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

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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.

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

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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, 10 min), 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:

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

$$\text{GOx}_{(\text{FADH2})} + \text{O}_2 \rightarrow \text{GOx}_{(\text{FAD})} + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{HRP}_{(\text{red})} + \text{H}_2\text{O}_2 \rightarrow \text{HRP}_{(\text{ox})} + \text{H}_2\text{O} \quad (3)$$

$$\text{HRP}_{(\text{ox})} + \text{ABTS} \rightarrow \text{HRP}_{(\text{red})} + \text{ABTS}^{\bullet+} \quad (4)$$

As shown in Fig. 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 (Fig. 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 Fig. 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