommended in order to simplify the experimental procedure and prolong the lifetime of the biosensor.

3.8 Analytical figures of merit of Au NPs/GO_x-based biosensor

In our study, the glucose biosensor could achieve 95% of the steady signal within 60 s. The inset of Fig. 2 displays the linear response range of the biosensor from 0.025 to 0.525 mM glucose: Decrease in oxygen (mg/L) = 15.78 [glucose] + 0.078 (r^2 = 0.9998) where the concentration of glucose is in mM. The biosensor has a LOD of 2.5 μM estimated at an S/N of 3. Satisfactory repeatability was obtained with an RSD of 3.6% by subjecting the biosensor repetitively to a 0.075 mM glucose standard for six times.

The shelf life of the biosensors with and without Au NPs was tested weekly over a 6-month period. The biosensors were stored at 4°C when not in use. The biosensor with Au NPs maintained 95% of its original response to 0.075 mM glucose over the period. However, the response of biosensor without Au NPs was 89% of its initial value. The better shelf life of the glucose biosensor using an Au NPs/GOx-immobilized eggshell membrane is possibly related to the biocompatibility of the eggshell membrane with the enzyme and Au NPs. However, the exact reason is still not clearly understood. The operation stability of the biosensors with Au NPs was evaluated by the repetitive measurements. The biosensor retained 99% of its initial response after over 300 measurements.

The analytical performance of our proposed glucose biosensor is compared with that of other glucose biosensors based on dissolved O_2 transducer. The results are summarized in Table 1. It can be seen that our biosensor has better analytical performance in terms of calibration sensitivity, detection limit, response time, and stability, possibly attributing to the catalytic effect of Au NPs on the enzymatic reactions and its biocompatibility with enzyme.

3.9 Interference test

The effect of potential interferents in real samples on the response of the glucose biosensor was evaluated by exposing it to the interferents in 50 mM PBS at pH 7.0. The results are summarized in Table 2. The decrease in O_2 level was calculated as the glucose concentration equivalence (i.e., the concentration of glucose that can produce the same decrease in dissolved O_2 concentration as the interferents). It was found that most potential interferents such as fructose, lactose, citric acid, sodium

Table 1. Comparison of analytical performance of various O_2-based glucose biosensors

Analytical performance	Au NPs/Eggshell membrane	Eggshell membrane	Substrate of immobilization Au NPs/Eggshell membrane	Chitosan	Nylon net
	(This work)	[36]	[35]	[45]	[46]
Linear range	5.0 μM–0.525 mM	10.0 μM–1.30 mM	8.33 μM–0.966 mM	16.0 μM–1.10 mM	18.0 μM–1.10 mM
Detection limit	2.5 μM	–	3.5 μM	8.0 μM	9.0 μM
Response time	< 60 s	100 s	< 30 s	< 60 s	80 s
Stability	95% after 6 months 99% after 300 measurements	85.2% after 4 months	87.3% after 10 weeks	86.5% after 3 months	83.0% after 50 days 86.3% after 200 measurements
Calibration sensitivity (mg/L O_2 depletion/ mM glucose)	15.8	8.34	9.42	6.75	6.28

Table 2. Results of the interference study

Interfering species added (in 0.075 mM glucose)	Concentration (mM)	Decrease in oxygen level equivalent to glucose (mM)
Fructose	20	0.001
Lactose	20	–0.001
Citric acid	10	0.002
Sodium benzoate	25	–0.001
Ascorbic acid	10	0.012
Sucrose	50	0.022
Urea	10	0.002
DL-cysteine	10	0.005
Soluble starch	8[a)]	0.008
KCl	10	0.006
NaCl	25	–0.007
$CaCl_2$	10	0.005
$MgSO_4$	10	0.003
NH_4Cl	20	0.008
$CuSO_4$	20	–0.002
$FeCl_3$	20	–0.008
$Na_3C_6H_5O_7$	20	0.004
$ZnSO_4$	20	–0.005
$NaHCO_3$	20	0.005
NH_4HCO_3	20	0.001

a) The concentration given in mg/mL.

benzoate, ascorbic acid, sucrose, urea, DL-cysteine, soluble starch, KCl, NaCl, $CaCl_2$, $MgSO_4$, NH_4Cl, $FeCl_3$, $Na_3C_6H_5O_7$, $ZnSO_4$, $NaHCO_3$ and NH_4HCO_3 did not interfere significantly with the response of the glucose biosensor.

3.10 Real sample analysis

The Au NPs/GO_x-based biosensor was applied to glucose determinations in some commercial food including liquid and solid samples such as fruit juice, milk, yoghurt, milk powder, chocolate, cake, biscuits and so on. All sample solutions were air-saturated before determination. The results are displayed in Table 3. All tested food samples contained various concentrations of glucose. Vita lemon tea had the highest content of glucose, while Lotte dreamy chocolate had the lowest content among these food samples. In addition, the recovery tests for glucose were done by adding known amounts of glucose standards in the real samples and then evaluated by the proposed glucose biosensor. The results are also summarized in Table 3. The range of recovery 92.1–104% demonstrates that the Au NPs/GO_x-based biosensor can offer excellent, accurate and precise determinations of glucose in real food samples.

4 Concluding remarks

A novel glucose biosensor based on an Au NPs/GO_x-immobilized eggshell membrane has been successfully constructed and applied to determine the concentration of glucose in commercial food samples. This biosensor is simple, cost-effective and user-friendly. It exhibits good stability with a shelf-life of at least 6 months. Au NPs can improve the sensitivity and stability of the glucose biosensor.

Table 3. Determination and recovery of glucose in commercial food samples using the proposed biosensor method

Real samples	Concentration of glucose (mM)	RSD. of glucose biosensor (n = 3) (%)	Concentration of glucose added (mM)	Concentration of glucose found (mM)	Recovery (%)	RSD. (n = 3) (%)
Vita lemon tea	506	4.6	150	655	99.3	1.5
Nescafe	4.65	3.7	1.00	5.58	93.0	2.6
Hot & In fresh orange juice	96.4	1.2	20.0	116.6	101	1.8
Water-soluble C100	113.7	0.5	150	257.2	95.7	4.6
American lemon & lime	370.5	2.0	400	736.9	94.6	3.9
Meng Niu red date yoghurt	45.3	1.3	50.0	97.0	103	2.4
Xiang Piao Piao dasheen milk tea juice	71.8	1.8	50.0	118.9	94.2	5.1
Wangzai milk	2.16	1.1	5.00	7.11	99.0	2.2
Gucheng full cream sugar milk powder	9.38[a)]	2.2	20.0[a)]	27.8[a)]	92.1	2.0
Hanbo date concentration with E-Jiao	44.6[a)]	2.6	50.0[a)]	94.5[a)]	99.8	1.9
Lotte dreamy chocolate	0.94[a)]	4.9	2.00[a)]	2.96[a)]	101	2.7
Hao Li You chocolate flavor pie	33.5[a)]	1.0	50.0[a)]	85.7[a)]	104	3.5
Ke Ke Ao layer cocoa cake	28.4[a)]	5.3	50.0[a)]	76.8[a)]	96.8	4.3
Xiang Piao Piao dasheen milk tea powder	15.3[a)]	0.5	20.0[a)]	35.4[a)]	101	2.9
Man Shi Ka layer wafer biscuit	44.5[a)]	2.1	50.0[a)]	91.8[a)]	94.6	5.1
Cola Cao solid nutritional drink	29.4[a)]	2.7	30.0[a)]	58.9[a)]	98.3	3.6

a) The concentration is given in mg/g.

It also produces a faster response to glucose with good reproducibility and selectivity. Our work proves that Au NPs/GO_x with eggshell membrane is a promising enzyme immobilization material for the fabrication of biosensors that has practical applications in biotechnology and food industry.

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The authors have declared no conflict of interest.

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