



A new electrochemically active–inactive switching aptamer molecular beacon to detect thrombin directly in solution

Guifang Cheng*, Bijun Shen, Fan Zhang, Jikui Wu, Ying Xu, Pingang He*, Yuzhi Fang

Department of Chemistry, East China Normal University, Shanghai 200062, China

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ABSTRACT

A new electrochemical aptamer molecular beacon (MB) was designed by the carminic acid (CA) covalently linking at the each end of a special single-stranded stem-loop shaped oligonucleotide and named as CAs-MB. CA is an electrochemically active molecule and two CA molecules at the ends of molecular beacon stem were closed enough to associate each other to be as CA dimer. The dimer was electrochemically inactive. It separated into two CA monomers and produced the electrochemical signal while CAs-MB combined with target. In this protocol, the detection strategy of CAs-MB for thrombin is based on electrochemical active–inactive switching between monomer and dimer forms of CA. In order to enhance the electrochemical signal, magnetic nanobeads (MNB) was applied by connecting CAs-MB with MNB through a duplex of DNA. With the magnetic enrichment, the detection limit for thrombin reached to 42.4 pM. The experiment results showed that this type of electrochemical active–inactive switching aptamer molecular beacon allowed the direct detection of target proteins in the solution with no requirement of removing uncombined CAs-MB. Besides, CAs-MB/MNB can be easily regenerated by using 2 M NaCl solution to cleave the thrombin from the aptasensor.

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1. Introduction

Molecular beacons (MBs) are the useful tool for the detection of DNA and proteins based on the high affinity of aptamer to proteins recently (Lee et al., 2007; Shlyahovsky et al., 2007; Wang et al., 2004; Liss et al., 2002). MBs are doubly end-labeled oligonucleotides that exist in solutions as stable stem-loop structures. The presence of target molecules leads to the change of MB conformation, and resulting in a fluorescent signal change (Fujimoto et al., 2004). Up to now, most of MB sensing methods are based on fluorescence including quencher–fluorophore pair mode (Yang et al., 2005; Hwang et al., 2004), donor–acceptor mode (Wabuyele et al., 2003), and excimer–monomer switches mode (Fujimoto et al., 2004). Most of the fluorescent MBs allow the homogenous detection in the solution. On the other hand, due to the unique advantages of electrochemistry such as convenience and sensitivity, MBs have also been adopted through electrochemical methods (Xiao et al., 2005; Radi et al., 2006). However, electrochemical MBs (E-MBs) were usually designed to be immobilized onto the electrode surface (Fan et al., 2003; Mao et al., 2003), instead of being in the solution as in the fluorescent MBs methods. The variation of E-MB signals owes to electrochemical labels close to or far from

the electrode surface corresponding to the conformational change of MBs in the absence or presence of their targets. However, these architectures often suffered from false positives or high background (Xiao et al., 2007; Tan et al., 2004). If there is a new kind of electrochemical MB, just like fluorophore excimer–monomer switches detection mode, it would be suitable for continuously direct electrochemical detection in the solution, thus resulting in a much simpler electrochemical detection procedure and comparable high signal-to-noise ratios. To get such new kind of E-MBs, the key issue is how to find out the suitable electrochemically active molecules to be labeled onto the ends of a MB sequence and then to exhibit different electrochemical characters corresponding to the variation of the MB conformation.

Aromatic compounds such as anthracycline can be oxidized or reduced and also have the tendency to self-association into dimers by H-bond or π – π electrons stacking (Jonathan et al., 1982; McLennan et al., 1985). The electrochemical activity of the dimer decreased as compared with that of the monomer form (Evstigneev et al., 2006; Qu et al., 2001). Carminic acid (CA) is such an anthraquinone compound. The monomer form of CA molecule displays electrochemical activity due to its hydroquinone and quinone groups (Domenech-Carbo et al., 2003), and its dimer form would turn off its electrochemical activity. Therefore, the electrochemical signal on or off switched by changing the state of CA molecule in dimer or monomer, which enable CA as the suitable label to develop the fluorescent-analogous electrochemical MB. This new kind of electrochemical MB was named as “electro-

* Corresponding authors. Tel.: +86 21 622 33510/33798; fax: +86 21 622 33798.
E-mail addresses: gcheng@chem.ecnu.edu.cn (G. Cheng),
pghe@chem.ecnu.edu.cn (P. He).

Table 1
The sequences of oligonucleotides used in this study.

	Sequence	Description
S1	NH_2 -5' GGTTGGTGTGGTTGG <u>TTTTTCTCTCTCTCT</u> TTTTTCAACC 3'- NH_2	Molecular beacon sequence, in which the bold faced letters are for the anti-thrombin aptamer sequence and the underlined letters are complementary with the sequence immobilized on magnetic nanobeads
S2	5' <u>AGAGAGAGAG</u> TTTTT3'- NH_2	The sequence immobilized on magnetic nanoparticles sequence in which the underlined letters are complementary with the sequence in MB
S3	NH_2 -5' GTTGGCACACCGACC <u>TTTTTCTCTCTCTCT</u> TTTTTCAACC3'- NH_2	Molecular beacon sequence, in which the bold faced letters are random sequence instead of anti-thrombin aptamer sequence
S4	5'GGTTGAAAAGAGAGAGAGAAAAACCAACCACCAACCC3'	The sequence is complementary with S1

chemically active–inactive switching MB” and has been utilized to directly detect DNA sequences in our previous study (Wu et al., 2009). Herein, we proposed a protocol for directly recognizing thrombin in the solution by CAs-MB. Thrombin is one kind of important enzyme acting as key clot promoter in human blood, and it has two of anti-thrombin aptamers (Ikebukuro et al., 1992). As shown in Table 1, S1 was selected as MB sequence in which 1st base of 5' end to the 15th base was the anti-thrombin aptamer sequence (colored blue in Fig. 1). Each end of the MB was labeled with CA molecules and named as CAs-MB. Magnetic nanobead (MNB) (Son et al., 2005) was employed to concentrate the CAs-MB in order to enhance the sensitivity of the sensor (Herr et al., 2006; He et al., 2007; Xu et al., 2009). S2 sequence was used as a linker to connect CAs-MB with MNB by one end of which immobilizing onto the MNB and the other end hybridizing with the 21st to 30th base sequences of the MB to become CAs-MB/MNB. In the absence of thrombin, CAs-MB/MNB retained the stem-loop structure and the dimer of CA was electrochemically inactive. After incubated with thrombin, the aptamer sequence bound thrombin by forming G-quadruplex and led to conformational change of the CAs-MB. As a result, the monomer displayed its electrochemical activity (Fig. 1). This new kind of E-MB (CAs-MB) would be as a more useful tool for bio-molecules detection especially in primitive and wild occasions.

2. Experimental

2.1. Reagents

The oligonucleotides (S1, S2, S3 and S4 shown in Table 1) used in this work were purchased from Invitrogen Biotechnology Inc. (Shanghai, China). The concentrations of all oligonucleotides were calculated from UV absorption at 260 nm with a correction for

the absorbance of the co-existence materials at 260 nm. Thrombin, bovine plasma albumin (BSA), lysozyme, pancreatic enzyme, Ethy-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and carminic acid (CA) were purchased from Sigma–Aldrich. Magnetic nanobeads (carboxyl functionalized, average diameter 100 nm) were purchased from Chemicell Inc. (Berlin, Germany). PBS buffer (pH 7.3) was purchased from Invitrogen Biotechnology Inc. (Shanghai, China) and diluted to 0.1 M prior to use. Ultrapure water was prepared from an Aquapro system (specific resistance is 13 MΩ cm).

2.2. Instrumentation

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on the CHI 660 Electrochemical Analyzer (CH Instruments Inc., USA). The spectrophotometric measurement was carried out using a Varian Cary-50 UV–visible spectrophotometer (Varian, USA) and a Mexus 670 FT-IR spectrometer (Nicolet, USA). A JB-1 stirring machine (Branson, Shanghai, China) and a super thermostatic bath 501 (Shanghai Laboratory Apparatus Factory, China) were used for facilitating the hybridization and incubation reactions.

2.3. Preparation of CAs-MB/MNB

The procedures of preparation of CAs-MB and S2-functionalized MNBs were listed in Supplementary material. The preparation of CAs-MB/MNB was carried out by hybridization CAs-MB with S2-functionalized MNB. The procedure was described as following. Briefly, 0.5 ml of 1.0×10^{-5} M CAs-MB solution was mixed with 1 ml of 1 mg/ml S2-functionalized MNBs suspended solution with 1.4 M NaCl. The mixture was gently stirred under 45 °C for 30 min. Unhybridized CAs-MBs were eliminated through magnetic separation.

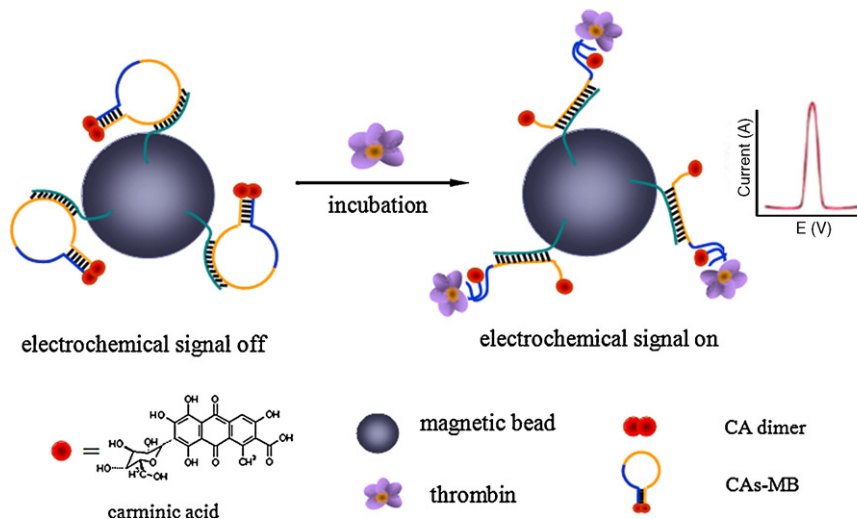


Fig. 1. Schematic representation of the thrombin detection by CAs-MB/MNB.

After washed by PBS, CAs-MB/MNB was re-suspended in 1 ml PBS and stored at 4 °C. CAs-S3-MB/MNB was prepared with the same procedures for control experiment.

2.4. Electrochemical detection

The procedures of CAs-MB or CAs-MB/MNB reacted with the protein were shown in [Supplementary material](#). Electrochemical measurement of CAs-MB and CAs-MB/MNB were individually carried out in a common cell and a special homemade electrolytic cell with a graphite electrode, a platinum wire counter electrode, an Ag/AgCl reference electrode and an inferior magnet (Zheng et al., 2007). The responses of CAs-MB/MNB were collected while CAs-MB/MNBs completely gathering onto the surface of working electrode (about 15 min after transfer). CV measurements were performed with the scan range from -1.0 to 1.0 V (vs. Ag/AgCl) at scan rate 0.1 V/s and the range of the DPV scanning was from 0.3 to 0.8 V (Incr E 0.004 V, amplitude 0.05 V, pulse width 0.05 s, pulse period 0.2 s) to measure the currents of the oxidation peak of CA.

3. Results and discussion

3.1. Formation and characterization of the CAs-MB

To confirm the formation of the CAs-MB, the purified CAs-MB solution was measured by IR and UV spectrophotometry and CV voltammetry. IR spectroscopy was applied first to validate the covalently linkage between CA and S1 sequence. Two distinguish absorption peaks were displayed in the IR spectrum of CA at 3127 and 1402 cm^{-1} , respectively assigning to the stretch vibration of OH (ν_{OH}) in carboxylic group and the asymmetry stretch vibration of CH₂ ($\nu_{\text{CH}_2}^{\text{as}}$). After the CA labeling onto the S1 sequence, the absorption at 1402 cm^{-1} dramatically depressed and shifted to 1383 cm^{-1} . Two strong absorption peaks representing the stretch vibration of $-\text{CH}_2-\text{O}-\text{P}$ ($\nu_{\text{P}-\text{O}}$) and the stretch vibration of $-\text{NH}-$ in $-\text{NH}-\text{CO}-$ group at 1079 and 3447 cm^{-1} raised with the emergence of a small peak at 1646 cm^{-1} represented the stretch vibration of $\text{C}=\text{O}$ ($\nu_{\text{C}=\text{O}}$) (see [SFigure 1, curve a and b in Supplementary material](#)). The variations of IR spectra of free CA and labeled CA indicated that the linkage between CA and S1 sequence was formed and CA has covalently linked onto the ends of S1 oligonucleotide by aminocarboxylation.

UV-visible spectrophotometry was secondly applied to identify the state of CAs onto both ends of S1 sequence. The monomers of CA exhibited a large absorption peak (513 nm) and a shoulder peak (560 nm). When CA was labeled at the end of S1 sequence formation of CAs-MB, its UV-visible spectrum was changed. The main and the shoulder absorption peak exchanged and exhibited a bathochromic shift to 532 and 580 nm, respectively (see [SFigure 2, solid and dotted lines in Supplementary material](#)). Almost 20 nm bathochromic shifts indicated that the π bond of anthracycline on CA molecule was conjugated, illustrating that two of the CA molecules at both ends of the MB stem formed into the dimer form.

And then, CV measurement was employed to demonstrate the difference in electrochemical activity between CA monomer and dimer. Free form of CA displayed a pair of quasi-reversible redox peaks ($E_{\text{pa}1} = -0.560$ V, $E_{\text{pc}1} = -0.629$ V) and an irreversible anodic peak ($E_{\text{pa}2} = 0.444$ V) (Fig. 2, dash line) corresponding to the redox of anthraquinone group and oxidation of hydroquinone group of CA, respectively. These redox peaks subsided while the CA labeled onto the S1 sequence (Fig. 2, solid line). According to the result obtained in UV-visible spectrophotometric study, two CA molecules at both ends of the S1 sequence were conjugated to the dimer form. Thus,

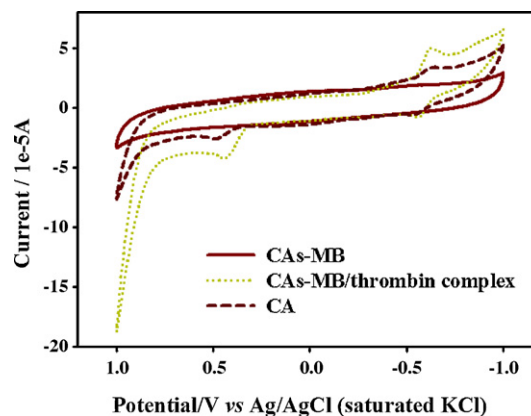


Fig. 2. Cyclic voltammograms of CA, CAs-MBs and CAs-MB incubated with thrombin ($c_{\text{CA}} = 1.2 \times 10^{-5}$ M, $c_{\text{CAs-MB}} = 8.0 \times 10^{-6}$ M, $c_{\text{thrombin}} = 4.0 \times 10^{-5}$ M in PBS with 1.0 M NaCl and 1.0×10^{-3} M MgCl₂, pH 7.3).

the decrease of redox peak current made it clear that the dimer form of CA was electrochemically inactive.

After CAs-MB was incubated with its corresponding target (complementary sequence or the thrombin), the stem-loop shaped CAs-MB was stretched and CA dimer split up. As a result, the absorption peak of IR at 1646 cm^{-1} shifted to 1633 cm^{-1} and became stronger ([SFigure 1, curve c in Supplementary material](#)) while the UV-visible spectrum was almost the same as that of free CA ([SFigure 2, dotted line in Supplementary material](#)) and its electrochemical activity restored (Fig. 2, dotted line). It can be concluded that the CAs in CAs-MB were at the monomer state in the presence of its corresponding target. Thus, the transformation of CAs-MB in electrochemically active or inactive depended upon the presence or absence of its corresponding targets.

3.2. Thermal behavior of CAs-MB

Thermal plot of CAs-MB was tested in a common cell with a bath for temperature controlling. As shown in [Fig. 3](#), CAs-MB gave no electric signal until the temperature rose to 48 °C. The sharp melting profiles denoted that CAs-MB was stable enough to be an element for thrombin recognition. The melting-point temperature (T_m) of CAs-MB was estimated to be 52 °C (curve CAs-MB). Thermal profile of CAs-MB/thrombin complex gave a typical curve (curve CAs-MB/thrombin complex) of combined MB ([Wang et al.](#),

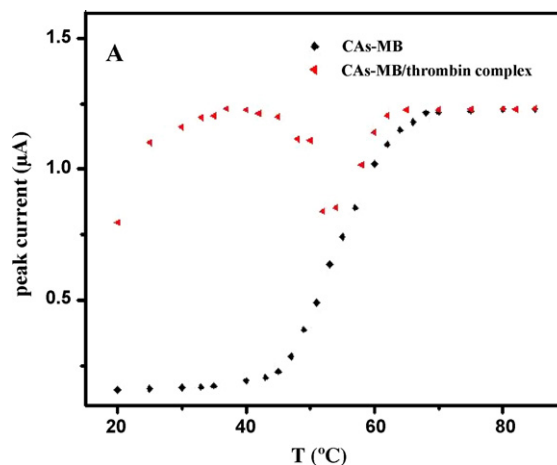


Fig. 3. The melting temperature curves of CAs-MB and CAs-MB incubated with enough thrombin ($c_{\text{CAs-MB}} = 6.0 \times 10^{-7}$ M, $c_{\text{thrombin}} = 3.0 \times 10^{-6}$ M in 0.1 M PBS with 1.0 M NaCl and 1.0×10^{-3} M MgCl₂, pH 7.3).

2008). In the presence of the thrombin, the higher electrochemical signals achieved at the range of 25–48 °C were contributed to the formation of CAs-MB–thrombin complex to break CA dimer into monomer. And lower electrochemical signals round 52 °C were for the destabilization of CAs-MB–thrombin complex; while at higher temperature (above 65 °C) the thrombin departed from CAs-MB, the destabilized stem-loop structure of CAs-MB was completely open as linear shape, which led to the enhancement of electrochemical signal. The thermal profiles of CAs-MB indicated the electrochemical signal switching on was contributed to the transformation of CAs-MB conformation from hairpin to line typed.

The conformation of CAs-MB also depended on pHs. CAs-MB is electrochemically active at pH 3–5 and then becomes electrochemically inactive at pH 6–8 (see SFigure 3 in Supplementary material). In an acidic medium, the stem duplex of MB was unstable; also the protonization of CAs prevented them from formation of dimer. However, at neutral and weak alkalinescent, these two effects upon the stability of CAs-MB were no longer existed so that both CA dimer and the stem-loop structure of CAs-MB were steady and no electrochemical signals given out.

3.3. The enrichment efficiency of MNBs

Separation and concentration of interested components by magnetic beads have been widely applied in bio-molecules analysis (Bergey et al., 2002; Wang et al., 2002; Hirsch et al., 2000; Cheng et al., 2005). Comparing with the strategy of immobilizing probe on the electrode surface (Loaiza et al., 2008; Laschi et al., 2009), the use of the functionalized magnetic nanoparticles have the superiorities not only in the prodigious surface area and fast assay kinetics but also the high target capturing efficiency as well. However, magnetic nanoparticles are not good electric conductor, which would block electron transmission and depress the current signal when they stacked on the electrode surface. On the other hand, some research works have proven that a few magnetic nanoparticles depositing on the electrode surface did not significantly affect electrochemical behaviors (Zacco et al., 2004). To minimize the obstruction of MNBs to electrochemical signal, the dosage of MNBs was tested by measuring the DPV responses of 75.9 μM $\text{K}_3\text{Fe}(\text{CN})_6$ solution mixed with MNBs from 1 to 240 μg (see SFigure 4 in Supplementary material). As the dosage of MNB used in the experiment was selected as 60 μg , the response of DPV signal was 97% of the original and 60 μg of MNB in 500 μl PBS would subside within 15 min. The concentration efficiency of MNB was also tested and the results were showed in Fig. 4. Without magnetic nanobeads, CAs-MB exhibited a low electrochemical response of 7.0×10^{-8} M thrombin (Fig. 4, curve b). After the magnetic enrichment, a much high electrochemical response was obtained even at 1.2×10^{-10} M of thrombin (Fig. 4, curve d). The employment of MNB greatly improved the sensitivity to thrombin of the assay.

3.4. Optimization of the incubation conditions

In order to block the nonspecific adsorption and promote the efficiency of incubation of aptamer to thrombin, it was necessary to control the temperature, time and ionic strength of the reaction. The experiment results showed that the CAs-MB/MNB could react with thrombin in the range of 25–50 °C and the higher reaction efficiency was within 32–40 °C for 30 min (see SFigure 5 in Supplementary material), which agreed with the result obtained in Fig. 3. Also, in the range of 0.5–2.0 M NaCl (the data not showed), the duplex between CAs-MB and MNB was stable enough to avoid releasing. Considering both the incubation efficiency and the stability of CAs-MB/MNB, the optimal incubation was selected in PBS with 1.0 M NaCl and 1.0×10^{-3} M Mg^{2+} at 32 °C for 30 min.

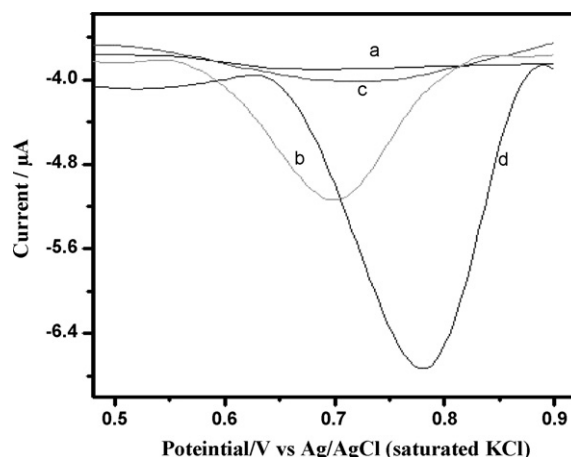


Fig. 4. DPV responses of 3.0×10^{-6} M CAs-MB in the absence of thrombin (a) and in the presence of 7.0×10^{-8} M thrombin (b); 3.0×10^{-7} M CAs-MB/MNB at the absence of thrombin (c) and in the presence of 1.2×10^{-10} M thrombin (d) in PBS with 1.0 M NaCl and 1.0×10^{-3} M MgCl_2 , pH 7.3.

3.5. Regeneration of the CAs-MB/MNB

Regeneration of CAs-MB/MNB was simple and easy, based on the reversibility of the thrombin–aptamer binding (Radi et al., 2005, 2006). The thrombin binding to the CAs-MB/MNB could be removed by putting the aptasensor into 2 M NaCl for about 10 min and washing with PBS several times. The renewed CAs-MB/MNB could be used for another round of protein detection. Additionally, the regenerative CAs-MB/MNB did not significantly lose its binding efficiency with the detection RSD of 2.2% (see SFigure 6 in Supplementary material). Besides, the CAs-MB/MNB could be stable for months if stored at 4 °C.

3.6. Direct thrombin recognition in the solution using CAs-MB/MNB

The specificity of CAs-MB recognition target molecules in the solution was measured by the following experiments: firstly, CAs-MB/MNB was incubated with 1.09×10^{-5} M bovine serum albumin (BSA), 3.75×10^{-6} M lysozyme (Lys), 1.18×10^{-10} M thrombin, and the mixture of these three kinds of proteins, respectively. Only the samples containing thrombin gave the remarkable responses, while the others resulted the signals almost equal to the background. Even the coexistence of other proteins over 105 times more than the thrombin did not interfere with the target determination. In another experiment CAs-S3-MB/MNB was used to incubate with the thrombin instead of CAs-MB/MNB, also a very small response resulted (see SFigure 7 in supplement material). Therefore, CAs-MB/MNB has favorable specificity to the thrombin.

The calibration curve of the thrombin detection was tested with and without the application of magnetic enrichment (Fig. 5, insert). CAs-MBs dispersed in solution and the DPV responses were very low. With the magnetic enrichment, those CAs-MBs attached on the surface of magnetic nanoparticles would be almost all gathered onto the working electrode, and consequently gave enhanced DPV signals. In the case of using magnetic enhancement, a linear response was obtained in the range of 1.18×10^{-11} to 1.41×10^{-10} M thrombin and the linear equation was $I_p (\mu\text{A}) = 0.156 + 1.89c_{\text{thrombin}} (10^{-10} \text{ M})$ with a correlation coefficient of 0.993. The detection limit of the protocol was 4.24×10^{-12} M determined by three times of standard deviation of a blank solution ($n = 11$). Therefore, it is sensitive enough for the thrombin determination in real samples.

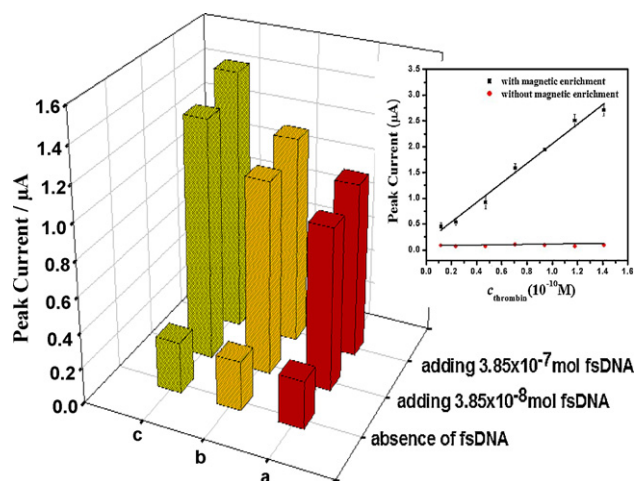


Fig. 5. The DPV response of CAs-MB/MNB for thrombin in 300-fold diluted (a), 200-fold diluted (b) and 100-fold diluted (c) plasma sample; the insert was the calibration plots of peak current of DPV vs. thrombin concentration in the range of 11.8–141 pM with magnetic enrichment (■) and without magnetic enrichment (●), $C_{\text{CAs-MB/MNB}} = 3.0 \times 10^{-7}$ M in PBS with 1.0 M NaCl and 1.0×10^{-3} M MgCl_2 , pH 7.3.

The experiment of the CAs-MB to detect target proteins in complex mixtures was important for whether CAs-MB could be applied in the science research and clinical diagnosis. The pretreatment of plasma was carried out according to the procedure as shown in our previous study (Zheng et al., 2007). Firstly a certain amount of fish sperm DNA (fsDNA) was added into the diluted plasma sample to deplete the degradation enzyme. And then, enough amounts of pancreatic enzymes were successively added to converted prothrombin to thrombin. The experimental data showed that reduction of the dilution times of the plasma sample resulted in a proportional increasing response of the thrombin (Fig. 5), and the reproducibility for the detection of thrombin in plasma was 87.3%. All the experiment (without sample preparation) could be done in 50 min and it was faster and more sensitive than the ELISAs (Bichler et al., 1991), which makes it possible for the proposed method applied in the fields requiring convenient, fast and sensitive detection.

4. Conclusions

Recently, the reusable, simple and convenient electrochemical molecular beacons have received particular attention for their high sensitivity, selectivity and simple instrumentation. The difference in electrochemical activity of CA monomer and dimer enables us to design a new type E-MB of electrochemically active–inactive switching MB (CAs-MB) for the direct detection of bio-targets in homogenous solution. Our study proves that the strategy of controlling electrochemical signal by conversion CA from monomer to dimer is feasible and reliable. Owing to the advantages of magnetic nanobeads, the protocol realized the ultra-sensitive target detection. Moreover, having the excellent specific affinity to targets and easy regeneration, this new electrochemically active–inactive switching MB opens a fire-new detective technique and can be expanded to more electrochemical biosensor systems to perform quick and convenient analyses.

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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.2010.03.008.

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