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Apoferritin protein nanoparticles dually labeled with aptamer and horseradish peroxidase as a sensing probe for thrombin detection

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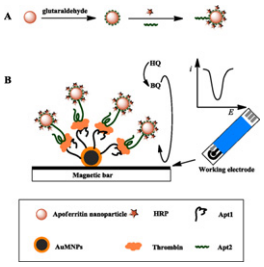
HIGHLIGHTS

- A novel apoferritin nanoparticles modified with HRP and aptamer was synthesized.
- Signal dual amplification was carried out by using the resulted nanoparticles as detection probe.
- Ultrasensitive and high specific thrombin detection was achieved.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel and ultrasensitive sandwich-type electrochemical aptasensor has been developed for the detection of thrombin, based on dual signal-amplification using HRP and apoferritin. Core/shell Fe_3O_4/Au magnetic nanoparticles (AuMNPs) loading aptamer1 (Apt1) was used as recognition elements, and apoferritin dually labeled with Aptamer2 (Apt2) and HRP was used as a detection probe. Sandwich-type complex, Apt1/thrombin/Apt2–apoferritin NPs–HRP was formed by the affinity reactions between AuMNPs–Apt1, thrombin, and Apt2–apoferritin–HRP. The complex was anchored on a screen-printed carbon electrode (SPCE). Differential pulse voltammetry (DPV) was used to monitor the electrode response. The proposed aptasensor yielded a linear current response to thrombin concentrations over a broad range of 0.5–100 pM with a detection limit of 0.07 pM ($S/N = 3$). The detection signal was amplified by using apoferritin and HRP. This nanoparticle-based aptasensor offers a new method for rapid, sensitive, selective, and inexpensive quantification of thrombin, and offers a promising potential in protein detection and disease diagnosis.

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1. Introduction

Recently, protein detection has been the subject of increasing interest in both basic discovery research and clinical diagnosis [1]. In many diagnostic applications, the molecular recognition of specific proteins is often accomplished through the use of antibodies. The preparation process of antibodies, however, is complex, costly, and difficult. Artificial nucleic acid ligands (aptamers), an molecular recognition element, have recently attracted great interest due to their capability of binding a variety of metal ions, amino acids, drugs, proteins, and other molecules with high affinity and specificity. In recent years, several aptamer-based biosensors (aptasensors) have been developed. Compared with antibody–antigen reaction-based immunoassay, aptasensors have the advantages of easy preparation, excellent stability and reusability, and general availability for almost any given protein [2]. In an aptasensing system, target recognition can generally be transduced into an electrical or optical readout [3–5]. Among these signal transduction protocols, electrochemistry detection has attracted

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considerable interest due to its intrinsic advantages, such as portability, low cost, high sensitivity, and low power requirements [6,7]. Ultrasensitive detection remains a challenge, however, mainly due to the low tagging efficiency of reporter molecules. To enhance the sensitivity of protein detection, various nanomaterials, including colloidal gold nanoparticles (AuNPs) [8], carbon nanotubes [9], graphene oxide (GO) [10], carbon nanospheres, silica nanoparticles [11,12], and magnetic beads [13] have proven to be excellent carriers for the preparation of labels via the loading of numerous signal tags such as quantum dots [14] and electroactive species [15] on these carriers. Some protein has been reported as carrier for loading molecular recognition compounds and signal compounds, such as Ru(bpy)(2)(dcbpy)NHS, Ferrocenes and digoxin hapten [16,17]. Such prepared labels can greatly amplify the transduction signal of a recognition event in bioassays. In this signal amplification research, one of the most popular strategies is the use of enzyme-functionalized nanoparticles as tracers, which enhances the sensitivity of detection by loading a large amount of enzymes in order to catalyze an individual sandwich immunological reaction. For example, Rusling et al. has achieved greatly enhanced sensitivity using bioconjugates that feature horseradish peroxidase (HRP) labels and signal antibodies linked to CNTs for immunodetection [9]. Mani et al. achieved highly amplified detection using multilabel bioconjugates for signal development by linking multiple horseradish peroxidase (HRP) and antibodies (Ab2) to carboxylated magnetic beads [13]. Wu et al. has presented a novel strategy for the sensitive detection of biomarkers using horseradish peroxidase (HRP)-functionalized silica nanoparticles as a label for the detection of α-fetoprotein [11]. Our group has developed a sensitive aptasensor for thrombin detection based on AuNPs/HRP labeling [18]. Nanostructured materials exhibit outstanding properties, such as a large surface-to-volume ratio, a high pore density, a high surface reaction activity and strong adsorption capability, which make them very promising materials for enzyme immobilization. The large surface area of nanomaterials is likely to provide a superior matrix for immobilizing enzymes, leading to an increased amount of enzyme loading per unit mass of particles. The available multi-binding sites for the attachment of enzyme molecules to nanomaterial surface reduce the degree of enzyme unfolding and result in enhanced stability of the enzyme. However, these approaches for the immobilization of enzymes on such nanostructured materials have disadvantages, because some of these nanomaterials exhibit low biocompatibility. Metallic nanoparticles, such as the nanometer gold and silver, the frequently used materials to immobilize enzyme, are able to denature enzyme and proteins. While carbon nanomaterials or silica nanoparticles are used to immobilize enzyme, e.g., carbon nanotubes and graphene, surface modification with hydrophilic materials and functionalization are needed which make the attachment of enzymes on such supports more complicated and time consuming. Furthermore, the attachment of enzymes in a heterogeneous environment makes them more prone to deactivate due to limited flexibility. In immunoassays, enzyme–nanoparticle conjugates used as a label might exhibit non-specific adsorption, which restricts their application to the improvement of sensitivity and signal amplification.

Among potentially feasible bionanomaterials, the iron storage protein ferritin has attracted great interest not only because of its nano scale, but also because of its high stability and special structure, which is a natural bionanomaterial with excellent biocompatibility. Apoferritin is composed of only a protein shell of ferrition, it has a hollow cage-like spherical shell structure with an outer diameter of 12 nm and an inner cavity diameter of 8 nm [19]. The protein cage of apoferritin can be disassociated into 24 subunits at a low pH (2.0), and the subunits can be reconstituted in a high pH (8.5) environment [20]. Because of its unique cavity structure, apoferritin has been used widely as a protein cage in the synthesis of size-restricted bioinorganic nanocomposites [21]. Moreover, the surface of the uniform size of individual protein shells with spherical structure, and excellent bio-compatibility may provide an excellent environment for enzyme immobilization. To our knowledge, however, there has been little research that has explored the use of this protein as a enzyme carrier for preparation of detection probe.

In this paper, we report an ultrasensitive electrochemical aptasensor for thrombin that is based upon enzyme-functionalized protein nanoparticles for signal amplification. As shown in Fig. 1, apoferritin was employed as the carrier for enzyme and aptamer coimmobilization. Greatly enhanced sensitivity is achieved by using HRP and amino-labeled aptamer linked to apoferritin via glutaraldehyde (GA) at a high HRP/aptamer ratio. Thrombin was employed as a model protein and the two aptamers (Apt1, 15 base sequence, and Apt2, 29 base sequence) [22] were used to recognize the different parts of thrombin in order to form a sandwich structure. For aptasensor fabrication, Apt1 was immobilized on magnetic nanoparticles as a capture probe. Upon the completion of sandwich reactions, the HRP-Aapt-apoferritin conjugate was captured onto the magnetic nanoparticles, and an AuMNPs–Apt1/thrombin/HRP–Apt–apoferritin sandwich-format system was fabricated. After magnetic separation, the resulting sandwich conjugate was immobilized onto a screen-printed carbon electrode (SPCE) surface via an external magnet. Highly specific and sensitive detection of thrombin was achieved by the electrochemical catalytic signals of HRP (as illustrated in Fig. 1B). The results demonstrated that the aptasensor based on this amplification strategy has a wide linear range and a low detection limit for thrombin. It thus shows great promise for application in biomedical research and clinical diagnosis.

2. Experimental

2.1. Apparatus and reagents

Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and differential pulse voltammetry (DPV) measurements were carried out on a CHI660A electrochemical workstation (CH Instruments Inc., USA). A disposable CNT/SPE (Dropensens, Spain) was employed as the working electrode, and Ag/AgCl and a carbon ring served as the reference and counter electrodes, respectively. A sensor connector was used to connect the CNT/SPE to the electrochemical analyzer. Transmission electron microscopy (TEM) images were collected on a JEOL-1230 transmission electronic microscope (JEOL, Japan). The UV–vis spectra were recorded on a UV-2450 spectrophotometer (Shimazu Co., Japan).

The oligonucleotides used in this study had the following sequences: thiolated 15-mer Apt1, 5′SH-$(CH_2)_6$-GGTTGGTGTGGTTGG; thiolated 29-mer Apt2 with polyT(20) tail, 5′NH_2-$(CH_2)_6$-TTTTTTTTTTTTTTTTTTTTAGTCCGTGGTAGG-GCAGGTTGGGGTGACT: were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Apoferritin, human α-thrombin, bovine serum albumin (BSA), human serum albumin (HSA), lysozyme, IgG, fibrinogen, HRP, and 6-mercaptohexanol (MCH) were purchased from Sigma (Milan, Italy). Hydroquinone (HQ) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from the Shanghai Chemical Reagent Co. All other chemicals were of analytical grade. Tris–HCl buffer (pH 7.4, 50 mM Tris, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$) was used as washing and binding buffer. The detection buffer was PBS buffer (pH 7.0, 0.1 M KH_2PO_4–K_2HPO_4, 0.1 M KCl). Milli-Q ultrapure water (Millipore, ≥18 MΩ cm) was used throughout the experiment.

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