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Monodispersed silica nanoparticles as carrier for co-immobilization of bi-enzyme and its application for glucose biosensing

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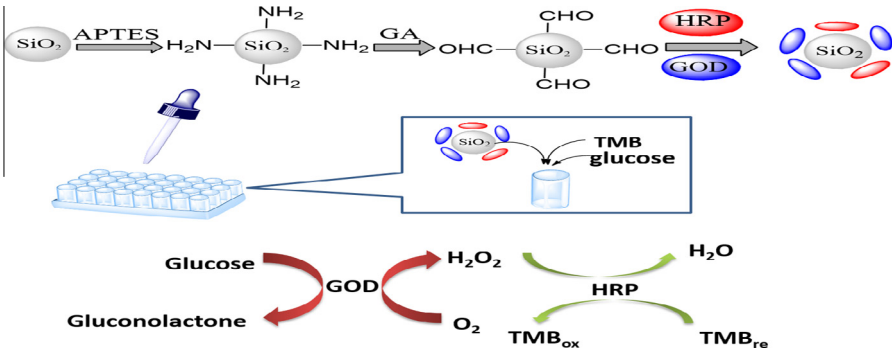
HIGHLIGHTS

- The bi-enzyme was easily and tightly immobilized on silica NPs.
- SiO_2-GOD/HRP was used to detect glucose by a visible method.
- The method is sensitive and reliable.
- It is possible to be applied in various fields by using SiO_2-GOD/HRP to make pills.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel glucose sensing strategy by using bi-enzyme coated monodispered silica nanoparticles (SiO_2) was proposed. The monodispered SiO_2 was synthesized according to our previously reported seed-growth methods. Glucose oxidase (GOD) and horseradish peroxidase (HRP) were simultaneously covalent immobilized on the surface of SiO_2 nanoparticles through the cross-linker of glutaraldehyde. The immobilized bi-enzyme remained their bioactivities well for the substrate reaction. Thus, the resultant SiO_2-GOD/HRP nanocomposites could be used as catalyst for enzymatic substrate reactions in the presence of 3,3′,5,5′-tetramethylbenzidine (TMB) as chromogenic reagent and glucose as substrate. The factors of affecting the catalytic activities of enzymes were optimized. Under optimal conditions, the absorbance at 450 nm in UV–visible spectra increased with the glucose concentration, which could be used for glucose detection with a linear range from 0.5 μM to 250 μM and a detection limit of 0.22 μM at a signal-to-noise ratio of 3σ. Considering the potential of making pills using this SiO_2-GOD/HRP, the present strategy has good prospect in the clinic science and other fields in future.

Introduction

With the excellent properties of high surface area, high chemical purity, good stability, well dispersion and ease of modification, nanosized silica has become one of the widely used nanomaterials [1]. It has been vastly applied in many aspects including paper, food painting, cosmetics, and so on. Furthermore, it has an important effect on the biochemical and medical fields. For example, SiO_2 as a perfect bio-carrier has been vastly occupied in biomedical engineering such as drug delivery, DNA transfection, cancer diagnosis, DNA mismatching, biomolecule detection and enzyme immobilization [2–6]. And the application of enzyme immobilization is outstanding in biomedical engineering.

Compared with the free enzymes, the enzymes immobilized on nanoparticles showed many advantages. The abilities of resisting some unusual conditions such as high temperature or extreme pH in the environment are improved. Basically, methods of enzymes immobilization can be divided into three categories: binding to a support, entrapment and cross-linking. Each of these ways has several characters, separately. The method of binding to a support

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can be included physical (such as hydrophobic and Vander Waals interactions), ionic, or covalent. Although the process is simple, it is usually sensitive to the synthesis conditions. Entrapment is via inclusion of an enzyme in a polymer network, typically organic/inorganic polymer matrices or a membrane device such as a hollow fiber. In general, additional covalent to attachment is often required to fix enzyme. Cross-linking, employing a bi-functional reagent, is a general method to fix enzyme [7]. It vastly improves the capacity to fix enzymes, but also cause some descents in enzyme activity.

The glucose detection has been deeply researched because of the increasing related diseases [8,9]. And the scientists have made many efforts to construct the bi-enzyme biosensors of glucose oxidase (GOD) and horseradish peroxidase (HRP) to detect glucose. When only GOD existed, glucose was converted into gluconic acid and O_2 changed to H_2O_2. So there are many experiments by detecting H_2O_2 to measure glucose in early work [10,11]. But they are usually unapparent and complex. When GOD and HRP are co-immobilized on the same carrier, the process would be altered. The product of H_2O_2 could be catalyzed to H_2O and O_2. If there were some chromogenic reductants, they can be oxidized by H_2O_2 and the color of the solution changed. In this way, the glucose detection would be visible and simplified. The common chromogenic reductant was 3,3′,5,5′-Tetramethylbenzidine (TMB) in practice experiments.

In this work, a simple and convenient approach to immobilize glucose oxidase (GOD) and horseradish peroxidase (HRP) on the surface of monodispersed SiO_2 to detect glucose in UV–visible spectra was proposed. The catalytic reactions of co-immobilized enzymes were investigated by using glucose and 3,3′,5,5′-tetramethylbenzidine (TMB) as substrates (Fig. 1). Through this method, the concentration of glucose could be monitored quickly and accurately in the serum samples, especially when there were plentiful specimens at the same time. Furthermore, considering the potential of making pills in industry, it would provide a lower-cost and more portable way to detect glucose in clinic and other fields.

Experimental

Chemicals and apparatus

Tetraethyl orathosilicate (TEOS), glucose oxidase (GOD, EC 1.1.3.4, from Aspergillus niger, 181.3 μ/mg) and (3-aminopropyl)-triethoxysilane (APTES) were purchased from Sigma–Aldrich Chemical Co., 3,3′,5,5′-Tetramethylbenzidine (TMB), horseradish peroxidase (HRP, EC 1.11.1.7, RZ ⩾ 3.0, 250 μ/mg), L-histidine, D-(+)-mannose, b-cyclodextrin, D-(−)-fructose, ascorbic acid and D-galactose were obtained from Nanjing Sunshine Biotechnology Co., Ltd. (Jiangsu, China). All other chemicals were of analytical grade and used without further purification. Phosphate buffer solution (PBS) was prepared by mixing NaH_2PO_4 and Na_2HPO_4. Twice-distilled water was used throughout the study.

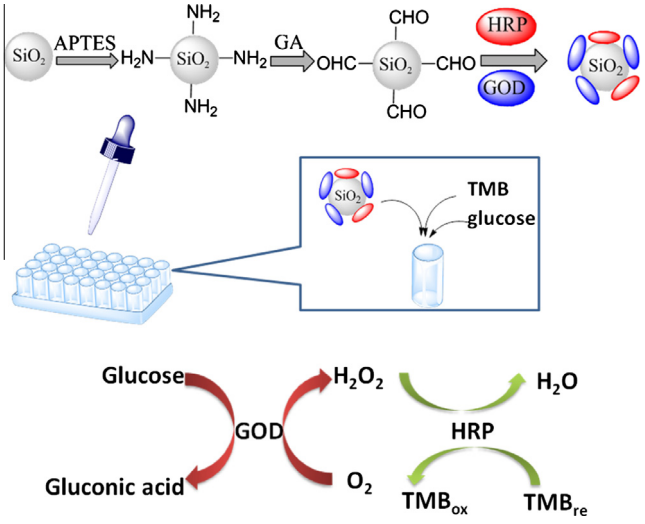

Fig. 1. Schematic illustration in colorimetric detection of glucose with SiO_2-GOD/HRP and TMB.

The morphology and size of mono-dispersed silica nanoparticles were analyzed with a transmission electron microscope (TEM, S-2400N, HITACHI, Japan). Before the measurements, 10 mL of silica suspension in water was dropped onto copper grids and dried in vacuum overnight. The optical density absorbance readings (450 nm) were taken by using the iMark Microplate Absorbance Reader (Bio-Rad, USA).

Synthesis of amino-functionalized silica particles (SiO_2–NH_2)

Monodispersed SiO_2 nanoparticles were synthesized according to our previously reported seed-growth methods [12–14]. The diameters of the as-synthesized silica nanoparticles were 130 ± 5.0 nm, determined by TEM microscopy. The synthesis of SiO_2–NH_2 was according to the literature with slightly modifications [15]. As-synthesized silica nanoparticles (0.04 g), anhydrous ethanol (6 mL) and 3-aminopropyltriethoxysilane (0.2 mL) were placed in a 10 mL glass tube. The mixture was stirred for 1 h at room temperature. Then, the solution was washed with dehydrous ethanol three times. Finally, the resultant product (SiO_2–NH_2) was dried under vacuum at room temperature for 12 h.

Co-immobilization of GOD and HRP on silica particles (SiO_2-GOD/HRP)

Typically, 4 mg amino-functionalized silica particles were ultrasonically dispersed in 500 μL glutaraldehyde (GA, 1%) followed by stirring at room temperature for 20 min. Then, the mixture was quickly centrifuged and cleaned up with PBS (0.01 M pH 7.4) several times. Next, the above mixture of SiO_2–GA was re-dispersed in 300 μL PBS containing a certain amount of GOD and HRP. The solution was then gently stirred overnight at room temperature. After that, the final product of GOD/HRP co-loading SiO_2 nanoparticles (SiO_2-GOD/HRP) was collected by centrifugation, washing and re-dispersing in a certain volume of 0.1 M pH 7.4 PBS.

Analysis procedure

0.5 μL 0.1 M TMB and 2 μL SiO_2-GOD/HRP suspension were added to 100 μL 0.1 M pH 6.0 PBS buffer. After a certain amount of glucose was added, the mixture was incubated at room temperature for 10 min. Then 50 μL 2 M H_2SO_4 was added to stop the enzymatic reaction as terminator. The absorbance at 450 nm of the resultant solution was measured. For glucose determination in human serum, the fresh serum samples were diluted 100 times using 0.1 M pH 6.0 PBS buffer. In control experiments, 2 mM L-histidine, D-(+)-mannose, b-cyclodextrin, D-(−)-fructose, ascorbic acid, D-galactose and PBS buffer were used instead of glucose for the experiments.

Results and discussion

Characterization of SiO_2-GOD/HRP nanocomposites

SiO_2 nanoparticles were employed as carriers for co-immobilization of glucose oxidase and horseradish peroxidase. Thus, silica nanoparticles with good monodispersion and similar surface morphology were vital for consistent loading of the same amount

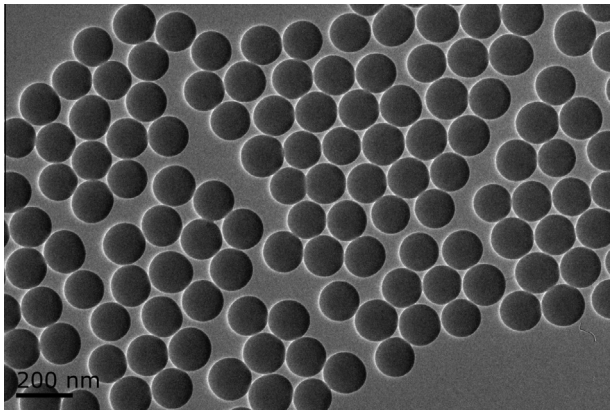

Fig. 2. TEM image of SiO_2.

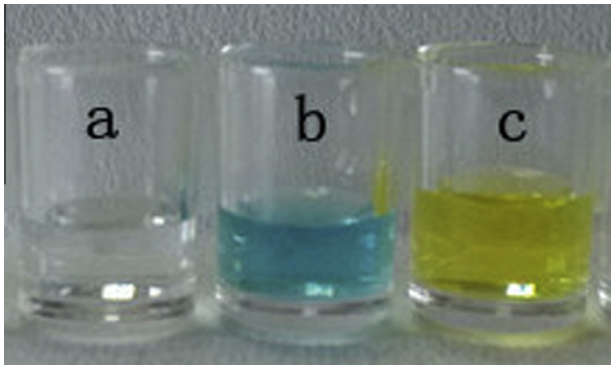

Fig. 3. The photos of chromogenic reaction added certain volume of SiO_2-GOD/HRP and TMB to 0.1 M pH 6.0 PBS buffer (a) after addition of glucose (5 min) (b) after addition of H_2SO_4 (10 min) (c).

of bi-enzyme on each microsphere. Herein, the silica nanospheres were synthesized with our previously reported seed-growth methods. The resultant silica nanospheres were chemically clean and displayed the uniform structure of nanospheres. The diameters of the nanospheres were about 130 ± 5.0 nm, which were demonstrated by TEM image (Fig. 2).

To investigate the bioactivity of SiO_2-GOD/HRP, the catalytic oxidation of peroxidase substrate TMB in the presence of glucose was tested. 2 μL of SiO_2-GOD/HRP suspension was added into 100 μL 0.1 M pH 6.0 PBS buffer containing 0.75 mM TMB. Upon addition of glucose, the mixture solution showed a maximum absorbance at 450 nm accompanied with an obvious color change from colorless to blue[1] (Fig. 3). Finally the color turned yellow after adding H_2SO_4 as terminator [16]. It was noted that the color change occurred only when both GOD and HRP existed (Fig. 4). This confirmed that both GOD and HRP were successfully co-immobilized on silica nanoparticles. Moreover, the bioactivities of immobilized bi-enzyme remained well. The enzymatic reactions could be summarized as follow: the immobilized GOD catalyzed glucose oxidation with the reaction given by GO $(FADH_2) + O_2 \rightarrow GO\ (FAD) + H_2O_2$ [17]. Then, the product of H_2O_2 will react with 3,3′,5,5′-tetramethylbenzidine in the presence of HRP that transfer H_2O_2 to H_2O meanwhile oxygenate TMB to TMB(ox). The oxidized TMB would present a blue color. And it would get a blue solution after reaction and then turn yellow when the strong acid as terminator was added.

[1] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

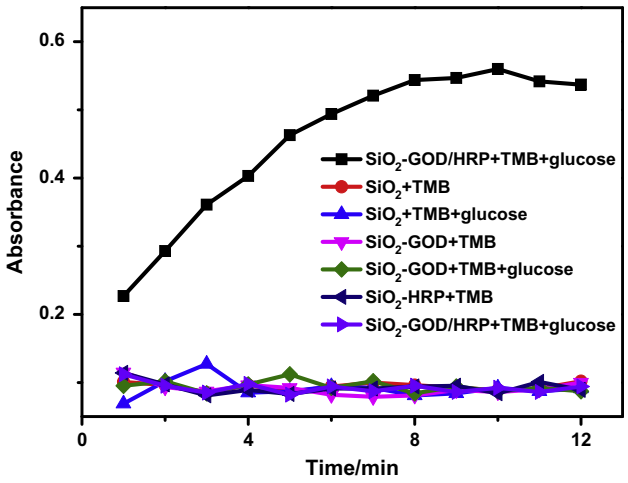

Fig. 4. Time-dependent absorbance changes at 450 nm of TMB in different reaction systems. Reaction condition: 0.1 M pH 6.0 PBS; glucose concentration: 200 μM; TMB concentration: 7.5×10^{-4} M.

Optimization of conditions for glucose detection

The efficiency and sensitivity of the glucose biosensor depend on the concentration ratio of bi-enzyme coated on nano-SiO_2 and GA activated time during the synthesis process of SiO_2-GOD/HRP. In addition, the effects of TMB concentration and pH on the activities of the SiO_2-GOD/HRP were also investigated. In the process of immobilizing bi-enzyme on SiO_2, the entire volume of reaction solution was constant. The original concentration of GOD and HRP were 5 mg/mL. When the concentration ratio of GOD/HRP was adjusted from 1:3 to 3:1 in the incubation solution, the absorbance at 450 nm increased with the increasing percentage of GOD, which indicated that the catalytic activities of bi-enzyme system increased. This tendency of increasing was reached maximum in the ratio of 2:1. When the ratio was further changed from 2:1 to 3:1, the catalytic activities decreased. However, either overmuch GOD or HRP would affect the efficiency of the color change. Therefore, the concentration ratio of GOD and HRP was ultimately determined 2:1 (Fig. 5a).

Glutaraldehyde as an excellent cross-linker was widely used in biochemical and biomedical fields [18–20]. If the activated time of GA was too short, fewer amounts of enzymes could immobilize on the nano-SiO_2 and worse catalytic activities were produced. The bioactivities of enzymes were harmed by excess GA because it destructed the protein structures. So, the final GA activated time was chose at 20 min with meticulous study (Fig. 5b).

Generally, the intensity of color change was in proportion to the concentration of the chromogenic materials when the other conditions were maintained invariable. The absorbance at 450 nm increased with the TMB concentration. However, the more TMB was not meant the better signal of catalytic process, for the solubility of TMB in PBS buffer was limited. When the TMB dosage was greater than itself solubility in the buffer, the excess TMB would be separated out and precipitated. So the absorbance at 450 nm would be quickly dropped, correspondingly. Therefore, the best concentration of TMB was measured as 0.75 mM (Fig. 6a).

The conformation and bioactivities of the enzymes were also affected by the pH. It was believed that the ionic state of an amino acid was pH-dependent. If the ionic state of amino acids in a protein was changed, the ionic bonds, hydrogen bond, and Vander Waals force that help to maintain the 3-D conformation of the protein would be altered, which may lead to its deactivation [21]. Herein, the catalytic activities of enzymes were sensitive to the

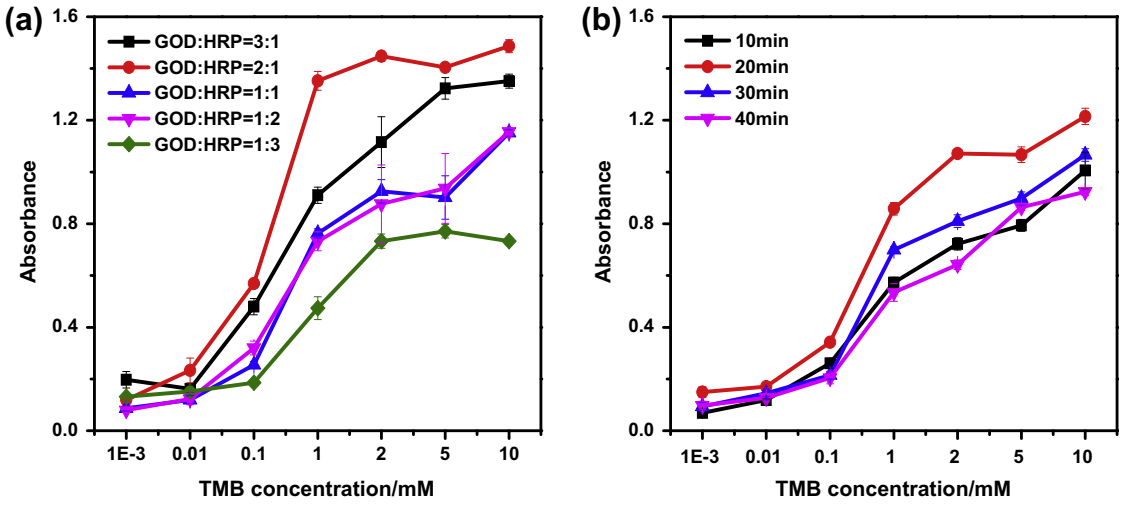

Fig. 5. Effects of the concentration ratio of GOD and HRP (a) the glutaraldehyde activated time (b) on the catalytic oxidation of TMB in the presence of SiO_2-GOD/HRP. Reaction condition: 0.1 M pH 6.0 PBS; Corresponding concentration of glucose is 0.001 mM, 0.01 mM, 0.1 mM, 1 mM, 2 mM, 5 mM, 10 mM, respectively; TMB concentration: 7.5×10^{-4} M.

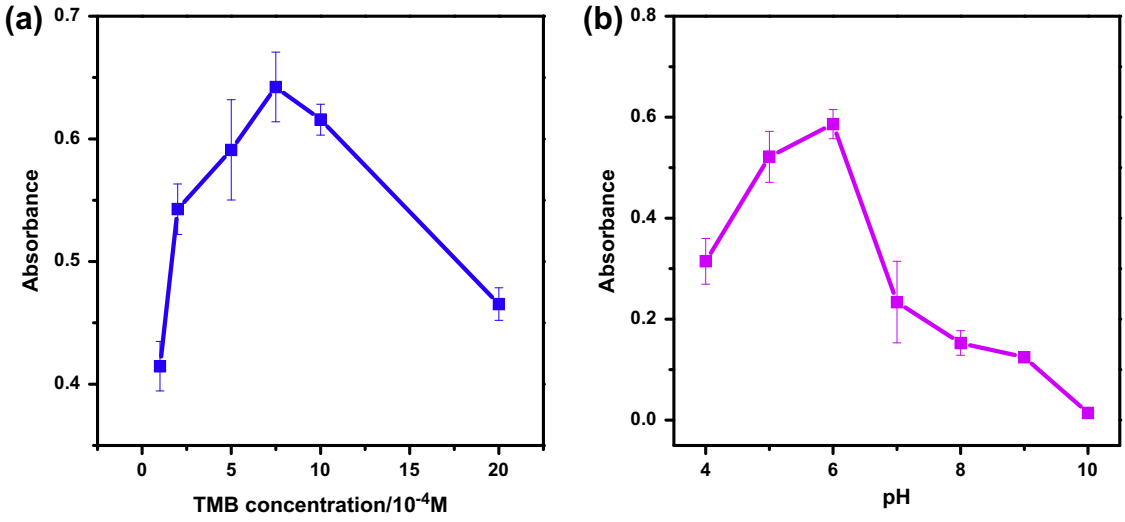

Fig. 6. Effects of TMB concentration (a) and solution pH (b) on the catalytic oxidation of TMB in the presence of SiO_2-GOD/HRP in PBS. The glucose concentration is 200 μM.

pH of buffer. The absorbance at 450 nm increased with the increasing of pH value in acidic condition and reached a maximum absorbance at pH 6.0. Previous studies demonstrated that the enzymes would be restrained or even inactivated with the pH condition of too acidic or too alkaline. Therefore, the optimal pH value of detection solution was ultimately selected 6.0 as working buffer (Fig. 6b).

Analytical performance of the glucose biosensor

Under optimal detection conditions, the absorbance at 450 nm increased with glucose concentrations (Fig. 7a). The calibration curve of the absorbance versus glucose concentration was linear in the range from 0.5 μM to 250 μM with a correlation coefficient of 0.991 (Fig. 7b). The regression equation of this method is $A = 0.00357 + 0.0034C$. In the equation, C stands for the concentration of glucose (μM) and A stands for the absorbance of TMB at 450 nm. The limit of detection for glucose was 0.22 μM at a signal-to-noise of 3, which was lower than that previously reported [1,16,22].

The apparent K_m^{app} and the apparent υ_{max} were calculated by using TMB and glucose as substrates, respectively. Table 1 shows the catalytic constants obtained from the equation of Lineweaver–Burk plots. In this work, the apparent K_m^{app} for bi-enzyme to glucose was up to 0.394 mM. The K_m^{app} is lower than the case of GOD immobilized on the Apoferritin with combination of biotin and streptavidin (7.54 mM) [23], as well as the experiment of HRP and GOD immobilized on GCE coated by multiwalled carbon nanotube (CNT) in Nafion (5.2 mM) [24]. Moreover, the K_m^{app} value in this paper is also lower than the results where bi-enzyme modified on single-wall carbon nanotubes (SWNT) coated with electropolymerized pyrrole (PPy) film (1.71 mM) [25], or in an Au electrode modified by multilayer films of sulfonate-capped gold nanoparticles/thionine (1.21 mM) [26]. Even when HRP and GOD were directly assembled in the Au electrode through disulfide groups [27], the K_m^{app} (1.05 mM) is higher than the value obtained in present work. Thus the smaller K_m^{app} value shows a higher affinity between the enzyme and the substrate, which meant that the bi-enzyme assembled on nano-SiO_2 has a higher activity in this paper compared with the previous work.

To test the selectivity of SiO_2-GOD/HRP to glucose, control experiments were carried out using L-histidine, D-(+)-mannose, b-myclodextrin, D(−)-fructose, ascorbic Acid, D-galactose and buffer (Fig. 8). 2 mM control samples were investigated and no

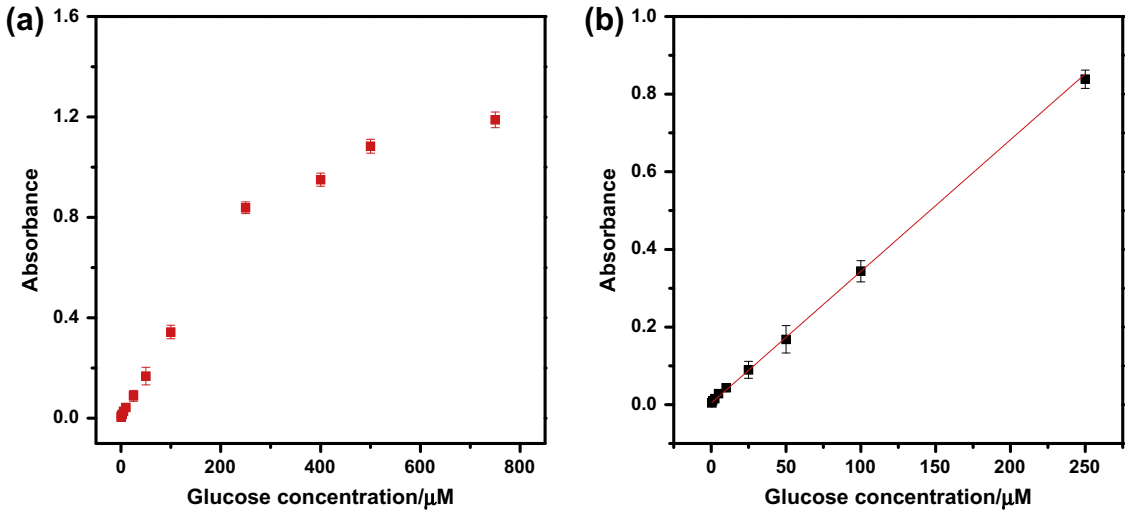

Fig. 7. A dose–response curve for glucose detection at 450 nm under the optimum conditions (a) The linear calibration plot for glucose (b). Reaction condition: 0.1 M pH 6.0 PBS; TMB concentration: 7.5×10^{-4} M.

Table 1
The apparent catalytic constants of immobilized enzymes at room temperature.

Substrate	Enzyme status	K_m^{app} (mM)	υ_{max} (μM s^{-1})
TMB	SiO_2-GOD/HRP	0.211	0.46
Glucose	SiO_2-GOD/HRP	0.394	0.97

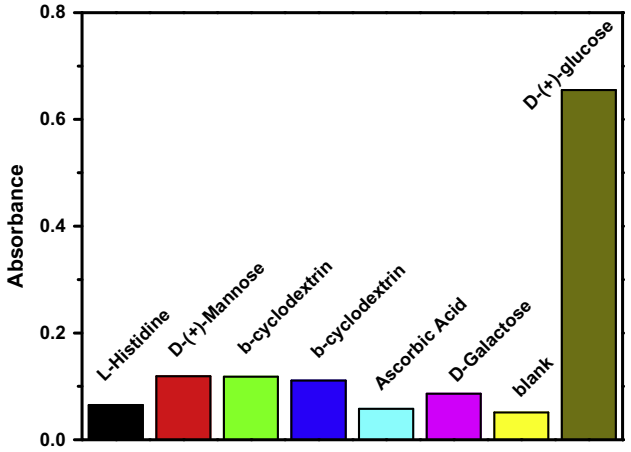

Fig. 8. Study of the selectivity of glucose detection. The concentration ratio of interfering substance and glucose is 10. Reaction condition: 0.1 M pH 6.0 PBS; glucose concentration: 200 μM; TMB concentration: 7.5×10^{-4} M.

detectable signals were obtained for glucose analogues. Thus, the developed colorimetric method has a high selectivity towards glucose detection.

At the same time, in order to demonstrate the stability of SiO_2-GOD/HRP, the same samples stored at 4 °C for different time were applied to the control experiments. With the time increased, the catalytic abilities of SiO_2-GOD/HRP were slightly decreased. When the time was up to 7 days, the absorbance was about 90% of fresh sample. It shows the stability of SiO_2-GOD/HRP was acceptable (Fig. 9). The same samples were tested in the same condition in 3 days. The RSD of the above parallel results was just 2.3%, which proved the good reproducibility of the method (Fig. 10).

Furthermore, bloody glucose level was also an indicator of human health conditions, so the determination of glucose in real serum samples is important in clinical diagnosis. Using this method,

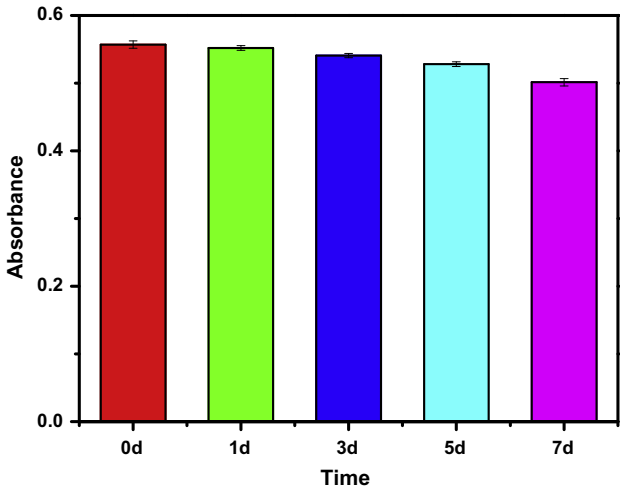

Fig. 9. Study of the stability of glucose detection. From left to right is the absorbance with same samples in different time after store at 4 °C. Reaction condition: 0.1 M pH 6.0 PBS; glucose concentration: 200 μM; TMB concentration: 7.5×10^{-4} M.

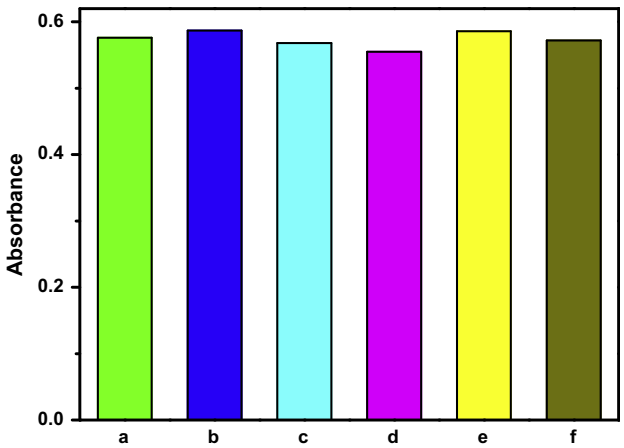

Fig. 10. Study of the reproducibility of glucose detection. From left to right is the absorbance with several same samples in different time in 3 days. Reaction condition: 0.1 M pH 6.0 PBS; glucose concentration: 200 μM; TMB concentration: 7.5×10^{-4} M.

Table 2
Determination of glucose in human serum samples. Each sample was tested for 5 times. Note: The sample was diluted 100 times before use in the experiments.

Samples	Glucometer (mM)	This work (mM)	RSD (%)
1	11.62	12.32	1.8
2	10.12	10.03	2.8
3	9.48	9.24	1.5
4	12.22	12.29	1.8
5	5.8	5.67	2.5
6	4.62	4.59	4.7

glucose in diluted human serum was detected. The serum samples were obtained from ZhongDa Hospital. After filtration, the samples were diluted 100 times for direct use. According to the calibration curve, the concentrations of glucose in the serum samples are recorded in Table 2. A good agreement between colorimetric method and glucometer detection (GB/T19634-2005) was observed, which indicated that the present method could be used in real samples for the determination of glucose.

Conclusion

Well monodispered SiO_2 nanoparticles co-immobilized GOD and HRP were prepared via a simple, low-cost, and convenient method. Based on the prepared SiO_2-GOD/HRP material, a highly sensitive and selective colorimetric method for glucose detection in buffer and dilute serum samples was developed. In addition, compared with other detection methods of glucose in previous work, the prepared SiO_2-GOD/HRP is more time-saving, convenient, simple and stable. It would have many important applications in the fields of biotechnology, medicine and others based on the potential to make pills of the SiO_2-GOD/HRP. And with adjusting the kinds and concentrations of multi-enzyme on the carrier, it could be applied to detect various substrates according to the practical needs.

Acknowledgements

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