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Magnetic nanoparticle-linked colorimetric aptasensor for the detection of thrombin

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ABSTRACT

Employed as a peroxidase mimic, chitosan modified Fe_3O_4 magnetic nanoparticles (MNPs) were synthesized via solvothermal method. This alternative model evidenced a remarkable catalytic activity in presence of hydrogen peroxide/3,3′,5,5′-tetramethylbenzidine (TMB) solution. Fe_3O_4 peroxidase mimic has the merits of stability, magnetic separation and enrichment. Enzyme-linked immunosorbent assay (ELISA) was used to detect thrombin by employing the catalytic properties of the as-synthesized chitosan modified Fe_3O_4 MNPs. The results showed that the absorption values at 652 nm increased with the thrombin concentrations, a linear range from 1 to 100 nM and 1 nM thrombin (S/N = 3) could be detected. The proposed approach confirmed that the Fe_3O_4 MNPs not only possessed peroxidase activity but also showed potential application in varieties of simple, robust, and cost-effective analytical methods in the future.

1. Introduction

There is currently intense interest in applying magnetic nanoparticles to the biomedical and technological applications, such as magnetic resonance imaging, drug delivery, biological separation, and even biological catalysis [1–4]. In biological catalysts, magnetic nanoparticles are coated with enzymes or metal catalysts to form dual-functional nanomaterials, thus incorporate both magnetic separation and catalytic features [5–8]. For example, horseradish peroxidase (HRP)-entrapped magnetic nanoparticles have been used for bioseparation and biocatalysis [4]. And the Fe_3O_4@SiO_2–polyamidoamine (PAMAM) dendrimer–Pd(0) composites use as magnetically recoverable catalysts for the hydrogenation of allyl alcohol [9]. Recently, Yan et al. have found that Fe_3O_4 magnetic nanoparticles (MNPs) exhibited an intrinsic enzyme mimetic activity similar to that found in natural peroxidases, though Fe_3O_4 MNPs are usually thought to be biological and chemical inert [10]. This discovery is of great significance, because MNP enzyme mimics have several advantages over traditional natural enzymes. (a) The catalytic property of MNPs is more stable than the peroxidases, such as HRP, which is susceptible to the reaction conditions. (b) MNPs have the properties of magnetic separation and enrichment, which can facilitate the biological applications. (c) The preparation of MNPs is simple and economical, while the preparation and purification of natural enzymes are usually time-consuming and expensive [11]. Therefore, the robustness of Fe_3O_4 MNPs peroxidase mimic makes them suitable for a broad range of applications in the environmental chemistry and biomedicine fields. For example, the ability to catalyze the oxidation of organic substrates to reduce their toxicity or to produce a color change is frequently used in wastewater treatment or as a detection tool.

Aptamers are nucleic acids (DNA or RNA) that are synthesized with systematic evolution of ligands by exponential enrichment (SELEX) through repetitive binding of target molecules. Their target molecules range from small inorganic and organic substances to proteins and even cells [12,13]. Aptamers have advantages over traditional recognition elements such as antibodies, including ease of synthesis, thermal stability and lack of immunogenicity [14,15]. Based on their high biomolecular recognition ability, a lot of optical and electrochemical aptasensors (aptamer biosensors) have been developed for the determination of thrombin, potassium ion, adenosine, etc. over the past decades [16]. Simple colorimetric sensors have the potential to eliminate the use of analytical instruments and are attracting more and more attentions. Now, a number of colorimetric aptasensors have been developed, most of which use gold nanoparticles (AuNPs) as sensing elements [17–20]. However, using Fe_3O_4 MNPs as peroxidase mimic for the construction of colorimetric aptasensors has never been explored.

Thrombin is a major stimulus of both procoagulant and anticoagulant reactions, and thus is a key element in various pathogenesis, including leukemia, arterial thrombosis and liver disease [21,22]. Therefore, the determination of thrombin

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level plays essential roles in fundamental research and clinical application [23]. In this paper, a magnetic nanoparticle-linked colorimetric assay for the determination of thrombin based on the enzyme-linked immunosorbent assay (ELISA) was developed. Biotinylated thrombin aptamer 1 was loaded into a 96-well plate by streptavidin binding. And then amino-terminal thrombin aptamer 2 was assembled onto the chitosan modified Fe_3O_4 MNPs through glutaraldehyde coupling. Subsequently, the sample solution and aptamer 2 modified Fe_3O_4 MNPs were successively added into the 96-well plate. If the sample contained thrombin, Fe_3O_4 MNPs would be linked to the 96-well plate. Moreover, the amounts of Fe_3O_4 MNPs were depended on the concentrations of thrombin. So when a chromogenic reagent, such as 3,3′,5,5′-tetramethylbenzidine (TMB), was used to produce detection signals, the concentrations of thrombin could be determined.

2. Experimental

2.1. Chemicals

Chitosan was provided by Generay Biotech Co., Ltd. Streptavidin was purchased from Calbiochem. Bovine serum albumin (BSA) was supplied by Beijing Like Biochemical Technology and Trade Co., Ltd. Glutaraldehyde (GA, 25% aqueous solution) was obtained from Acros. 3,3′,5,5′-Tetramethylbenzidine (TMB) and horseradish peroxidase (HRP, 300 u/mg) were purchased from Bio Basic Inc. α-Thrombin from bovine plasma (1000 u) and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were provided by Sigma. The thrombin aptamers were supplied by Shanghai Sangon biological engineering technology and services Co., Ltd, and have the following sequences: biotinylated 29 mer aptamer 1, 5′-biotin-$(CH_2)_6$-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′; amino-modified 15 mer aptamer 2, 5′-NH_2-$(CH_2)_6$-GGT TGG TGT GGT TGG-3′. All other chemicals were of analytical grade and were used as received. Doubly distilled water was used throughout.

2.2. Instrumentation

Scanning electron microscopy (SEM) images were taken using a Philips XL-30 FE-SEM. X-ray diffraction (XRD) patterns of the prepared samples were recorded on a Rigaku-Dmax 2500 diffractometer equipped with graphite monochromatized Cu Kα (λ = 0.15405 nm) radiation at a scanning speed of 4°/min in the range from 10° to 80°. Hysteresis loops were collected on a Quantum Design superconducting quantum interference device (SQUID) magnetometer (LakeShore 7307) at 300 K. FT-IR spectra were recorded on a Bruker Vertex 70 FT-IR spectrometer. UV–vis absorption was characterized by Cary 50 UV–vis NIR spectrometer (Varian, USA). The absorption values of the 96-well plate were obtained using a microplate spectrophotometer (Bio Tek, Powerwave) and the Gen5 Data Analysis software. The Fe concentrations in the washing solutions were determined using an iCAP 6000 inductively coupled plasma optical emission spectroscopy spectrometer (ICP-OES, Thermo).

2.3. Synthesis of chitosan modified Fe_3O_4 MNPs (CS-MNPs)

The chitosan modified Fe_3O_4 MNPs were prepared via a previously reported solvothermal method [24]. 0.41 g $FeCl_3$ was firstly dissolved in 20 ml of ethylene glycol under vigorous stirring. After the solution became colorless, 1.8 g NaAc and 0.5 g chitosan were added with continuous stirring for 30 min. Then the mixture was sealed in a teflonlined stainless-steel autoclave. The autoclave was heated to and maintained at 200 °C for 12 h, and allowed to cool to room temperature. The formed black Fe_3O_4 nanoparticles were collected by a external magnetic field, rinsed with ethanol for three times, dried at 60 °C and made into powder.

2.4. The color reaction of Fe_3O_4 MNPs as peroxidase mimic

The catalytic property of the as-synthesized Fe_3O_4 MNPs was characterized by the color reaction of ABTS and TMB in the presence of H_2O_2. In a catalytic experiment of ABTS, the mixed solution of 600 μM ABTS, 1 mM H_2O_2 and 20 μg/ml as-prepared Fe_3O_4 MNPs dissolved in 0.2 M acetate buffer (pH 4.0), and incubated in a 45 °C water bath for 10 min. Then the Fe_3O_4 MNPs were removed from the reaction solution with an external magnetic field. 100 μl of the reaction solution was added to 900 μl of water for the adsorption spectroscopy measurement. In a typical color reaction of TMB, briefly TMB was firstly dispersed ultrasonically in 0.2 M acetate buffer (pH 3.5) for at least 30 min in a concentration of 200 μg/ml (832 μM). Then 60 μl of 30% H_2O_2 was mixed with 1 ml of TMB solution, the concentration of H_2O_2 was 530 mM. Finally, 50 μg of Fe_3O_4 MNPs was added in the 1 ml of TMB/H_2O_2 mixed solution, and reacted for 10 min. The Fe_3O_4 MNPs were removed from the reaction solution. After the data was recorded, the reaction was terminated by adding 200 μl of 1 M H_2SO_4.

2.5. Determination of thrombin

2.5.1. The assembly of the 96-well plate

The 96-well plate used in this work can realized the high throughput determination of thrombin. 100 μg/ml of streptavidin in 0.1 M $NaHCO_3$ was firstly loaded into a 96-well microtiter plate for 6 h, washed with water and 0.02 M phosphate-buffered saline (PBS, pH 7.0). The plate was subsequently blocked with 1 mg/ml of BSA in PBS for 4 h and washed with PBS. The wells were then assembled with 5 μM of biotinylated 29 mer aptamer 1 PBS solution for 6 h, washed with water and 0.02 M Tris–HCl buffer (containing 140 mM NaCl, 5 mM KCl and 5 mM $MgCl_2$, pH 7.4). Finally, the plate was incubated in the Tris–HCl buffer at 4 °C until use.

2.5.2. The aptamer 2 modified Fe_3O_4 MNPs

2 mg chitosan modified Fe_3O_4 MNPs were dispersed ultrasonically in 1 ml of 5% glutaraldehyde PBS solution. Then 1 ml of 5 μM amino-modified 15 mer aptamer 2 PBS solution was mixed with the MNP solution and shaked for 4 h. Then the 2 ml of aptamer 2 modified Fe_3O_4 MNPs was washed with 2 ml of water and 2 ml of Tris–HCl buffer using a magnet, respectively. The 4 ml of washing solution was collected for the inductively coupled plasma (ICP) experiment. The precipitate was dispersed in 2 ml of Tris–HCl buffer for use.

2.5.3. The color reaction for the determination of thrombin

The different concentrations of thrombin in Tris–HCl buffer and aptamer 2 modified MNPs solution were successively added into the blank modified 96-well plate for 1 h at room temperature, the plate was washed with water and acetate buffer. Then the mixed solution of 1 ml of 200 μg/ml TMB in acetate buffer and 60 μl of 30% H_2O_2 was added into the 96-well plate (100 μl/well) for 10 min. The reaction was stopped by the addition of 1 M H_2SO_4 (20 μl/well).

2.6. The ELISA reaction for the detection of thrombin using HRP as the catalyst

The plate was assembled with streptavidin, BSA and biotinylated 29 mer aptamer 1, as mentioned in Section 2.5.1. Then adding 50 nM thrombin and amino-modified 15 mer aptamer 2 in Tris–HCl buffer in the well for 2 h, the plate was subsequently fabricated with 5%