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Gold nanoparticles conjugates-amplified aptamer immunosensing screen-printed carbon electrode strips for thrombin detection

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ABSTRACT

Thrombin plays the role in cardiovascular diseases and regulates many processes in inflammation and could be a feature of many pathological conditions, including the thromboembolic disease, cancer and neurodegenerative diseases. An ultrasensitive and amplified electrochemical sandwich assay using screen-printed carbon electrode (SPCE) strips for thrombin detection was established in this study. The conductivity and sensing performance of the carbon electrodes were enhanced by using gold nanoparticles (AuNPs). The aptamer addressed on the strips was used as a primary probe to capture thrombin in the detected samples. An amplifier was invented for recognizing thrombin captured on the SPCE, which is the multiple molecules of anti-thrombin antibody (Ab) and horseradish peroxidase (HRP) co-modified AuNPs (AuNPs/Ab–HRP). Hydrogen peroxide was used as the substrate for HRP and then the response current (RC) could be detected. The optimization of these AuNPs conjugates-amplified aptamer immunosensing SPCE strips was conducted for thrombin detection. The detection sensitivity showed a linear relation between RC and thrombin concentration in the range of 10 pM–100 nM, and limit of detection (LOD) was 1.5 pM. The fabricated AuNPs/Ab–HRP-amplified aptamer immunosensing SPCE strips were further used to detect thrombin in human serum with a linear range of 100 pM–100 nM. This study provided the promising SPCE strips with highly sensitive and rapid detection for thrombin by the electrochemical aptasensor combined with AuNPs conjugates for amplifying the detection signal.

1. Introduction

Thrombin plays a central role in blood coagulation and in various proinflammatory activities (Esmon, 2008; Strukova, 2001). Thrombin is shown to promote thrombosis, angiogenesis and inflammation and link the development of a variety of diseases (Kitamoto et al., 2008; Nierodzik and Karpatkin, 2006; Yin et al., 2010). It is essential to develop sensors to monitor thrombin in blood with high sensitivity, selectivity and simplicity for clinical practice and diagnostic applications.

While evaluating clinical hemorrhagic risk or thrombosis, the formation of a fibrin clot occurs at 10–30 nM thrombin or approximately 3% of the total amount of thrombin produced in the generation of a fibrin clot (Mann et al., 2003). Therefore, a direct assay for the detection of the presence of thrombin in blood could have an important diagnostic value for diseases associated with coagulation abnormalities. Colorimetric (Chen et al., 2010) and amperometric (Escosura-Muñiz et al., 2013; Thuerlemann et al., 2009) biosensing platforms using antibodies for the detection of thrombin in blood have already been reported.

In recent years, many biosensors based on the thrombin-binding aptamer have been developed for thrombin detection, such as the colorimetric aptasensing model (Oroval et al., 2013; Razquin et al., 2012) and electrochemical aptasensing model (Kang et al., 2008; Li et al., 2010; Zhang et al., 2014). Aptamer can immobilize various types of solid surface (beads, silica particles, glass, platinum, gold, etc.) for capturing target molecules. The thrombin-binding aptamer (15-mer, 5′-GGT TGG TGT GGT TGG-3′) of its G-quartet structure (Macaya et al., 1993; Padmanabhan et al., 1993) and the binding site to thrombin fibrinogen-recognition exposits have been identified subsequently (Paborsky et al., 1993; Tang et al., 2007).

In order to improve the sensitivity for thrombin detection, the enhanced detection signals via the use of nanoparticles have been reported (Ding et al., 2010; Oaew et al., 2012; Zheng et al., 2013). In comparison to enzyme-linked immunosorbent assay (ELISA), simple biosensing platforms being able to rapidly detect thrombin in human blood at pM levels have been of considerable interest. A few biosensing platforms have been used as an analytical method for the detection of thrombin in real samples (Escosura-Muñiz et al., 2013). Gold nanoparticles (AuNPs) are efficient nanomaterial for biomolecules immobilization and have been widely used for

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fabricating biosensing platforms (Zhang et al., 2002; Zhao et al., 2011). Therefore, we intended to compose AuNPs conjugates as detection signal amplifier, since it plays an essential role in biosensing platforms.

For the combination of AuNPs conjugates as detection signal amplifier, the idea of sandwich-type electrochemical and immune-aptasensor was brought in and performed in this study. An ultrasensitive and amplified electrochemical sandwich assay by using screen-printed carbon electrode (SPCE) strips for thrombin detection was proposed. In this case, AuNPs were used to modify the electrode and were also used as labels and bridges between the anti-thrombin antibody and horseradish peroxidase (HRP). HRP has also been used as a label for signal amplification and catalyzing the oxidation of o-phenylenediamine by H_2O_2 (Hu et al., 2014; Nam et al., 2004; Zhang et al., 2004). Hence, an antibody–AuNPs–HRP conjugate (AuNPs/Ab–HRP) was prepared and applied to fabricate the AuNPs conjugates-amplified aptamer immunosensing SPCE strips (also named AuNPs-amplified aptasensing strips in this study) that provided an efficiently alternative tool for clinical thrombin monitoring.

2. Materials and methods

2.1. Apparatus

Cyclic voltammetric and amperometry were performed with a CV50W voltammetric analyzer (Bioanalytical Systems, West Lafayette, IN, USA) connected to a personal computer and a PalmSens/Personal Digital Assistant, hand-held, battery-powered instrument (Palm Instruments, BZ Houten, Netherlands) for data collection and calculation. The absorption spectrum of AuNPs and absorbance values of ELISA test were recorded by a UV spectrophotometer (Molecular Devices, Sunnydale, CA, USA). Investigation of modified-AuNPs was performed with a NanoWizard II AFM (JPK instruments, Berlin, Germany).

2.2. Materials

G-451 carbon/graphite ink and silver-resin inks were purchased from ERCON (Wareham, MA, USA). Sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) and hydrogen peroxide (H_2O_2) were obtained from Merck (Darmstadt, Germany). Ferrocenedicarboxylic acid (FeDC), hydrogen tetrachloroaurate(III) ($HAuCl_4 \cdot 3H_2O$, 99.9%), ammonium hydroxide (NH_4OH), glutaraldehyde, 3,3′,5,5′-tetramethylbenzidine (TMB) enzyme substrate, human α-thrombin, bovine serum albumin (BSA), streptavidin, HRP, Triton X-100, PD-10 desalting column and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-thrombin antibodies were purchased from Abcam (Cambridge, UK). The buffer and solutions used in this study were prepared as follows: PBS buffer (PBS, pH 7.4), PBST (PBS containing 0.1% Tween 20), blocking buffer (150 μM BSA in PBST, pH 7.4), substrate solution [0.3% (w/w) H_2O_2 in PBS buffer, pH 7.4], FeDC solution (5 mM FeDC in PBS, containing 3% DMSO). Agarose and 10 × Tris–Borate–EDTA buffer (TBE) were purchased from Amresco (Cleveland, OH, USA); 40% acrylamide/Bis solution (37.5: 1) was purchased from Bio-Rad (Hercules, CA, USA); Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm; Millipore, MA, USA).

2.3. Aptamers

A thrombin-binding aptamer sequence (biotinylated 15-mer aptamer with polyT tail; 5′-biotin-T TTT TTT TTT TTT TTT TTT TTT GGT **TGG** TGT **G**GT **TG**G-3′) and non-specific aptamer with the scrambled sequence (biotinylated 5′-biotin-T TTT TTT TTT TTT TTT TTT TTT GGT **GG**T TGT **T**GT **GG**T-3′) were synthesized by Applied Biosystems (Foster, CA, USA). The thrombin-binding aptamer has a higher affinity reported to form an intermolecular quadruplex structure and recognize thrombin at anion-binding exosite I (Tang et al., 2007).

2.4. Synthesis of AuNPs conjugates

2.4.1. Synthesis of AuNPs

13 nm AuNPs were prepared by citrate reduction of $HAuCl_4 \cdot 3H_2O$ (Chuang et al., 2010; Lin et al., 2008). A 50 mL aqueous solution consisting of 1 mM $HAuCl_4 \cdot 3H_2O$ was brought to a vigorous boil with stirring in a conical flask, and then 38.8 mM sodium citrate (5 mL) was added to the solution rapidly. The solution was boiled with stirring and the color changed from the yellow to wine red. The solution was cooled to room temperature with continuous stirring, of which the colloidal AuNPs with an average diameter of 13 nm were produced and stored at 4 °C.

2.4.2. Synthesis of AuNPs/Ab–HRP

The approach used to conjugate AuNPs with antibody and HRP was based on previous methods (Pissuwan et al., 2007; Wu et al., 2009). The synthesis of AuNPs/Ab–HRP was according to the following procedures: 50 μL of HRP (500 U mL^{-1}), 1 μL of anti-thrombin antibody (Ab; 1 mg mL^{-1}) and 49 μL of PBS were mixed with 900 μL AuNPs (13 nm) to compose a mixture. After gently mixing, the mixture was incubated for 1 h at 4 °C and then it was centrifuged for 6 min at 10,000 × *g* at 4 °C to remove the excess HRP and antibody in the supernatant. In total, two centrifuge/wash cycles were conducted and the sediment of AuNPs/Ab–HRP was resuspended in 1 mL of PBST with 1% BSA for 12 h and stored at 4 °C.

2.5. Procedure of fabricating and using the AuNPs-amplified aptasensing strips

Fabrication of AuNPs/FeDC–SPCE strip was according to the protocol reported by Lin et al. (2008) with slight modifications and the strips were further applied to compose the antibody for specific detecting thrombin. The procedure of fabricating and using AuNPs-amplified aptasensing strips is shown in Fig. 1 and the working electrode was washed twice with PBST in between steps. Detailed fabrication procedures are described in Supplementary material (S1). The general procedure of thrombin detection was briefly described as follows: a volume of 10 μL of the sample containing thrombin was dropped onto the electrode of AuNPs/FeDC–SPCE aptasensing strip for 1 h at 4 °C and then 10 μL of AuNPs/Ab–HRP was applied to the thrombin-captured strip for 40 min at 25 °C.

2.5.1. Measurement of cyclic voltammetry

The measurements were performed in 50 μL of substrate solution dropped onto the electrochemical reaction area of the SPCE strip. Cyclic voltammetry was carried out using a signal generator and a potentiostat.

2.5.2. Measurements of amperometry

All amperometric measurements were performed in 50 μL of substrate solution dropped onto the electrochemical reaction area of the SPCE strip and incubated for 40 s at 25 °C. A fixed potential of +300 mV (counter/reference) was applied after the incubation for amperometric detection. The current signal defined in this study: response current (RC) was the sum of current signals collected per 0.1 s during the following 50 s.

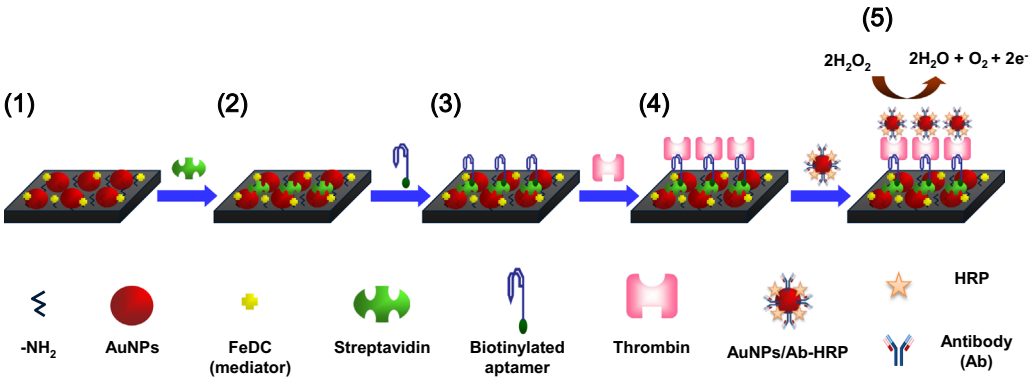

Fig. 1. Schematic illustration of the fabrication of AuNPs-amplified aptasensing strips for thrombin detection. Step (1), the surface of working electrodes of SPCE strip was pretreated with NH_4OH, and the AuNPs as well as FeDC were sequentially modified. Step (2), streptavidin was immobilized on the SPCE surface. Step (3), the aptamer sequence that functionalized with biotin at 5′ terminal was immobilized on the SPCE surface via biotin–streptavidin affinity binding. Step (4), thrombin in sample could be captured by aptamer on the SPCE surface. Step (5), the AuNPs/Ab–HRP was applied which could amplify the detection signal, i.e., response current (RC, μA).

2.6. Procedure of plate ELISA

The plate ELISA procedure was performed according to the indirect sandwich method (Kim et al., 2005) with slight modifications. Each well of a Maxisorb plate (Nunc, IL, USA) was coated with 100 μL glutaraldehyde (0.25%) at 25 °C for 30 min. After washing with PBST, 100 μL sample containing thrombin was added for 1 h at 25 °C, and 300 μL BSA (100 μg mL^{-1}) was added to block for 1 h at 25 °C. A volume of 100 μL of the detecting regent (AuNPs-amplified ELISA: AuNPs/Ab–HRP; classical immune-based ELISA: anti-thrombin antibody) was added and incubated for 1 h at 25 °C. For classical immune-based ELISA, another 100 μL of HRP-conjugated polyclonal antibody (1 μg mL^{-1}) prepared in PBS (50 mM, pH 7.4) was added and incubated at 25 °C for 1 h. Both systems completing above procedures were then washed with PBST, and the enzymatic reaction was developed by adding TMB (100 μL) and mixing at 25 °C for 5 min (AuNPs-amplified ELISA) or 20 min (classical immune-based ELISA). The stop solution (100 μL; 0.18 M H_2SO_4) was added and the absorbance at 450 nm was measured by a UV spectrophotometer.

2.7. Thrombin generation assays

To avoid fibrinogen which leads to the formation of fibrin and results in thrombin embedded in fibrin clot, fibrinogen was precipitated before the activation of thrombin in plasma. According to Centi's protocol (Centi et al., 2007), such selective precipitation was based on the use of ammonium sulfate as precipitant: 250 μL of plasma was treated with 1.25 mL of 2 M ammonium sulfate and 1 mL of 0.1 M sodium chloride. The solution was mixed for 5 min and then centrifuged (3000 × *g*, 5 min), and the supernatant was eluted in a PD-10 desalting column for a rapid desalting and buffer exchange. The protein amount of the raw plasma and of the eluted solution was evaluated by spectrophotometric measurements at $\lambda = 280$ nm. A loss of protein content (40%) was detected after precipitation of fibrinogen.

Thrombin is present in the form of its precursor in plasma, which is prothrombin and converts to thrombin via proteolytic processing by factor Xa (FXa). To generate thrombin, 8 nM FXa (in 6 mM $CaCl_2$ and 50 μM phospholipids) was added in pretreated plasma (without fibrinogen). The generation of thrombin was monitored using the AuNPs-amplified aptasensing strips.

2.8. Statistical analysis

All data were reported as the mean ± standard deviation (SD) for the specified number of replicates indicated in the caption. Statistical significance was determined by two-tailed Student's *t*-test with 95% confidence for unpaired observations. A *p* value less than 0.05 was considered to be significant.

3. Results and discussion

3.1. Immobilization of antibody and HRP onto AuNPs

The AuNPs immobilized with anti-thrombin antibody and HRP were prepared and named as AuNPs/Ab–HRP. Different methods, including UV-spectra, gel electrophoresis and AFM, were used to confirm the modification of AuNPs/Ab–HRP. Fig. 2A shows the UV-spectra of AuNPs and AuNPs/Ab–HRP. The 13 nm AuNPs has surface plasmon resonance (SPR) band (λ_{SPR}) at 520 nm. The effect of immobilization causes λ_{SPR} of AuNPs/Ab–HRP redshift from 520 to 525 nm. In addition, the absorption peak around 280 nm is the characteristic of the functional group of amino acid; hence, the peak around 280 nm corresponds to the existence of HRP and antibody. The gel electrophoresis was used to identify the effect of immobilization, according to the results of nanoparticle mobility. Fig. 2B obtains the mobility in the following decreasing order: AuNPs > AuNPs–HRP > AuNPs/Ab–HRP. The mobility of particles is only related to particle size due to the SDS pretreatment of particles (Parak et al., 2003; Zanchet et al., 2001). Fig. 2C and D shows the AFM images of AuNPs and AuNPs/Ab–HRP, respectively. Size analysis showed that the AuNPs were around 11–13 nm, whereas the AuNPs/Ab–HRP was around 17–23 nm.

The optimized composition of AuNPs/Ab–HRP was described in detail in Supplementary material (S2). The optimal condition of AuNPs/Ab–HRP was using above 25 U mL^{-1} HRP and 0.5–2 μg mL^{-1} antibody to compose with 13 nm AuNPs.

3.2. Effects of AuNPs modification on electrochemical characters of SPCE

The electrochemical behavior of the modified SPCE was characterized by cyclic voltammetry and amperometry by using H_2O_2 as the substrate. The effects of AuNPs and FeDC modification on a typical cyclic voltammogram (CV) were evaluated. Fig. 3A shows that bare SPCE (curve “a”) and SPCE pretreated with NH_4OH (curve “b”) exhibited low background current with no cathodic and anodic peaks. The immobilization of AuNPs and mediator FeDC could remarkably increase the peaks of oxidation and reduction current (curves “c” and “d” in Fig. 3A). Results indicate that streptavidin and aptamer immobilized onto the AuNPs/FeDC–SPCE strip only slightly decrease the redox current (curves “e” and “f” in

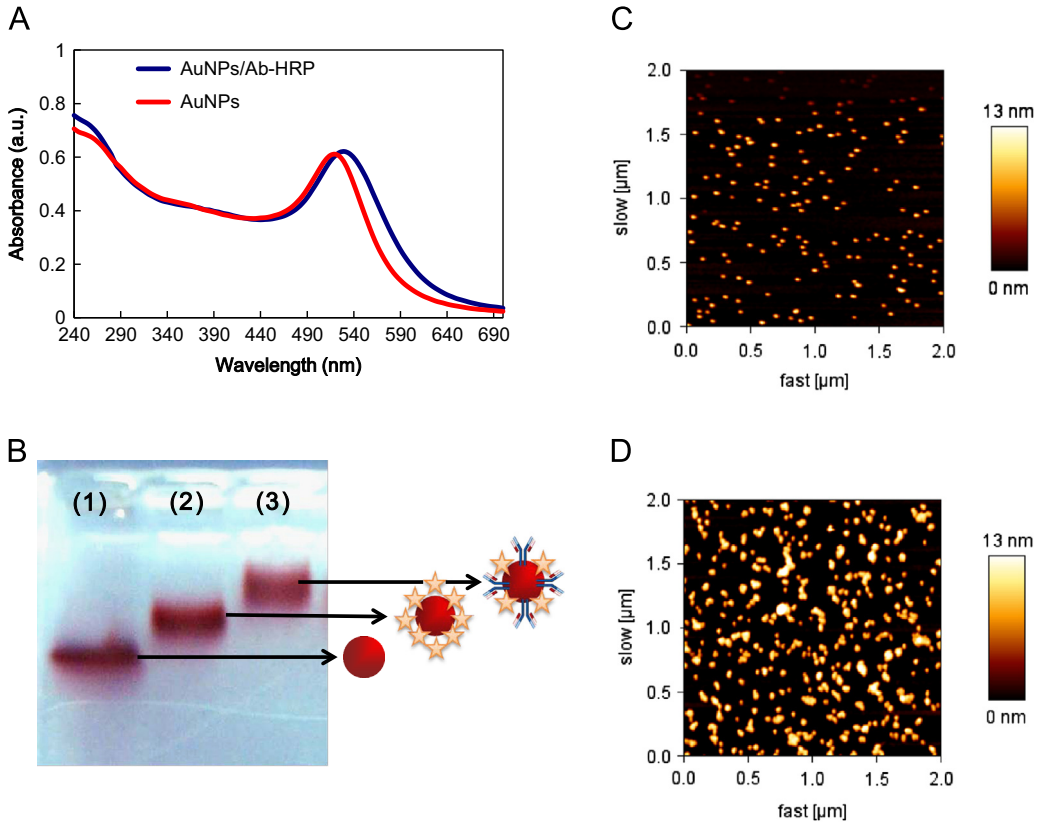

Fig. 2. Characterizations of AuNPs conjugates (AuNPs/Ab–HRP). UV-spectra analysis (A), λ_{max} of 13 nm AuNPs (red curve) is located at 520 nm, whereas λ_{max} of AuNPs/Ab–HRP (blue curve) is red-shifted from 520 to 525 nm, and exhibit absorption peak around 280 nm. Gel electrophoresis analysis (B), lane (1), AuNPs; lane (2), AuNPs–HRP; lane (3), AuNPs/Ab–HRP. AFM images of AuNPs (C) and AFM images of AuNPs/Ab–HRP (D) on the electrodes of SPCE strips. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3B), which mainly attribute to the non-electroactivity of the protein molecules.

The series of modified SPCE strips were used to test by amperometry, and the detected RC was the sum of current signal collected per 0.1 s during the following 50 s after H_2O_2 substrate addition. Fig. 3C and D indicate that SPCE strips modified with AuNPs and FeDC (curves or histograms "d, e and f") are essential and only if the thrombin is recognized by the aptamer, the RC would remarkably increase (curve or histogram "g").

Optimizations of the AuNP-amplified aptasensing strips with respect to different variables, including concentrations of streptavidin and aptamer and the composition of AuNPs/Ab–HRP, were performed and described in detail in Supplementary material (S3). Hence, the optimal condition of AuNPs-amplified aptasensing strips was the AuNPs/FeDC–SPCE strip modified with 5 μg mL^{-1} streptavidin and then modified with 1 μM aptamer. After capturing the thrombin in sample, the AuNPs conjugates (composed with 25 U mL^{-1} HRP and 1 μg mL^{-1} anti-thrombin antibody) were applied to amplify the detection signals.

The application of AuNPs in electrode/conductors has been known (Lin et al., 2008; Merkoçi, 2007; Yi et al., 2000) which is AuNPs not only provide large specific surface area but also process high conductivity. The low electron-transfer rate of redox enzyme was contributed to its redox center usually lacks direct electrical contact with electrodes. The redox center of the enzyme and the electrode acts as a donor–acceptor pair, and thus, metallic nanoparticles may act as nano-electrodes that decrease the steric separation between the redox center of the enzyme and electrodes (Willner et al., 2007). Lin et al. (2008) showed 13 nm AuNPs immobilized on the SPCE improved electrochemical ability. Our results also confirmed that AuNPs and FeDC immobilized on the electrodes of SPCE could increase the peak current remarkably. Therefore, the cooperation of AuNPs and FeDC can facilitate the electron transfer between the electrolyte and electrode surface, and then enhance the RC dramatically.

3.3. *Specificity and sensitivity of AuNPs-amplified aptasensing strips*

In order to confirm the specificity of aptasensing SPCE strips with AuNPs/Ab–HRP amplification, the non-specific aptamer (with scrambled sequence of 5′-biotin-$(T)_{25}$ GGT **GGT** TGT **TGT** **GGT**-3′) was compared to the thrombin-binding aptamer (5′-$(T)_{25}$ GGT **TGG** TGT **GGT** **TGG**-3′) (Tang et al., 2007) by amperometry. Fig. 4 indicates that the non-specific aptamer leads to a little RC change that could be viewed as background current, whereas the thrombin-binding aptamer shows obvious RC change related to the thrombin concentrations. In the amperometry the AuNPs-amplified aptasensing strips showed a linear relation between RC and thrombin concentration in the range of 10^{-11}–10^{-7} M, of which RC (μA) = 6.46 × thrombin (log M) + 97.97 (R^2 = 0.998) and the limit of detection (LOD) was 1.5×10^{-12} M. Threshold for the LOD is defined by the signal-to-noise (S/N) characteristics as S/N ≥ 3 and the 95% confidence intervals (CI) for the thrombin detection with linear range are calculated and illustrated in Supplementary material (S4).

An electrochemical method for the detection of thrombin based on an AuNPs sensing platform and usage of stripping voltammetry technique was developed (Kerman and Tamiya, 2008; Suprun et al., 2008). In these studies, a linear range corresponding from 10^{-8} to 10^{-5} M of thrombin concentration and LOD of 10^{-9} M were reported. Compared to the previous study, it is noted that approximate 100 folds of sensitivity for

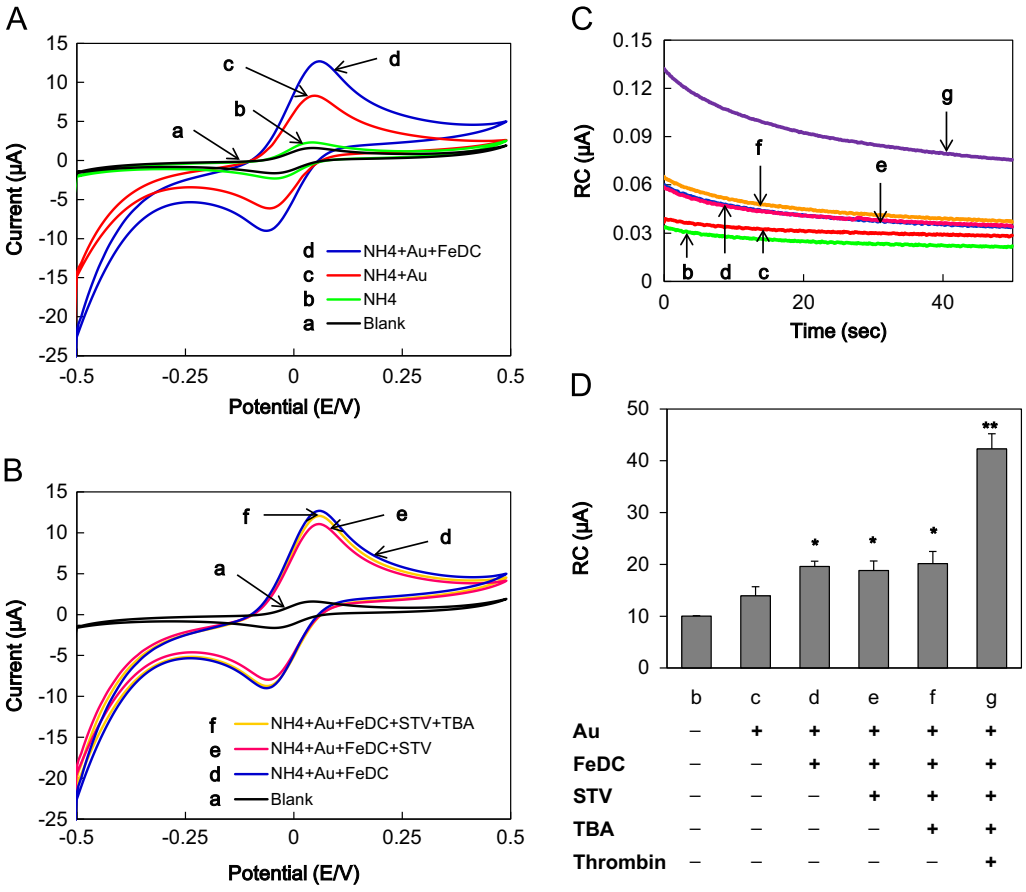

Fig. 3. Characterizations of AuNPs-amplified aptasensing strips. Cyclic voltammetry (A and B) and amperometry measurements (C and D) were performed. In the measurements, 3% H_2O_2 was used as a supporting electrolyte for the SPCE formed under the following conditions: (a) bare SPCE; (b) SPCE/NH_4OH; (c) SPCE/NH_4OH/AuNPs; (d) SPCE/NH_4OH/AuNPs/FeDC (i.e., AuNPs/FeDC–SPCE); (e) streptavidin–AuNPs/FeDC–SPCE; (f) aptamer–streptavidin–AuNPs/FeDC–SPCE (i.e., AuNPs/FeDC–SPCE aptasensing strip); (g) AuNPs/FeDC–SPCE aptasensing strip used to detect thrombin. NH_4 represents NH_4OH; Au represents AuNPs; STV represents streptavidin; TBA represents thrombin binding aptamer. * and ** indicate statistical significance as $p < 0.05$ and < 0.01 compared with that of SPCE/NH_4OH (b), respectively. Each value was derived from five independent detections and error bars mean standard deviation (SD).

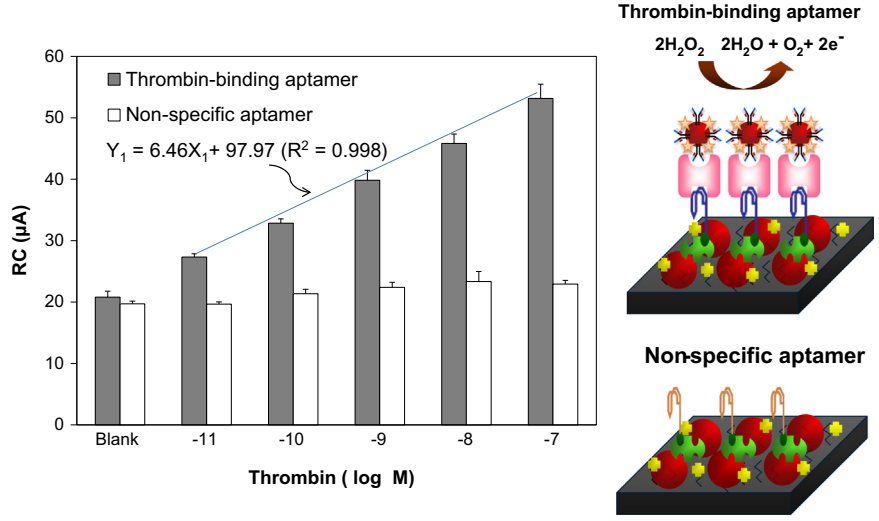

Fig. 4. Specificity and sensitivity of AuNPs-amplified aptasensing strips for thrombin detection. Thrombin-binding aptamer that could recognize thrombin (gray histograms) and a non-specific aptamer that could not recognize thrombin (white histograms) were designed and synthesized. The thrombin-binding aptasensor used for thrombin detection in buffer solution showed a positive linear relationship with thrombin concentration ranging from 1×10^{-11} to 1×10^{-7} M. Each value was derived from five independent detections and error bars mean standard deviation (SD).

thrombin detection using the novel AuNPs conjugates, i.e., AuNPs mounted with multiple detecting molecules, to amplify the detection signal in the electrochemical aptasensing SPCE strips was presented in this study, and also confirmed by the study reported by Escosura-Muñiz et al. (2013). The levels of sensitivity and LOD for thrombin detection demonstrated in the present study are comparable to other aptasensors that needed precision instruments for signal detection, e.g., an impedimetric aptasensor

based on the enlargement of surface-charged AuNPs with linear range of 0.05–35 nM (Deng et al., 2008), an aptasensor based on dye trapped inside mesoporous silica particles with dynamic range of 2–1700 nM (Oroval et al., 2013) and an aptasensor based on porphyrin–graphene modified glassy carbon electrode with linear range of 5–1500 nM (Zhang et al., 2014).

3.4. Comparison of thrombin detection by ELISA using AuNPs-amplified and classical immune-based method

Thrombin was also analyzed and compared using the methods of AuNPs-amplified ELISA developed in this study and classical immune-based ELISA (i.e., using the first anti-thrombin antibody to recognize thrombin and then using the HRP modified secondary antibody to recognize the first anti-thrombin antibody). Fig. 5 demonstrates that the AuNPs/Ab–HRP amplified ELISA exhibits the higher signals, of which the thrombin concentration showed a positive linear relationship with thrombin ranging from 10^{-9} to 10^{-6} M. The immune-based ELISA assay showed much lower absorbance but also acquired a positive linear relationship with thrombin ranging from 10^{-8} to 10^{-6} M. The thrombin detection using AuNPs/Ab–HRP as a detected amplifier showed a pretty high sensitivity and a reliable detected range for thrombin assay compared with those of classical immune-based assay. The sensitivity of thrombin by using AuNPs conjugates is amplified 1 order in ELISA platform (Fig. 5); meanwhile using AuNPs conjugates amplified 2 orders in aptasensing strips platform (Supplementary material Fig. S5). Moreover, the total detection time by using the AuNPs/Ab–HRP as a detecting regent was significantly shortened by more than 1 h compared with the classical immune-based platforms, because only one immune-recognizing step of AuNPs/Ab–HRP applications is necessary. It is noted that the sensitivity of thrombin detection by the AuNPs-amplified aptasensing strips was approximately 2 orders higher than that of the AuNPs-amplified ELISA (Fig. 4 vs. Fig. 5).

On account of the large surface area to volume ratio of AuNPs, a large amount of antibody and HRP could feasibly immobilize onto AuNPs surface to form AuNPs conjugates (Oaew et al., 2012). Once the antibody on AuNPs conjugates recognized the captured thrombin on AuNPs/FeDC–SPCE aptasensing strip, a lot of HRPs would immobilize on AuNPs/FeDC–SPCE aptasensing strip. Hence, the HRP enriched on the electrode surface catalyzed the reduction of H_2O_2, which produced an amplified reduction peak through an electron-transfer reaction (Gan et al., 2011).

The use of signal amplifier in biosensing methods usually achieved lower LOD (Ding et al., 2010; Escosura-Muñiz et al., 2013; Tennico et al., 2010), and our work's LOD for thrombin is 1.5 pM and the linear detection range is 10 pM–100 nM. Our works provide a promising method for sensitive and rapid proteins detection by using disposable electrochemical aptasensing SPCE strips.

3.5. Thrombin detection in human blood samples by using AuNPs-amplified aptasensing strips

Serum has the chemical composition similar to plasma, but without coagulation proteins such as thrombin or other factors (Cho et al., 2008). For the real sample application, serum was used to evaluate the efficiency of the thrombin detecting platform developed in this study. Fig. 6A shows that the AuNPs-amplified aptasensing strips acquired a linear correlation between RC and thrombin spiked in the serum ranging from 10^{-10} to 10^{-7} M, of which RC (μA)$=6.08\times$thrombin (log M)$+84.80$ ($R^2=0.964$). Compared with the thrombin detection performed in buffer shown in Fig. 4, matrix effect was observed that led to the RC decrease. However, the data indicated that the developed AuNPs-amplified aptasensing strips used for thrombin detection of human sample could reach to almost pM level. It is possible and potential for further development of disposable point-of-test strips for thrombin detection based on the experimental results presented herein.

Thrombin is usually not present in serum under normal conditions. A thrombin concentration ranging from 50 pM to 100 nM mediates the neuroprotection against ischemia and environmental insults is detected. High concentration of thrombin causes degeneration and cell death in both astrocyte and hippocampal neuron (Striggow et al., 2000). The generation of thrombin is a key event in hemostasis which is stemmed from the exposure of tissue factors after vascular injury, and results in the conversion of fibrinogen to fibrin, as a central component of clot formation (Crawley et al., 2007). Prothrombin, the precursor of thrombin, is present in plasma and can be converted to thrombin via a

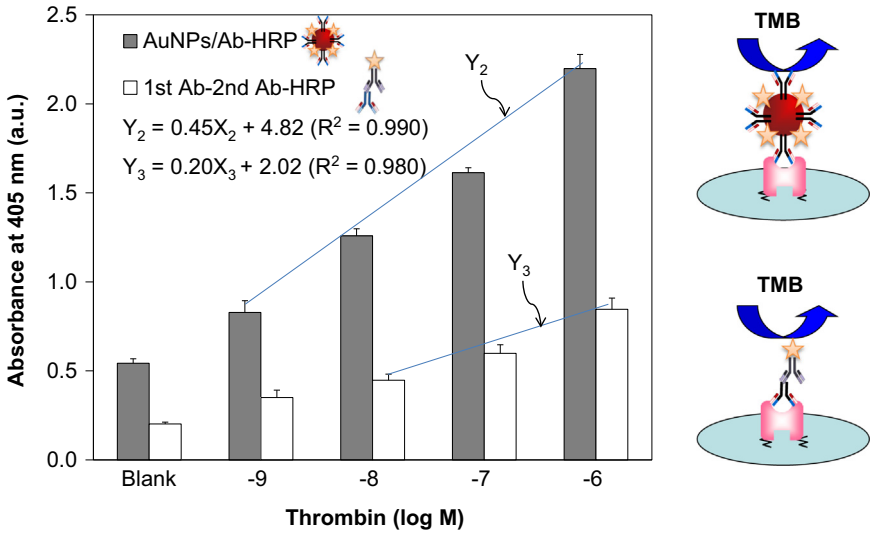

Fig. 5. Comparisons of thrombin detection by AuNPs-amplified and immune-based ELISA detection. The AuNPs-amplified (gray histograms) and classical immune-based (white histograms) ELISA were performed for thrombin detection in a 96-well ELISA plate, respectively. In the detections, different concentrations of thrombin were immobilized onto ELISA plates. The colorimetric signals were developed by enzyme reaction using TMB substrate. Each value was derived from five independent detections and error bars mean standard deviation (SD).

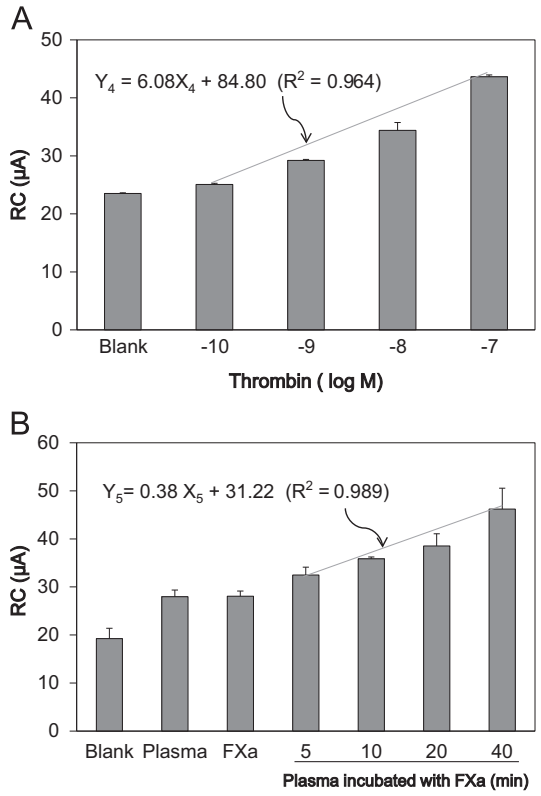

Fig. 6. Thrombin detection in human blood samples by AuNPs-amplified aptasensing strips. Thrombin in human serum was detected using AuNPs-amplified aptasensing strips (A). In the detection, 10% dilution of human serum was applied and different concentrations of thrombin were spiked in the 10% serum. The assay of plasma thrombin generation was performed (B). In the detection, de-fibrinogen plasma of human was used and the plasma prothrombin would be converted to thrombin by treating with the activator, FXa. The detected results show a little increasing RC detected in the samples of plasma and FXa only. The de-fibrinogen plasma was treated with FXa for different incubation times (5–40 min) and detected by the AuNPs-amplified aptasensing strips. Each value was derived from five independent detections and error bars mean standard deviation (SD).

proteolytic process by activated factor X (Heyduk and Heyduk, 2005). Human factor Xa (FXa), that physically cleavages prothrombin to thrombin, was used for the test of thrombin generation in human plasma using the fabricated aptasensing SPCE strips (Fig. 6B). The results indicate that little increasing RCs were detected in the samples of human plasma or FXa only compared with the blank. An increasing RC was measured when FXa was mixed with human plasma and then incubated for various times (Fig. 6B); the results indicate that the conversion of prothrombin to thrombin is time dependent. These findings demonstrate the ability of the AuNPs-amplified aptasensing strips applied in monitoring thrombin under physiological conditions.

4. Conclusion

An AuNPs-amplified aptasensing strip was fabricated for thrombin detection that AuNPs were conjugated with multiple molecules of anti-thrombin antibody and HRP. Compared with conventional sandwich-type immunoassay, the presence of AuNPs increased the amount of antibody and HRP, which enhances the sensitivity of the electrochemical detection. The LOD is 1.5 pM in thrombin under experimental condition, whereas the LOD is around 100 pM in serum, which is practical for clinical thrombin detection. Our works provided a promising sensitive and rapid method for thrombin detection by using disposable electrochemical aptasensing SPCE strips that possess the potential to be developed as a point-of-test assay for thrombin detection.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.05.007.

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