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Cascade enzymatic catalysis in poly(acrylic acid) brushes-nanospherical silica for glucose detection

*Yan Zhao, Ying Wang, Xiaobin Zhang, Rongmei Kong, Lian Xia, Fengli Qu†**

The Key Laboratory of Life-Organic Analysis, College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu, Shandong 273165, P. R. China

*Corresponding author information: Prof. F. Qu, Tel/fax:(+86) 537 4458301. E-mail:fengliquhn@hotmail.com

Abstract

The ultrasensitive monitoring of glucose with a fast and accurate method is significant in potential therapeutics and optimizes protein biosynthesis. Incorporation of enzyme into matrix is considered as promising candidates for constructing highly sensitive glucose-responsive systems. In this study, three-dimensional poly(acrylic acid) brushes-nanospherical silica (PAA-nano silica) with high amplification capability and stability were used to covalently immobilize bienzymes for cascade enzymatic catalysis. The major advantages of PAA-nano silica-bienzyme co-incorporation is that the enzymes are proximity distribution, and such close confinement both minimized the diffusion of intermediates among the enzymes in the consecutive reaction and improve the utilization efficiency of enzymes, thereby enhancing the overall reaction efficiency and specificity. Thus, this present bienzymatic biosensor shows robust signal amplification and ultrasensitivity of glucose-responsive properties with a detection limit of 0.04 μM.

Keywords: Bienzyme catalysis; glucose detection; poly(acrylic acid) brushes-nanospherical silica; horseradish peroxidase; glucose oxidase

1. Introduction

Glucose, as a major component of animal and plant carbohydrates[1, 2], has caused great interesting in diagnosing metabolites[3, 4] and food analysis [5]. Additionally, the monitoring of lower levers of glucose, especially in cell cultures and microbial fermentation processes, is significant for potential therapeutics and optimize protein bio-synthesis[6-8]. Its lack or excess could produce detrimental influence on metabolism functions[9]. The monitoring of glucose levels with faster and more accurate methods has become an increasingly active area of research. As one kind of important biomolecules, enzymes have been widely used in the determination of glucose with their high substrate specificity and high catalyzing activity[10-12].

Incorporation of enzymes into matrix to improve the stability of the immobilized enzymes is a prolific area applied to proteomic analysis [13], antifouling [14], biofuel cells [15], and tissue engineering [16]. Compared with single-enzyme immobilization, the bienzymes co-incorporated nanomaterials enable the combination of cascade reactions in one pot, either by running the cascade reactions simultaneously or in a stepwise fashion without isolating the intermediates [17, 18].

Compared to the immobilization of enzymes onto substrate surface [19, 20], incorporation of enzymes into matrix could increase the amount of enzyme loading and protect the enzyme from the surrounding environment, thus ensuring good enzyme stability (such as: thermal and pH stability) over long period of time [21, 22]. Coupled polymer-enzyme co-incorporated structures, which provide favorable microenvironment because of the flexibility and functional groups of the polymer, have been attracting increased attention [23, 24]. Some conventional intermixed structure or self-assembled polymers like chitosan, agarose, poly(acrylamide), and poly(propyleneimine) were widely used in biomedical fields including protein adsorption, biomolecule immobilization, controlled drug release and so on [25-27]. Among these functional polymers, poly(acrylic acid) (PAA) is a water-soluble biocompatible polymer for its dispersity and capacity properties [28, 29].

Herein, three-dimensional PAA-nanospherical silica (PAA-nano silica) was used

to covalently immobilize bienzymes for cascade enzymatic catalysis. In brief, glucose oxidase (GOx) oxidizes glucose to generate H_2O_2, and then H_2O_2 could reacts with the adjacent horseradish peroxidase (HRP) to oxidize the chromogenic substrates, resulting in an apparent color change. In this study, we describe a facile method to enhance the stability of bienzyme-loaded bioassays. The success of this work relies on the use of PAA [30] to modify the surface of SiO_2 nanoparticles. The resultant PAA-nano silica serves as an ideal matrix for stable and large-quantity enzyme loading while preserving their biological activities. In this structure, the SiO_2 nanoparticles serve as a robust and versatile core for surface modification [31, 32]. Surface-initiated reversible addition-fragmentation chain transfer (RAFT) polymerization process of PAA on nano silica provides a good control of molecular weights, content of carboxyl groups and thickness of the PAA brushes. As a result, PAA-nano silica nanoparticles exhibit many superior properties over conventional nanoparticles, such as the three-dimensional structure, flexibility, biocompatibility and high-abundant capacity of enzyme. Given the close proximity of the bienzyme in a scaffold structure, this novel sensor greatly reduces the diffusion and the decomposition of H_2O_2, and most of the enzyme could be involved in the enzymatic reaction.

2. Experimental section

2.1 Reagents and apparatus

HRP (250 U/mg) was purchased from Sinopharm Chemical Reagent Co. LTD. (Shanghai, China). GOx (150 U/mg) was obtained from Sigma-Aldrich Co. LLC. (St. Louis, USA). *N*-(3-dimethylaminopropyl)-*N′*-ethyl-carbodiimidehydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Aladdin Industrial Inc. (Shanghai, China). Glucose and sodium dodecyl sulfate (SDS) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All aqueous solutions were prepared with deionized water (resistivity > 18 MΩ•cm) produced using a Millipore system.

Transmission electron microscope (TEM) images were obtained using a JEM-

2100 microscope operated at 200 kV. UV/vis measurements were performed using a Varian Cary-300 bio UV/vis spectrophotometer (USA). Thermo gravimetric analysis (TGA) measurements were performed using a Hitachi DMA7100 Thermo gravimetric analysis at a heating rate of 10°C/min. Gel permeation chromatography (GPC) results were obtained by using a Polymer-GPC220. FT-IR spectrum was performed using a Thermo Nicole IS5.

2.2 Preparation of PAA-nano silica

PAA-nano silica was synthesized based on the substrate of SiO_2 NPs via surface-initiated reversible addition–fragmentation chain transfer (RAFT) polymerization [30]. SiO_2 NPs with a diameter of 40 nm were prepared by the Stöber method [33]. Table S1 lists the main parameters for the synthesis of PAA-nano silica.

2.3 Preparation of PAA-nano silica-bienzyme

PAA-nano silica-bienzyme was synthesized by covalently immobilizing enzymes into PAA-nano silica using the "chemical conjugation after electrostatic entrapment" (CCEE) process. 1.5 mg of PAA-nano silica was suspended in 5 mL of 10 mM 2-(N-morpholino) ethanesulfonic acid buffer containing 0.05 wt% Tween-20 (MES buffer, pH = 5.0), then mixed with 200 μL of HRP (15 mg/mL) and 400 μL of GOx (15 mg/mL) with gentle stirring for 5 h at 4 °C to form the spatially co-localized PAA-nano silica-bienzyme complexes. After removing the excessive enzymes by centrifugation (5000 r/min, 10min), 500 μL of 0.5 mM EDC was added for the electro statically adsorption of HRP and GOx, and this conjugation process was conducted for 2 h at 4 °C. After centrifugation and discarding the supernatant, the PAA-nano silica-bienzyme was obtained and stored at 4 °C with a concentration of 1.5 mg/mL (in terms of PAA-nano silica).

2.4 Procedure of glucose detection

100 μL of 100 mM ABTS and 75 μL of prepared PAA-nano silica-bienzyme complexes were added into 10 mL of phosphate saline buffer (PBS buffer, pH = 7.0) containing various concentration of glucose. The mixed solution was shaken at room temperature for 5 min to hold the reaction and then 1 mL of SDS solution (1 wt%) was added to stop the reaction completely. The resultant solution was used for the

absorbance measurement at wavelength 420 nm using a UV/vis spectrometer.

For comparison, carboxylated SiO_2-bienzyme complexes were obtained by NHS/EDC method for cascade catalyzed reaction.

3. Results and discussion

3.1 Sensing mechanism and the characterization of the PAA-nano silica-based glucose biosensor

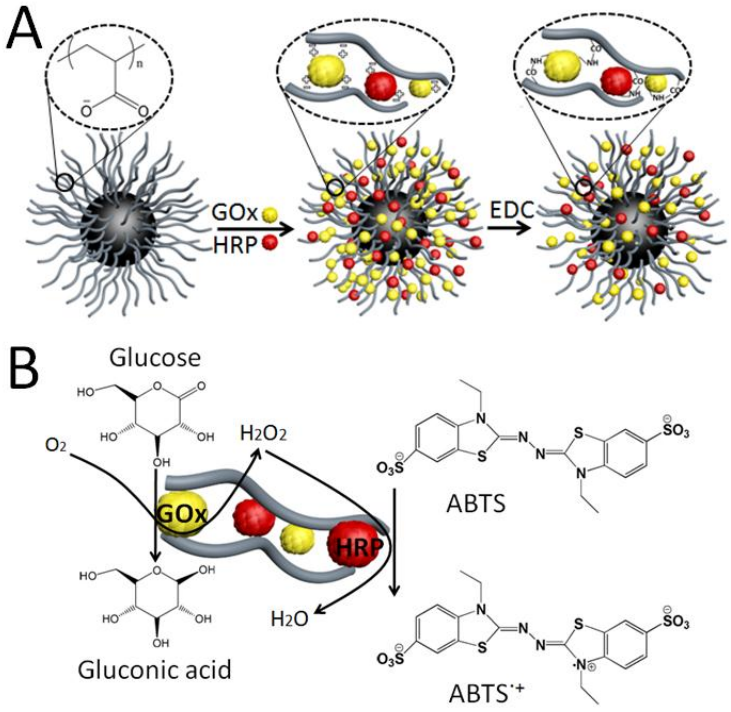
The as-prepared PAA-nano silica-bienzyme complex demonstrated high stability and reactivity for the detection of glucose via 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) oxidation. PAA-nano silica-bienzyme complex was prepared by covalent immobilization of HRP and GOx in PAA-nano silica via the "chemical conjugation after electrostatic entrapment" (CCEE) [34] process (Scheme 1A) through two sequential steps: tandem electrostatic entrapment and chemical conjugation. While entrapment leveraged the unique "Donnan effect" [35] for PAA-nano silica to achieve a high enzyme binding capacity, conjugation effectively turned labile electrostatic interaction into stable covalent binding, allowing resuspension of PAA-nano silica-bienzyme into the biological medium or buffer used in bioassays. In the presence of dissolved oxygen, a cascade reaction occurred. The first reaction was the conversion of glucose by GOx into gluconic acid and H_2O_2. Then the amount of H_2O_2 could be determined through oxidizing ABTS to ABTS radical cation ($ABTS^{\bullet+}$) using HRP as catalysis (Scheme 1B). The cascade reaction could be easily monitored using UV/vis spectrophotometre due to a shift in the UV/vis absorption spectrum at 420 nm when ABTS was oxidized to $ABTS^{\bullet+}$. The co-incorporated PAA-nano silica-bienzyme have proximity distributed enzymes, which not only minimized the diffusion of intermediates among the enzymes in the consecutive reaction, but also improve the utilization efficiency of enzymes, thereby enhancing the overall reaction efficiency and specificity. The detailed reaction mechanism [36, 37] is presented below:

$$\text{Glucose} + GOx_{(FAD)} \rightarrow \text{gluconic acid} + GOx_{(FADH2)} \quad (1)$$

$$GOx_{(FADH2)} + O_2 \rightarrow GOx_{(FAD)} + H_2O_2 \quad (2)$$

$$HRP_{(red)} + H_2O_2 \rightarrow HRP_{(ox)} + H_2O \quad (3)$$

$$HRP_{(ox)} + ABTS \rightarrow HRP_{(red)} + ABTS^{\bullet+} \quad (4)$$



Scheme 1. Schematic illustration of the colorimetric bioassay protocol: (A) Covalent immobilization of HRP and GOx into PAA-nano silica by CCEE process, (B) the cascade reaction for glucose detection using PAA-nano silica-bienzyme amplified biosensing strategy.

As shown in Figure 1B, the PAA-nano silica was monodispersed colloidal particles with a SiO_2 core and densely grafted PAA chains. The SiO_2 NPs were quasi-spherical with an average diameter of around 40 nm (Figure 1A). The core-spherical structures of PAA-nano silica were clearly shown that the SiO_2 core was capped by the spherical PAA brushes with an average thickness of 20 nm. FT-IR spectrum of the PAA-nano silica is represented in Figure S1, A strong absorption at 1716 cm^{-1} belongs to C=O stretching of carboxylic acid group. The same group shows a medium peak at 1410 cm^{-1} corresponding to the plain deformation of C-O-H. Ring stretching at 1570 cm^{-1} is masked by the CO_2^- absorption of PAA brushes. The PAA brushes, with abundant carboxyl groups, were considered particularly suitable for the CCEE process. In aqueous solution, the PAA-nano silica feature long-stretching PAA chains with high grafting density, abundant carboxyl groups, uniform distribution and excellent

dispersability.

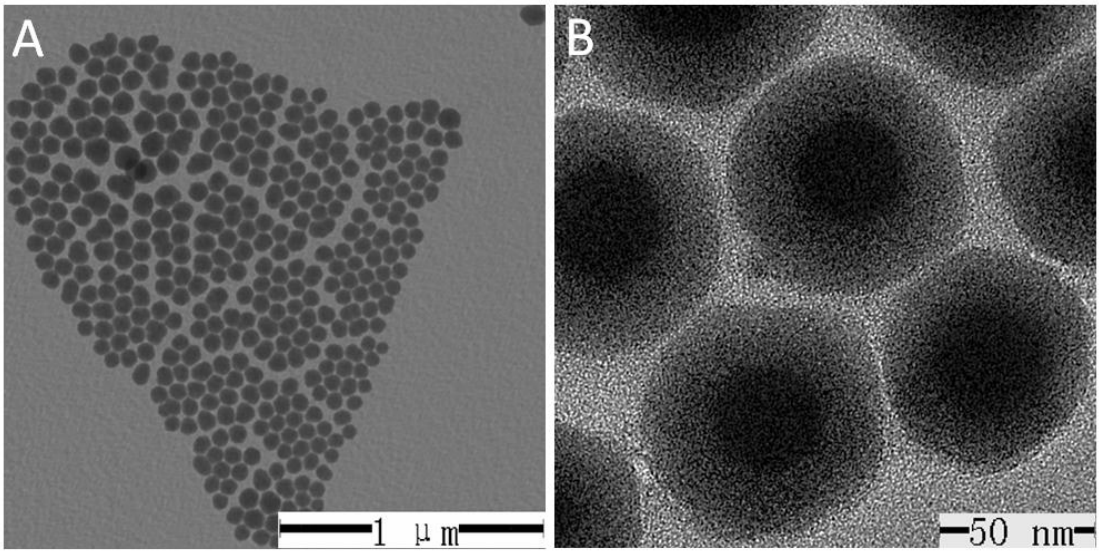


Figure 1. TEM images of (A) SiO_2 NPs and (B) PAA-nano silica.

Successful immobilization of enzymes into PAA-nano silica could be intuitively visualized by eye (Figure 2A). PAA-nano silica possessed a characteristic brownish color of HRP and a characteristic yellowish color of GOx after immobilization with HRP and GOx, respectively. A transitional brownish yellow was observed for PAA-nano silica-bienzyme. Immobilized enzymes could be separated by centrifugation along with PAA-nano silica, leaving the supernatant colorless. The color change of nanoparticles and their response to centrifugation indicated the success of enzymes immobilization. For further verification, as shown in Figure 2B, the characteristic absorption of HRP and GOx were clearly seen in PAA-nano silica-bienzyme. In the presence of glucose/ABTS, only PAA-nano silica- bienzyme caused dramatic UV/vis absorbance increase. PAA-nano silica, PAA-nano silica-HRP and PAA-nano silica-GOx did not induce peak intensity change, demonstrating this glucose-responsive assay required bienzyme. The inset graph showed this result intuitively, indicating the feasibility of this colorimetric assay of glucose (Figure 2C).

Note-worthily, excellent dispersability of PAA-nano silica was maintained after immobilization of enzymes, as PAA-nano silica-bienzyme could be stored in a dispersion state for weeks without notable precipitation. This property of bienzyme-loaded PAA-nano silica was extremely important for their practical applications.

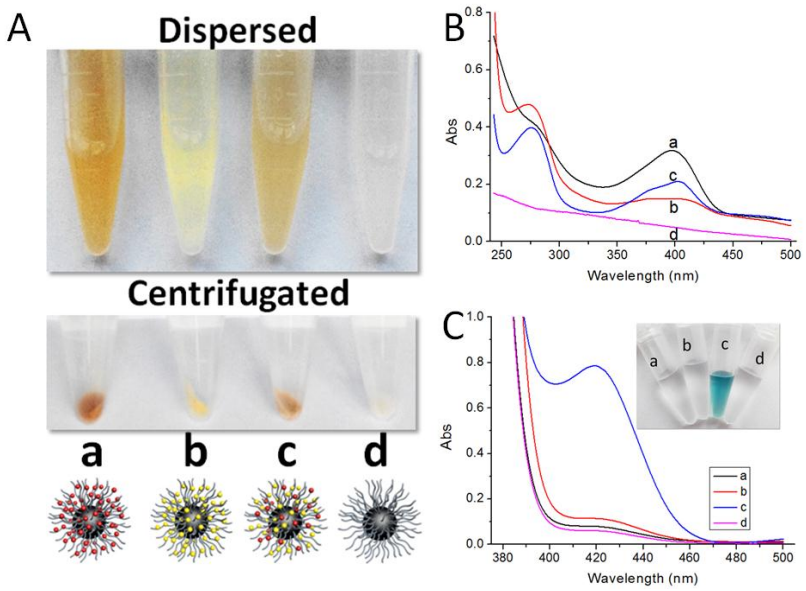


Figure 2. (A) Pictures of aqueous suspensions of (a) PAA-nano silica-HRP, (b) PAA-nano silica-GOx, (c) PAA-nano silica-bienzyme and (d) PAA-nano silica; (B) UV/vis spectra of aqueous suspension of a, b, c and d; (C) UV/vis spectra of aqueous suspension of a, b, c and d with glucose (10 μM)/ABTS (0.1 M). The inset picture shows the corresponding color change.

3.2 Optimization of the bioassay conditions

In the dissolved oxygen solution, glucose could be oxidized to gluconic acid and H_2O_2 by GOx, then HRP catalyzed the colorless ABTS to the colored $ABTS^{\bullet+}$ in the presence of H_2O_2. Since bienzymes (HRP/GOx) play different roles, the ratio of both two enzymes on the biosensor system may determine the sensor performance. The effect of bienzyme (HRP/GOx) volume ratio (from 1:0 to 0:1 with a total volume of 600 μL) was examined under UV/vis absorbance at 420 nm when the concentration of glucose was set to 10 μM (Figure S2A). With the increased amount of GOx, the cascade reaction was triggered and the catalytic activity steadily increased. While in the presence of excessive GOx, lower catalytic activity was observed. It could be due to local overproduction of H_2O_2 by GOx that rapidly saturates and inhibits the enzymatic activity of HRP. In short, the prepared HRP/GOx at a volume ratio of 1:2 yields the highest catalytic ability.

Temperature and pH could also influence the detection sensitivity of the assay. Considering the practical application of proposed system in clinical testing, all the

experiments were carried out at 25 ± 1.0°C. The effect of pH on the bienzymatic activity of the PAA-nano silica-based assay was investigated by varying buffer pH from 3 to 10 for the detection of 10 μM of glucose (Figure S2B). Under UV/vis absorbance at 420 nm, the maximum absorbance intensity occurred at pH 7, which indicating the neutral environment was more conductive for the bienzymatic assay to be in operation [38].

The activity of HRP and GOx was probed by procedures based on the classical bienzymatic assay for glucose exploiting the reaction (2) and (4). Under the optimal condition and the same initial bienzyme concentration, the reaction in solution was triggered by the addition of a suitable amount of glucose (10 μM) and the increase in absorbance at 420 nm due to the ABTS oxidation was followed. As shown in Figure S3, activity of immobilized bienzyme relative to SiO_2-bienzyme (HRP/GOx at a volume ratio of 1:2 with a total volume of 600 μL) was estimated from the initial slope of catalysis kinetics. This method led to the conclusion that relative activities of immobilized bienzyme as compared to SiO_2-bienzyme was ~ 25.0 % for PAA-nano silica-bienzyme. The remarkable enhancement in enzyme activity clearly demonstrated the advantage of PAA-nano silica as efficient enzyme carriers.

3.3 Analytical performance of the biosensor

Using the optimized working parameters, high sensitivity of the PAA-nano silica-bienzyme toward glucose detection was achieved. As concentration of glucose increased, the UV/vis signal intensity increased (Figure 3). Good correlation between the glucose concentration and the absorbance was observed with a wide dynamic range (from 0.167 μM to 20 μM) (Figure 4A line a). The linear regression equation is $A = 0.060C + 0.077$ ($R^2 = 0.993$), where A was the absorbance response and C (μM) was the concentration of the glucose. The limit of detection (LOD) concentration was 0.04 μM at S/N = 3.

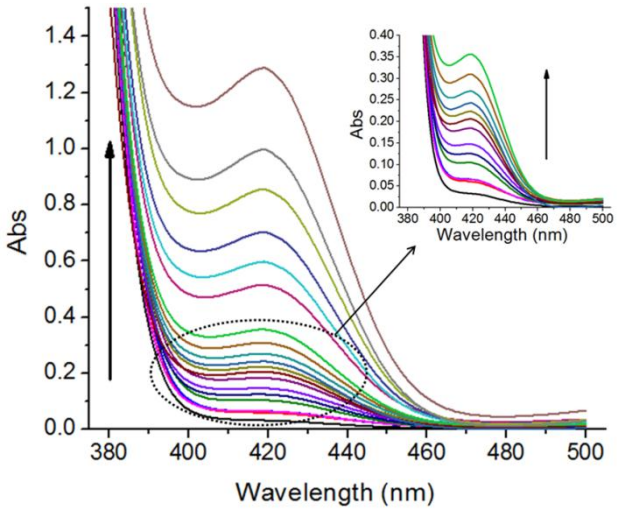


Figure 3. The UV/vis spectra of ABTS oxidation toward different concentrations of glucose. The inset shows the magnified spectra of low glucose concentrations.

To validate the contribution of the unique structure of PAA-nano silica, SiO_2-bienzyme-based structure was used to perform control experiments. Comparatively lower response was obtained, indicating that the presence of PAA-nano silica-bienzyme efficiently enhanced the overall signal detected. As indicated in Figure 4A (line b), SiO_2-bienzyme-based system exhibited linearity for glucose concentration with linear regression equation of $A = 0.019C + 0.023$ ($R^2 = 0.999$). By comparing the slope values of these linear curves, the UV/vis response of PAA-nano silica based label exhibited a response rate nearly 3.15-fold higher than SiO_2 labeled bienzyme, the dramatic amplification of signal was intuitive visually in Figure 4B.

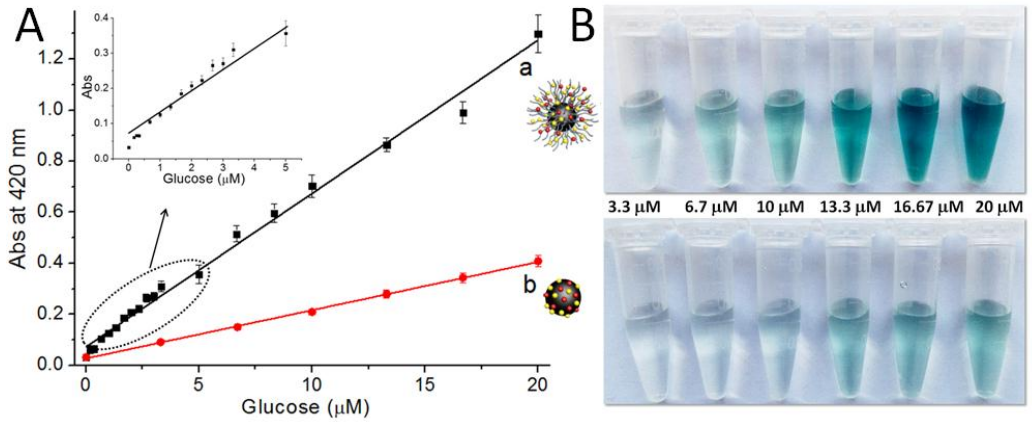


Figure 4. (A) Calibration curve toward different concentrations of glucose by using various elements of (a) PAA-nano silica-bienzyme and (b) SiO_2-bienzyme; (B) the corresponding color

variation. Error bar = SD (n =5).

This signal enhancement result could be summarized as follows: First, the PAA-nano silica structure had high-abundant enzyme capacity and could maintain enzyme activity. Second, this PAA-nano silica scaffold structure kept enzyme loosely distribution, thus most of the enzyme could be involved in the enzymatic reaction. Compared with quasi enzyme[39, 40], cascade enzymatic reaction could guarantee good biocompatibility, leading less degeneration production and isolated impurities. The high sensitivity indicated the success of this signal amplification strategy for the detection of glucose. These readily prepared PAA-nano silica-enabled method exhibited an ultrasensitive linear dynamic range than those for glucose detection documented in literatures (Table S2). Moreover, compared with electrochemical assays[41-44], which could achieve lower limit of detection, this proposed simple configuration glucose detection method could obtain more stable signal output and better reproducibility. Due to the designed signal amplification, the proposed immunoassay not only avoided the spilling of enzyme substrate in the detected environment and exhibited high enzyme activities for a long time, but also proposed an ultrasensitive detection range with a low detection limit.

3.4 Specificity and stability

The determination of glucose was of great importance in food and pharmaceutical industry as well as clinical testing. Good specificity for glucose was necessary for a new designed sensing system with potential applications in real samples. To evaluate the specificity of the developed signal amplified bioassay for glucose, several potential interfering compounds were studied under the same conditions. The 10 μM of glucose containing 100 μM interference carbohydrates including D-Fructose, galactose, D-Sucrose, maltose, lactose, xylose, dextrin and starch were measured using the developed bioassay. As shown in Figure 5A, the mixed compound caused dramatic UV/vis response while these potential interferences triggered negligible signal change. The interference from other carbohydrate should be negligible since GOx was highly specific. The inset graph showed this result

intuitively, indicating that the selectivity of the bioassay was quite acceptable and the common component existed did not interfere the detection of glucose.

The long-term stability of the bienzymatic activity has been checked daily and weekly by measuring the initial rate of $ABTS^{\bullet+}$ formation at a constant glucose concentration (10 μM). When not in use, the bienzymatic PAA-nano silica were dispersed in buffer and kept at 4 °C. The long-term storage stability of enzymes was improved upon incorporation into the complexes. As shown in Figure 5B, the PAA-nano silica-bienzyme retained ~ 70 % initial activity after incubating in buffer solution at 4 °C for 30 days. In contrast, the SiO_2-bienzyme system in solution lost nearly 85 % of the overall activity within 30 days. The enhanced enzyme stability was due to the confined configuration of the incorporated proteins, which prevent protein denaturation and leakage.

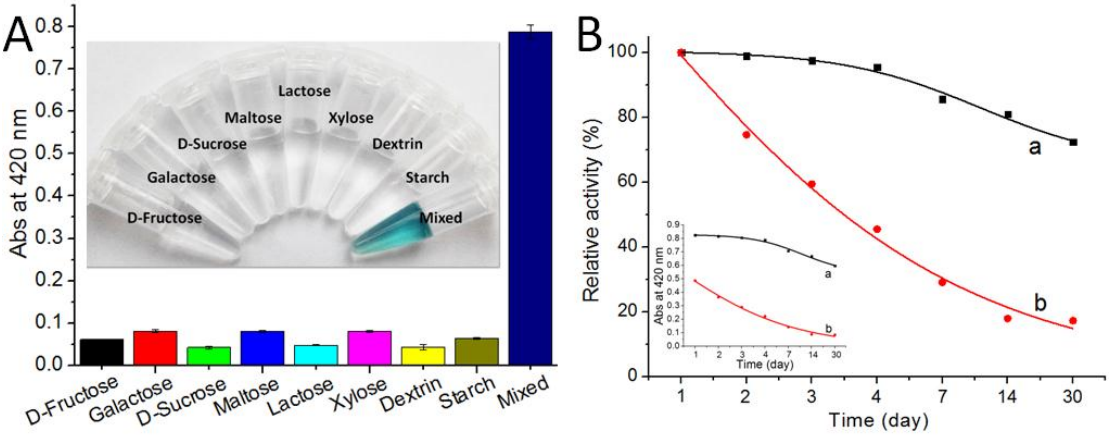


Figure 5. (A) The specificity of the bioassay using 10 μM of glucose containing 100 μM interference carbohydrates. The inset is the corresponding photographs of color change for the specificity. (B) The stability of (a) PAA-nano silica-bienzyme and (b) SiO_2-bienzyme in aqueous solution, the inset figure demonstrates the UV/vis performances of a and b.

3.5 Analysis of complex biological samples

In order to evaluate the potential application of the bioassay in complex biological samples, the glucose detection was also conducted in human serum samples. Three diluted human serum samples (0.1 %) were performed for the detection of glucose using the standard addition method. As indicated in Table S3, 5 μM, 10 μM, and 15 μM of glucose were added into the diluted serum

samples, respectively. The recovery percentage of glucose was ranged from 99.1 % to 117.4 %, and the relative standard deviation (RSD) was ranged from 0.7 % to 3.8 %. These results revealed that the proposed bioassay could be applicable for practical glucose detection in real biological samples with other potential interference co-existed.

4. Conclusion

We developed a facile synthesis route of bienzyme co-incorporated PAA-nano silica for cascade enzymatic reactions. The prepared PAA-nano silica-bienzyme complex amplified the signal of the catalytic oxidation and thus resulted in significant color change and excellent linearity signal variations for glucose with satisfactory results, exhibiting its promising practical applications in biotechnology, biomedical and environmental chemistry.

Acknowledgements

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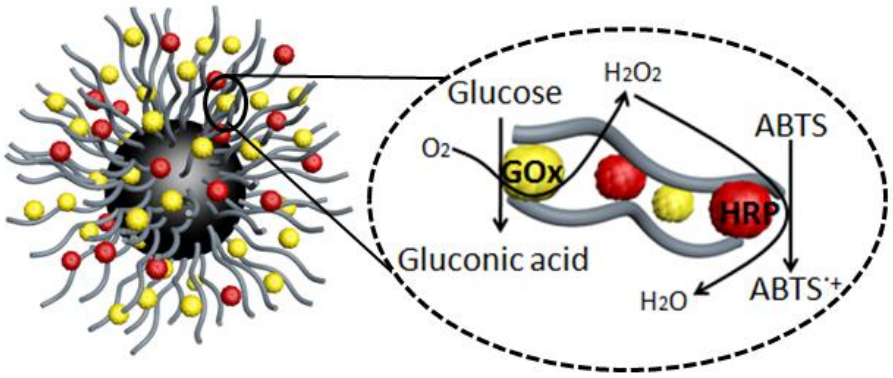
Highlights

- Colorimetric bioassay was designed for the ultrasensitive detection of glucose.
- Signal amplification was achieved using PAA-nano silica-bienzyme.
- Good performance was obtained with a detection limit of 0.04 μM.
- Favorable results were achieved for detecting glucose in complex biological samples.



Graphical abstract