Aptamer based electrochemical assay for the determination of thrombin by using the amplification of the nanoparticles

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A B S T R A C T

A novel electrochemical assay based on the aptamer and the signal of amplification of nanoparticles (NPs) was constructed for the determination of thrombin. Aptamers immobilized on the electrode and Au NPs could be assembled with the target protein to form a sandwich structure in the presence of the latter. Differential pulse voltammetry (DPV) was employed to detect the CdS NPs loaded on the surface of the Au NPs through the linker DNA, which was related to the concentration of the target protein. The assay took advantage of the amplification ability of Au nanoparticles carrying multiplex CdS NPs and the specific affinity of aptamers. Thrombin was detected in this assay in the linear range of $1.0 \times 10^{-15}$ to $1.0 \times 10^{-12}$ M with the detection limit of $5.5 \times 10^{-16}$ M of target protein. In addition, the assay could be used to detection thrombin in real samples with high sensitivity and good selectivity.

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1. Introduction

Detecting specific proteins in biological samples is one of the foundations in biomedical research. Thrombin, an important physiological protease found in the blood, is composed of two polypeptide strands through crosslink by disulfide bonds. It plays an essential role in several physiological and pathological processes, such as blood coagulation, incrustation and inflammation. Therefore, recognition and quantification of thrombin are crucial in fundamental research as well as in clinical practice (Stubbs and Bode, 1993; Centi et al., 2007). While antibody-based immunoassay systems are versatile and powerful tools for various molecular analyses in research and medical diagnostics, they are not well so adapted to rapid, high throughput parallel protein detection.

Aptamers are single-stranded nucleic acids which bind highly specifically to a variety of targets ranging from small molecules (Huizenga and Szostak, 1995; Wang et al., 2005; Sankaran et al., 2006), proteins (Bock et al., 1992) to cells (Shangguan et al., 2006; Colin et al., 2008), and thus are potential alternatives for well established antibody-based assay (Gold et al., 1995; Brody and Gold, 2000; Wilson and Szostak, 1999; Jayasena, 1999).

So far various aptameric sensors based on electrochemistry (Polsky et al., 2006; Xiao et al., 2005; Radi et al., 2005; Li et al., 2008; Pavlov et al., 2004; Pavlov et al., 2005), optics (Heyduk and Heyduk, 2005), fluorescence (Xiao et al., 2007) electrochemiluminescence, and other assays have been developed for thrombin detection. King’s group has reported a protein detection method with a highly sensitive using the intrinsic compatibility of aptamers (Di Giusto et al., 2005). The circular DNA architecture facilitated the integration of multiple functional elements into a single molecule: aptameric target recognition, nucleic acid hybridization specificity and rolling circle amplification. Successful exploitation of these properties was demonstrated for the molecular analysis of thrombin, delivering a detection limit nearly three orders of magnitude below the dissociation constants of the two aptamer–thrombin interactions (Di Giusto et al., 2005). He et al. constructed a novel label-free bioelectrochemical assay based on the amplification of aptamer-nanoparticle bio bar codes for thrombin determination, which was realized through the highly sensitive redox activity of adenine (A) nucleobases on a pyrolytic graphite (PG) electrode. Because the nanoparticle carries a large number of aptamers per thrombin binding event, there was substantial amplification and thrombin can be detected at a very low level of detection (0.1 ng/mL) (He et al., 2007). Although a lot of aptameric sensors for the detection of thrombin have been reported, the detection limits were not as low as respected. Alternative simple and sensitive aptameric sensors are thus highly desirable. The combination of the advantages of electrochemical assay with the specificity of the aptamers has enabled the investigations on electrochemical aptasensors, which are both theoretically interesting and practically useful (Hansen et al., 2006;...
In the present work, a novel electrochemical protocol based on aptamer and the amplification of nanoparticles has been constructed for protein determination, which was realized by the indirect quantification of the dissolved cadmium by differential pulse voltammetry (DPV). The CdS-modified on the surface of Au NPs through linker DNA served not only as a tool of amplification but also as a target recognition element. Because one AuNP could be modified with hundreds of CdS NPs as described in our former work (Ding et al., 2009a,b), the sensitivity of the method was improved dramatically. The indirectly detection process of Cd2+ performed by anodic stripping voltammetry (ASV) technology further increased the performance of the design. Besides the high sensitivity, the selectivity of the strategy was excellent due to the specific affinity of the aptamer. With thrombin as a model, the electrochemical signals was proportional to the concentration of target protein in the range of $1.0 \times 10^{-15}$ to $1.0 \times 10^{-11}$ M. A detection limit of $5.5 \times 10^{-16}$ M of target protein was achieved.

2. Experimental

2.1. Reagents

Oligonucleotides were synthesized by SBS-bio Genetech Co. Ltd. (China). Their base sequences were as follows:

Aptamer: 5′-SH-GGT TGG TGT GGT TGG-3′

Linker DNA sequence: 5′-SH-ATG GAG ATG ATG CTC ANH2-3′

Purified thrombin (10 μg/mL, lyophilized powder) was purchased from dingsguo biological Technology Corporation (Beijing, China). Mercaptoacetic acid (MAA) was obtained from Yuhang Chemical Technology Company (China), 6-Mercapto-1-hexanol (MCH) was obtained from Fluka (USA). Tri(2-carboxyethyl)phosphine (TCEP), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), hydrogen tetrachloroaurate(III) trihydrate (HAuCl4·3H2O), imidazole, bovine hemoglobin (BHb), lysozyme and bovine plasma albumin (BSA) were purchased from Sigma (USA). All the reagents were of analytical grade and used without further purification. 0.1 M PBS buffer (pH 7.4), 0.1 M Tris–HCl buffer (pH 6.8) and 0.1 M phosphate buffer containing 0.1% SDS (pH 7.0) were prepared according to standard procedures. Deionized and autoclaved water was employed to prepare all solutions.

2.2. Apparatus

All electrochemical measurements were performed on CHI 660C electrochemical working station (CH Instrument Company, USA). The three-electrode system consisted of a glassy carbon electrode (GCE, Ø4 mm, used for DPV measurements) as working electrode, a platinum wire as an auxiliary electrode, and Ag/AgCl electrode as reference electrode. The Au electrode (Ø = 2 mm), was used for ssDNA immobilization, the CV and the EIS measurements. UV–vis absorption spectra were carried out on a Cary 50 UV–Vis–NIR spectrophotometer (Varian Co., Australia). TEM was recorded with a JEM-2000EX transmission electronmicroscope (JEOL Ltd., Japan).

2.3. Preparation of Au NPs

Au NPs were prepared according to a previously reported method with a slight modification (Grabar et al., 1996). Solutions of HAuCl4 and trisodium citrate were filtered through a 0.22 μm microporous membrane filter prior to use. 1.0 mL of 1% trisodium citrate was added to 100 mL of boiling 0.01% HAuCl4 solution and stirred for 10 min at the boiling point. The freshly prepared nanogold electrode was incubated for about 16 h to produce an aptamer attached electrode in 1 mL 0.1 M PBS buffer containing 1.0 μM aptamer. Then the modified electrode

2.4. Preparation of water-soluble CdS NPs

MAA-modified CdS NPs were prepared according to the literature (Milica et al., 1990). Briefly, 2 μL MAA (99%) was added to 100 mL of 1 mM CdCl2 solution under vigorous stirring, and the pH of the mixture was adjusted to ~11 with 0.5 M NaOH solution. After bubbled with N2 for 30 min, 50 mL of 1.34 mM Na2S solution was added dropwise to the solution. The reaction was carried out for 24 h under N2 atmosphere, and a yellow colloid was formed gradually. The mixture was centrifuged for at least 30 min at 15,000 rpm to remove the supernatant, and the precipitate was washed and redispersed in 30 mL of water and stored at 4 °C. The MAA-modified CdS NPs possessed an average diameter of about 10 nm according to TEM as shown in Figure S1B.

2.5. Preparation of ssDNA-labeled CdS nanoparticle

Linker DNA sequences were covalently linked to MAA-modified CdS nanoparticles using the following procedure. 2.0 mL CdS colloid was centrifuged for 30 min at 10,000 rpm, and the precipitate was redispersed in 500 μL water. 100 μL of 0.1 M imidazole solution (pH 6.8) was added to 600 μL 1.0 × 10−5 M linker DNA sequences for 30 min and the mixture was stand for 30 min (Xu et al., 2004). Then 50 μL 0.1 M EDC solution and 500 μL of CdS colloid (pH 4.0) were added. The mixture was stirred for 12 h at room temperature to allow the labeling reaction to proceed, and then centrifuged at 10,000 rpm for 30 min at 4 °C to remove unbound oligonucleotides. The CdS–ssDNA precipitate was washed twice with PBS. Finally, the obtained nano-CdS–ssDNA conjugate was resuspended in PBS and stored at 4 °C before use.

2.6. Preparation of the Au NP probe

The Au NP probe was prepared following an established procedure (Nam et al., 2004; Storhoff et al., 1998). First, the 25 nm Au particles were added to a solution containing the aptamer and CdS–ssDNA (alkyl-thiol capped oligonucleotides should be activated with 10 mM TCEP before use) at appropriate ratio. After shaking gently for 16 h, the solution was allowed to stand for another 40 h, followed by centrifugation for at least 30 min at 10,000 rpm to remove excess reagents. Following removal of the supernatant, the precipitate was washed with PBS, recentrifuged, and redispersed in 4 mL PBS and stored at 4 °C.

2.7. Fabrication of the sandwich complex

The process for the fabrication of the sandwich complex was shown in Scheme 1. The surface of the gold electrode was pretreated step by step in the following order: heated in a piranha solution (30% H2O2:concentrated H2SO4 3:7) for about 5 min to remove any previous organic layer, polished with alumina slurries (1, 0.3, 0.05 μm in turn) to obtain amirror-like surface, washed ultrasonically with deionized and doubly distilled water, dried with nitrogen gas and cyclcd in 0.5 M H2SO4 aqueous solution scanning between 0.3 and 1.5 V until a stable gold oxide formation/reduction cyclic voltammogram was obtained. The electrochemical deposition of Au nanoparticle was carried out in the HAuCl4 solution containing 0.1 M KNO3 as electrolyte at −400 mV by single potential mode. The freshly prepared nanogold electrode was incubated for about 16 h to produce an aptamer attached electrode in 1 mL 0.1 M PBS buffer containing 1.0 μM aptamer. Then the modified electrode
was immersed in 1.0 mM MCH for 1 h to block the uncovered gold surface.

The fabrication of sandwich complex for detection consisted of two steps. First, the aptamer-modified electrode was interacted with different concentration of thrombin in 0.1 M PBS for protein–aptamer interaction at room temperature. After 90 min, the modified electrode was taken out and immersed into 0.1 M PBS buffer containing Au NPs probe for 150 min at room temperature. The electrode surface was rinsed with 0.1 M phosphate buffer containing 0.1% SDS (pH 7.0) after each step to remove nonspecifically adsorbed species.

2.8. Dissolution of CdS nanoparticles and electrochemical detection

The resulting sandwich complex was treated with 200 μL of 1.0 M HNO₃ solution for 5 min to dissolve the CdS NPs. Then 1.8 mL of 0.1 M HAC–NaAC buffer (pH 5.3, containing 200 μL of 0.1 g.L⁻¹ Hg²⁺) was added. Control experiments were performed in a similar fashion but without adding the thrombin.

The DPV measurements of the dissolved Cd²⁺ were performed (in a stirring HAC–NaAC buffer solution) using an in situ prepared mercury film on a glassy carbon electrode with a deposition time of 300 s and deposition potential of −1.1 V. The positive DPV scan was performed after a 15 s rest period (without stirring), with a pulse amplitude of 50 mV and a pulse width of 50 ms. An anodic stripping peak current (i_p,a) at ca. −0.67 V was taken as the analytical response.

3. Results and discussion

3.1. The fabrication of the sandwich complex and the detection process

Scheme 1 showed the procedure of the amplified sensing of target protein. Aptamers immobilized on the nanogold electrode and the surface of Au NPs was used to construct the sandwich-type detection strategy. Since a single Au NP could be loaded with many CdS NPs labeled linker DNA, a significant amplification for the detection of thrombin was obtained. The concentration of thrombin was monitored based upon the concentration of dissolved Cd²⁺ formed in the dissolution of CdS by acid treatment and quantified by DPV.

3.2. UV–vis spectra of the DNA-NPs conjugate

The UV–vis spectrum of the CdS colloid, Au colloid, linker DNA, linker DNA-labeled CdS nanoparticle and the nanocomplex were shown in Fig. 1. Compared with the CdS colloid (curve d), a new band at about 260 nm appeared, which was attributed to the absorbance of DNA labeled on the CdS NPs (curve c). It was similar to curve a of linker DNA with a certain shift to the section of long wavelength. Curve e exhibited both the characteristic of DNA (curve a) and the characteristic absorbance of Au colloid (curve b) at about 520 nm. The results indicated that the CdS and Au NPs had been successfully labeled on the linker DNA.
3.3. Optimization of the electrochemical detection conditions

To improve the sensitivity of electrochemical quantification of thrombin, the incubation time of the aptamers immobilized on the electrode was investigated as shown in Figure S2A. The response current increased at first and reached an equilibration step after 90 min. Thus, the incubation time of 90 min was adopted for the subsequent assays.

The pH of the buffer solution in which the thrombin combine with aptamers was another influence condition for the assay. The electrochemical signals were investigated against the pH in the range of 6.0–9.0 as shown in Figure S2B. The current response reached the maximum at pH 7.5. Thus, the pH 7.5 was used as a optimal condition.

3.4. Electrochemical characterization of the sandwich complex fabrication

The fabrication process of the sandwich complex was characterized by EIS as shown in Figure S3A. Curve a showed the EIS of the nanogold electrode. An almost straight line was exhibited, indicating its property of conductivity was better than the bare electrode (curve b). After the electrode was assembled with aptamer (curve c) and treated with MCH (curve d) in turn, the EIS of the assembled monolayer showed a large interfacial electron-transfer resistance (R_set). After incubation with thrombin, the formation of the aptamer–thrombin complex on the electrode surface induced a larger R_set (curve e). It is consistent with the fact that the layer of the protein molecules perturbed the interfacial electron transfer between the electrode and the electroactive species in solution and thus being insulated (Wang et al., 2007). After the further recognition of target thrombin by Au NPs probe (curve f), the R_set became much larger. As described above, aptamer, Au and CdS functionalized aptamer poised difficult in the electron transfer. Similarly, CV analysis was used to provide further evidence for interface assembly on the electrode. As seen from Figure S3B, stepwise modification on the electrode was accompanied by a decrease in the amperometric response and an increase in the peak-to-peak separation between the cathodic and anodic waves of the redox probe, showing that the electron-transfer kinetics of Fe(CN)_6^{3−}/4− is obstructed. These results were consistent with the fact that the electrode was fabricated as expected.

3.5. The detection of thrombin based on DPV

Under the optimal conditions, the current increased with the increasing thrombin concentration. As shown in Fig. 2, the dose–response curve for thrombin showed a liner range from 1.0 to 20 fM with a correlation coefficient of 0.9973. The regression equation was I (µA) = 0.73848 + 0.17551 C (fM). The detection limit of 0.55 fM of thrombin could be estimated using 3σ rule (where σ is the relative standard deviation of a blank solution, n = 11). A series of eleven duplicate measurements of 2.0 fM gave a relative standard deviation (RSD) of 6.7%. The sensitivity was significantly improved by the nanogold immobilized on the surface of the Au electrode as shown in the Figure S4.

3.6. Selectivity of the sandwich complex

The selectivity of current assay was also studied as shown in Fig. 3. The peak current with the possible interfering substances of 1 µM BSA (b), 1 µM BHb (c) and 1 µM lysozyme (d) were almost the same with the blank solution (a). While the peak current in the presence of 1.0 pM thrombin (e) was much higher than b, c and d. When 1 µM BSA, 1 µM BHb and 1 µM lysozyme were coexisted in 1.0 pM thrombin, the signal did not changed much (f) compared with e.

Fig. 2. Dose–responses (A) and calibration curves (B) for the electrochemical detection of thrombin (insert was amplifications of the dots 1.0–20 fM). The concentrations of thrombin (a–j): 0, 0.001, 0.002, 0.004, 0.006, 0.01, 0.02, 0.1, 1.0, and 10 pM.

The result demonstrated that the current assay could response to target protein specifically.

3.7. Detection of thrombin in clinical serum samples

The clinical serum samples were pretreated with salt solution (2 M ammonium sulfate and 0.1 M NaCl) to avoid the formation...
of fibrin and the rapid sample clotting according to the previous reports (Bini et al., 2007; Centi et al., 2007). The blood and stop solution mixture was then immediately centrifuged for 5 min at 10,000 rpm at 4 °C. The serum was removed and frozen at −70 °C until used. It has been reported that the physiological concentrations of thrombin in resting and activated blood range from the grade of nanomole to micromole (Becker and Spencer, 1998). The concentrations of thrombin in three serum samples obtained using the proposed method are shown in Table 1, which were compared with the results obtained by the other electrochemical method (Zhang et al., 2008). The results indicated that the presented method is in good agreement with Zhang’s method. The thrombin concentration in some samples was beyond the kinetic range of the proposed method, thus appropriate dilution before assay was necessary.

3.8. Reproducibility and stability of the sandwich complex

The reproducibility of the sandwich complex was evaluated by assaying the peak current at three prepared electrode for three replicate measurements. The relative standard deviation of this method was 9.7%, showing an acceptable reproducibility.

When the aptamer-modified electrode was stored in pH 7.4 PBS at 4 °C, it retained 96% of its initial current after 7 days storage. 92% of its initial current retained when stored 15 days, and 87% retained after 30 days. The Au NP probe was also stored in pH 7.4 PBS at 4 °C. Be stored 7 days, it retained 97% of its initial current. After 15 days and a month, 82% and 64% retained, respectively. This indicated that the aptamer assembled on the electrode was very toughly and the Au NP probe has a good stability.

4. Conclusions

In this work, an electrochemical aptasensor for the detection of thrombin was constructed. This assay could directly detect thrombin with a low detection limit of 0.55 fM. High sensitivity was achieved due to the amplification of nanoparticles and the usage of DPV for the detection of dissolved Cd^{2+} in the solution. Thrombin was detected in this assay in the linear range of 1.0 × 10^{-15} to 1.0 × 10^{-11} M. This assay was also proved to be able to distinguish the target protein from the interferents, and can be used in the detection of specific protein species in real samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.10.017.

References


<table>
<thead>
<tr>
<th>Sample</th>
<th>Proposed method (nM)</th>
<th>Zhang’s method (nM)</th>
<th>Relative deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.3 ± 0.1</td>
<td>6.5 ± 0.1</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>10 ± 0.2</td>
<td>11.1 ± 0.3</td>
<td>9.9</td>
</tr>
<tr>
<td>3</td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>3.8</td>
</tr>
</tbody>
</table>

When the thrombin concentration was more than 0.1 nM, an appropriate dilution of sample was needed in the pre-incubation step. *Each value is the average of three measurements.*