Biosensors and Bioelectronics 66 (2015) 363–369

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Background eliminated signal-on electrochemical aptasensing platform for highly sensitive detection of protein

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ARTICLE INFO

Article history:
Received 8 September 2014
Received in revised form
20 November 2014
Accepted 21 November 2014
Available online 24 November 2014

Keywords:
Endonuclease
Aptamer
Electrochemical
Biosensor
PDGF-BB

ABSTRACT

Using platelet-derived growth factor B chain dimer (PDGF-BB) as the model target, a background current eliminated electrochemical aptameric sensing platform for highly sensitive and signal-on detection of protein is proposed in this paper. Successful fabrication of the biosensor depends on ingenious design of aptamer probe, which contains the aptamer sequence for PDGF-BB and the recognition sequence for *Eco*RI endonuclease. In the absence of PDGF-BB, the ferrocene labeled aptamer probe folds into a hairpin structure and forms a recognition site for *Eco*RI. By treatment with endonuclease, the specific and cleavable double-stranded region is cut off and redox-active ferrocene molecule is removed from the electrode surface, and almost no peak current is observed. When binding with target protein, the designed aptamer probe changes its conformation and dissociates the recognition double strand. The integrated aptamer probe is maintained when exposing to *Eco*RI endonuclease, resulting in obvious peak current. Therefore, a signal-on and sensitive sensing strategy for PDGF-BB detection is fabricated with eliminated background current. Under the optimized experimental conditions, a wide linear response range of 4 orders of magnitude from 20 pg mL^{-1} to 200 ng mL^{-1} is achieved with a detection limit of 10 pg mL^{-1}. Moreover, the present aptameric platform is universal for the analysis of a broad range of target molecules of interest by changing and designing the sequence of aptamer probe.

1. Introduction

Early diagnosis for malignant tumor is of significant importance since it helps disease therapy and prolonging lifetime of the patients (Mukundan et al., 2009). Despite advances in powerful imaging techniques such as endoscopic ultrasound (EUS), computed tomography (CT) and magnetic resonance imaging (MRI), most tumors are not detected sufficiently early to benefit from surgery (Bezabeh et al., 2009; Chakraborty et al., 2011; Chang et al., 2013; Pliarchopoulou and Pectasides, 2009). Moreover, current methods for early diagnosis of malignant tumor are often associated with a high incidence of false positives and negatives, or requiring elaborate infrastructure and trained personnel for operation (Mukundan et al., 2009). Therefore, there is an immediate need for specific, sensitive and rapid detection methods that can complement existing technology for early diagnosis of malignant tumor.

Tumor markers are biomolecules differentially expressed in organ hosting a tumor and may be secreted in blood or interstitial fluids (Wu et al., 2007; Lin et al., 2013). For example, platelet derived growth factor (PDGF) plays an important role in vascular proliferation and cell transformation, which is closely related to tumor growth and progression (Huang et al., 2008; Wang et al., 2015). The content of platelet-derived growth factor B chain dimer (PDGF-BB), an important hypotype of PDGF, is very low in normal cells, even undetectable. But in glioma and sarcoma tumors, the level of PDGF-BB will rise sharply. Tumor marker proteins are important indicators for detecting and staging tumors, tracking tumor recurrence or metastasis, determining responses to therapies, and estimating the prognosis of cancer patients. Therefore, developing highly sensitive biosensor to detect such tumor marker protein is an effective means for the early diagnosis of cancer.

Traditionally, antibody–antigen specific reaction based immunoassay is the main analysis technique for protein (Wilson and Hu, 2000; Andreotti et al., 2003; Clerico et al., 2000). However, due to the inherent drawbacks such as the weak stability for preserving of antibody and the long term for synthesis, the application of immunoassay technique is limited to some extent. In

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http://dx.doi.org/10.1016/j.bios.2014.11.044
0956-5663/

the past decades, a new class of recognition element for protein named aptamer has been widely studied. Aptamers are artificially selected single stranded DNA or RNA oligonucleotides from random-sequence nucleic acid libraries by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) with characteristic spatial structures and are able to bind with their target with high specificity and affinity (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Nutiu and Li, 2003; Shangguan et al., 2006). Moreover, aptamers exhibit many other advantages such as good stability, easy modification, simple synthesis and wide applicability. Therefore, lots of research efforts have been focused on the development of aptamer based biosensing strategy, including colorimetric (Liu and Lu, 2006; Zhao et al., 2008; Li et al., 2014), fluorescent (Stojanovic et al., 2000; Nutiu and Li, 2004) and electrochemical (Wu et al., 2007; Zhang et al., 2010) methods and so on. Among these techniques, electrochemical method is the most frequently studied due to its significant advantages over other approaches, such as high sensitivity, low sample volume, short detection time, simple pretreatment procedure, inexpensive instrumentation, automated detection, amenable to miniaturization and not affected by sample turbidity (Zhang et al., 2010).

However, since most aptamer-involved electrochemical sensing strategies are based on conformation transformation of aptamer probe according to reported papers, electrochemical aptasensors are often facing the problem of background current for not only the signal-off type but also the signal-on type. For example, Wu et al. (2007) reported a reusable electrochemical sensing platform based on structure-switching signaling aptamers for small molecules detection. Ferrocene-labeled aptamer probe is designed to hybridize with capture probe and specifically recognize adenosine. The introduction of adenosine triggers structure switching of aptamer, forcing the signaling aptamer probe dissociates from the sensing interface and resulting in a decrease in redox current of ferrocene. These signal-off type of electrochemical aptameric sensors generally accompany with high background current of blank sample, which influencing the detection sensitivity of the sensor. Many signal-on type of aptamer involved electrochemical biosensors also suffer from high background current (Baker et al., 2006; Lai et al., 2007; Radi et al., 2006). For example, Baker et al. (2006) developed a structure switching based signal-on electrochemical aptameric sensor for cocaine detection. The aptamer probe is modified with electroactive methylene blue and immobilized on the electrode via thiol group. Signal generation is based on binding-induced folding of aptamer probe. However, background current can still be detected even in the absence of target cocaine, which reduced the detection sensitivity to some extent. Therefore, developing background current-eliminated or depressed analytical technique is of significant importance for improving analytical performances of electrochemical aptameric sensors.

Herein, a background current-eliminated electrochemical aptameric sensing platform is proposed for sensitive detection of protein using PDGF-BB as the model target. A ferrocene modified thiolated aptamer is adopted and immobilized on electrode surface. Introduction of a recognition site with palindrome structure for endonuclease *Eco*RI not only eliminates the peak current corresponding to blank sample but also provides a signal-on response mechanism. The aptamer probe folds into a hairpin structure and form a cleavable double-stranded palindrome. By treatment with endonuclease *Eco*RI, ferrocene is capable of being removed from the electrode surface and almost no peak current is observed. Introduction of target protein PDGF-BB triggers structure switching of the aptamer probe and the cleavable double-stranded segment is dissociated. As a result, the aptamer probe is still maintained on the electrode surface when exposing to *Eco*RI, generating an obvious peak current. Making use of the high digestion efficiency and remarkable precision of *Eco*RI endonuclease, the proposed strategy proved to be an efficient method for elimination of background current for blank sample, which can be utilized in fabricating highly sensitive electrochemical aptameric sensors for not only proteins but also small molecules. The operation principle and the fabrication of the biosensor are shown in Scheme 1.

2. Materials and methods

2.1. Chemicals

The designed aptamer oligonucleotide was obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China) and used as received with the sequences as follows:

5′-HS-$(CH_2)_6$-
*GAATTC*CAGCAGGCTACGGCACGTAGAGCATCACCATGATCCTG
GAATTC-$(CH_2)_6$-NH_2-3′

The aptamer oligonucleotide is modified with thiol group in the 5′ end and amino group in the 3′ end, respectively. The italic portion refers to recognition site of *Eco*RI endonuclease with a GAATTC palindrome structure. The underline part is the sequence of original aptamer for PDGF-BB.

*Eco*RI endonuclease set, including *Eco*RI endonuclease and 10 × *Eco*RI endonuclease digest buffer, was bought from Takara Biotechnology Co., Ltd. (Dalian, China). PDGF-BB, PDGF-AA, PDGF-AB, IgG, anti-IgG, BSA and Tris were all obtained from Dingguo Biotechnology Co., Ltd. (Changsha, China). Ferrocene monocarboxylic acid and 6-Mercaptohecanol (MCH) were obtained from Acros organics, while *N*-(3dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) were received from Sigma. All other chemicals were of analytical-reagent grade and used as received. Triply distilled water (resistance $> 18\ M\Omega\ cm$) was used throughout the experiments.

Several buffers were used in the present study: stock and dilution buffer (shortened form as 0.3 M buffer) contained 50 mM Tris–HCl (pH 7.8), 300 mM NaCl and 1 mM $MgCl_2$; digestion buffer (shortened form as 0.1 M buffer) contained 50 mM Tris–HCl (pH 7.8), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT.

2.2. Apparatus

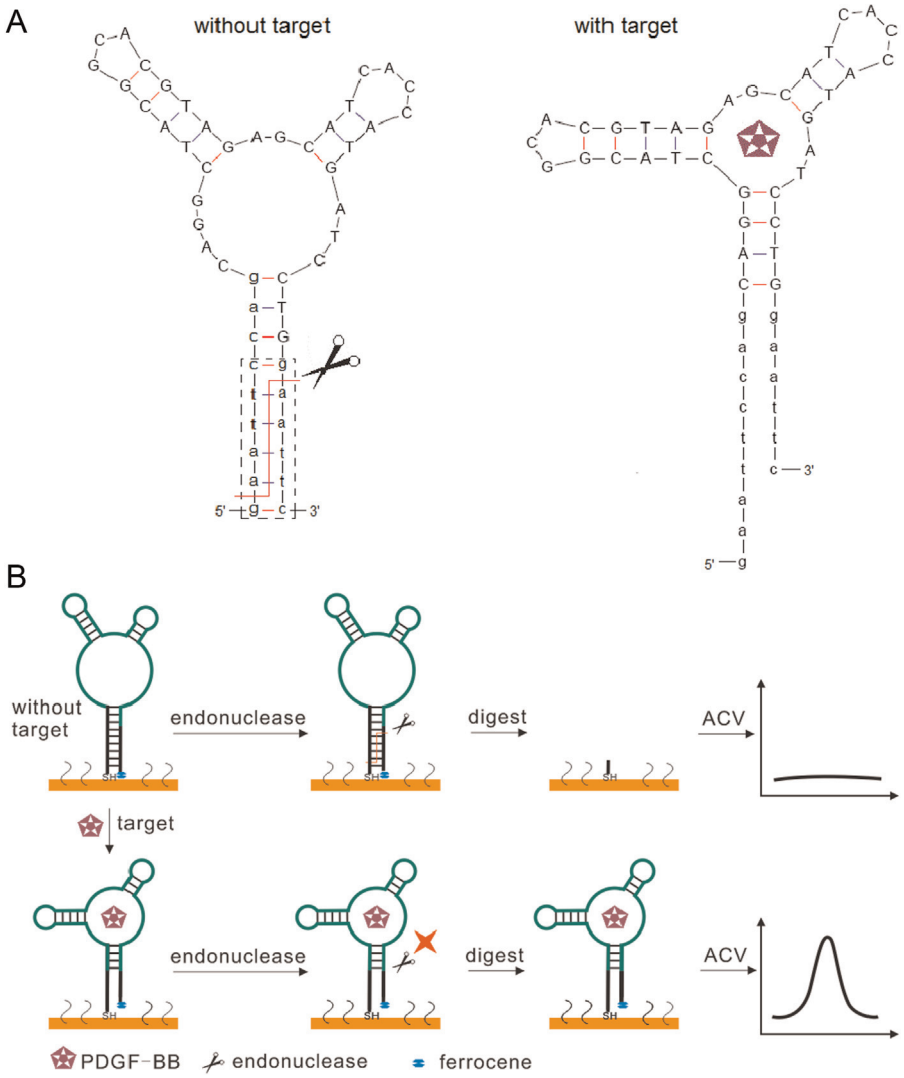
All electrochemical measurements were conducted on CHI 660E electrochemical workstation (Shanghai Chenhua Instruments, China) in a self-made measuring cell at ambient temperature. A conventional three-electrode system was employed: a gold electrode (polycrystalline gold rod, 99.99%, 2 mm diameter) of interest as working electrode, a platinum foil as auxiliary electrode and KCl saturated calomel electrode (SCE) as reference electrode. All potentials were referenced to the SCE reference electrode.

2.3. Preparation of ferrocene modified aptamer probe

The ferrocene labeled aptamer probe was prepared according to the reported literature (Wu et al., 2007) with a minor modification. Briefly, 1 mg of ferrocene monocarboxylic acid was added to 1 mL of fresh prepared 0.3 M buffer solution containing EDC and NHS (0.1 M each), and followed by being mixed immediately. Then 100 μL of 2.0 μM aptamer was injected into the mixture followed by stirring at room temperature for 2 h. Subsequently, the resulting solution (ferrocene modified aptamer solution) was stored in refrigerator at 4 °C until use.

2.4. Fabrication of aptasensor

The gold electrode (2 mm diameter) was polished to mirror smoothness with 0.3 and 0.05 μm alumina slurries sequentially on



Scheme 1. Design of aptamer probe and the working principle of the electrochemical aptasensor. (A) Predicted secondary structure of the aptamer probe without (left panel) and with (right panel) target molecules. The sequence in capital letter is the original aptamer sequence for PDGF-BB and that in the dotted rectangle is the cleavable palindrome for *Eco*RI restriction endonuclease. The folded line indicates the cuts made by *Eco*RI. (B) Schematic diagram of the detection principle for PDGF-BB. In the absence of PDGF-BB, the *Eco*RI endonuclease cleaves the recognition site of palindrome, resulting in the fact that the redox-active ferrocene removes from electrode and the background current for blank sample is completely eliminated as shown in the upper half. On the contrary, the target binding event deforms the *Eco*RI recognition site and the aptamer probe is still integrated when exposing to endonuclease. As a result, an obvious peak current is observed as shown in the lower half.

microcloth pads. After rinsing successively in an ultrasonic bath with distilled water, absolute alcohol and distilled water for 5 min respectively, the gold electrode was dipped in piranha solution (a bath of 7 parts H_2SO_4 to 3 parts 30% H_2O_2, ***Caution!*** *Piranha solution is highly corrosive and reacts violently with almost all organic materials. Extreme care should be taken when handling Piranha solution, and only small quantities should be prepared.*) for 15 min, and rinsed with distilled water. Then, the electrode was electrochemically treated by cycling the potential between −0.3 and +1.5 V in 0.1 M H_2SO_4 until a reproducible cyclic voltammogram was observed. The cleaned electrode was rinsed with distilled water and dried in a nitrogen stream. Subsequently, 20 μL of ferrocene modified aptamer probe was dropped onto the freshly cleaned gold electrode and allowed to incubate for 2 h in a water-saturated atmosphere. After rinsing with distilled water to remove the unbound oligonucleotides, 20 μL of 1 mM 6-mercaptohexanol solution was pipetted onto the resulting electrode and maintained for 10 min to block the unreacted sites. Finally, the electrode was rinsed with distilled water and ready for target protein detection.

2.5. Electrochemical measurement and analytical performances assessment

For target protein detection, 20 μL of PDGF-BB solution at specific concentration (diluted with 0.3 M buffer) was placed on the prepared sensing interface and allowed to incubate at 37 °C for 2 h. Then, 20 μL of endonuclease solution containing 0.9 U μL^{-1} *Eco*RI was dropped on the resulting electrode surface followed by digestion at 37 °C for another 2 h. Subsequently, the electrode was rinsed in 0.3 M buffer solution under stirring for 30 s to remove the physically adsorbed molecules. To evaluate the response characteristics of proposed aptasensor, ac voltammogram (ACV) measurements were carried out from 0.0 to 0.6 V (versus SCE) at a specific frequency in 5 mL of 1.0 M $NaClO_4$ solution. The peak current observed at 0.27 V was used to estimate the amount of PDGF-BB in sample and to assess the analytical performances of the aptasensor. For characterization of the sensor fabrication process, cyclic voltammetry (CV) was performed at a scan rate of 100 mV/s in 5 mL of 10 mM PBS (pH 7.4) containing 5 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ and 0.1 M KCl.

3. Results and discussion

3.1. Design principle of sensing strategy and improved signaling capability

Most of reported aptamer-involved electrochemical sensing strategies are based on structure switching of aptamer probe upon binding with target molecules. However, these sensing methods are often accompanied by high background current of blank sample for both signal-off type and signal-on type of electrochemical aptasensors, which depressed the detection sensitivity of the sensor to some extent (Zhang et al., 2010). On one hand, most signal-off type of electrochemical aptasensor is based on signal probe's removing from or far away from the electrode triggered by configuration transformation of aptamer probe. That's to say, for blank sample, signal probe is placed on the electrode or close to the electrode surface, which resulting in background current obviously. On the other hand, for many signal-on type of electrochemical aptasensor (Baker et al., 2006; Lai et al., 2007; Radi et al., 2006), the signal probe is originally far away from the electrode surface. Upon introduction of specific target, the aptamer probe switching its structure to bind with target molecule, which making the signal probe be in close proximity to the electrode surface. In these sensing strategies, a certain background current can still be detected though the signal probe is formerly far away from the electrode surface.

It is well known that sensing system with high signal-to-noise ratio is generally predicted to offer attractive analytical characteristics. We once developed a blank current-suppressed aptasensing platform based on restrict endonuclease for small molecules detection and good analytical performances were achieved (Zhang et al., 2010). However, this unique sensing strategy has never been applied for protein detection to our knowledge. Since tumor marker proteins are important indicators for detecting and staging tumors, it is worth expanding this interesting sensing strategy for protein analysis. Therefore, in the present contribution, we proposed an endonuclease based electrochemical aptasensor for protein detection (using PDGF-BB as the model target) to solve the problem of background current and to improve the analytical performances. The design of aptamer probe and the detection principle of the electrochemical aptasensor are shown in Scheme 1. The origin aptamer sequence for PDGF-BB contains 35 bases as shown in capital letters in Scheme 1A. The aptamer probe used in this study is designed with 9 bases extension at the 5′ end and 6 bases extension at the 3′ end, respectively. In the absence of PDGF-BB, the aptamer probe adopts a relatively big hairpin structure as predicted by 'mfold' program (http://mfold.rit.albany.edu/?q=mfold/DNA-Folding-Form). As shown in the left panel of Scheme 1A, a complementary stem with nine pairs of bases is formed which containing a cleavable palindrome structure with the sequence of GAATTC. This special double-stranded structure can be specifically recognized by endonuclease *Eco*RI and cut off between guanine (G) and adenine (A). After enzyme digestion, the cleavage products are moved from the electrode surface and almost no peak current is observed as shown in the top panel of Scheme 1B. On the contrary, introduction of PDGF-BB triggers the structure switching of aptamer probe to specifically bind with target protein, resulting in the deconstruction of palindrome structure and the deformation of recognition site for EcoRI as shown in the right panel of Scheme 1A. Therefore, the aptamer probe cannot be cut off by the endonuclease and the ferrocene tag is still close to the electrode surface. Accordingly, a high peak current (see bottom panel of Scheme 1B) of ferrocene can be obtained and the current intensity is directly related to the concentration of target molecules. By this way, an interesting and effective electrochemical aptameric sensing platform is proposed with two key characteristics. First, the often encountered background current of blank sample for electrochemical aptasensor is completely eliminated, which enhances the signal-to-noise ratio and improves the detection sensitivity. Second, a signal-on signaling mechanism is simultaneously achieved, which further avoids the drawbacks of high background current as well as high detection limit for signal-off types of biosensors.

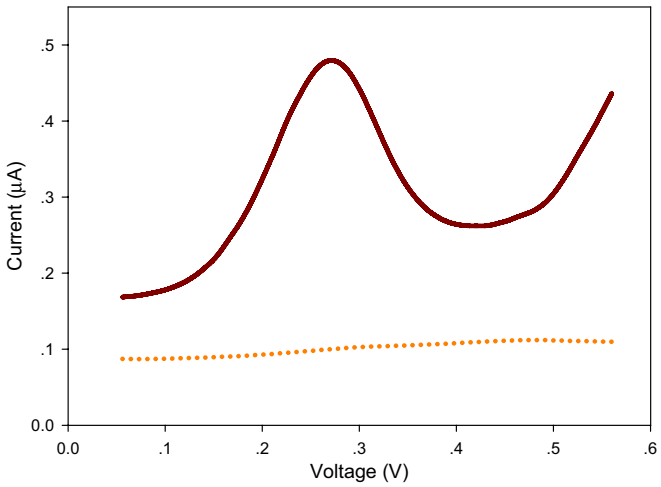


Fig. 1. Control experiments for electrochemical response of the aptameric sensing system without (dotted line) and with 20 μg/mL PDGF-BB (solid line). All ac voltammogram measurements were performed in 5 mL of 1.0 M $NaClO_4$ solution.

A control experiment confirms the feasibility of the present sensing system as shown in Fig. 1. No peak current of ferrocene is detected by ac voltammogram measurements for blank sample (dotted line), indicating the palindrome structure is successfully formed and completely digested by endonuclease *Eco*RI. In contrast, a high concentration sample of PDGF-BB causes a striking peak current of about 250 nA (solid line). The obtained current intensity which is almost equal to the peak current of freshly prepared sensing interface, indicating the deformation of recognition site and the success of the sensor fabrication. The elimination of background current makes the signal-to-noise ratio an extremely large value, promoting the improvement in analytical characteristics of aptasensor.

3.2. Sensing interface fabrication and electrochemical characterization

In order to monitor the sensor fabrication procedure and further check the validity of the proposed sensing strategy, cyclic voltammetry for each stage of sensor fabrication was performed. Cyclic voltammetry of electroactive species in conducting aqueous solution is a helpful method for probing the electrochemical characterization of the modified gold electrode (Wu et al., 2007). In the present study, cyclic voltammetry of a fairly reversible redox couple ($K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$) in PBS (pH 7.4) was carried out in the sweeping range from −0.2 to 0.6 V as shown in Fig. 2. During cycling of potential, relatively higher reversible peaks for oxidation and reduction of the redox probe on the bare gold surface can be observed (line a), reflecting excellent electrochemical conductivity of the treated electrode. Line b represents the cyclic voltammetry of aptamer probe immobilized and 6-mercaptohexanol blocked electrode. Immobilization of the aptamer probes via self-assembly significantly induced the decrease of the peak current of the redox couple. This should be attributed to the fact that the negatively charged phosphate backbone hinders the electron transfer. Treatment with 6-mercaptohexanol induced a further little decrease of the current peak, indicating the unreacted sites of the gold

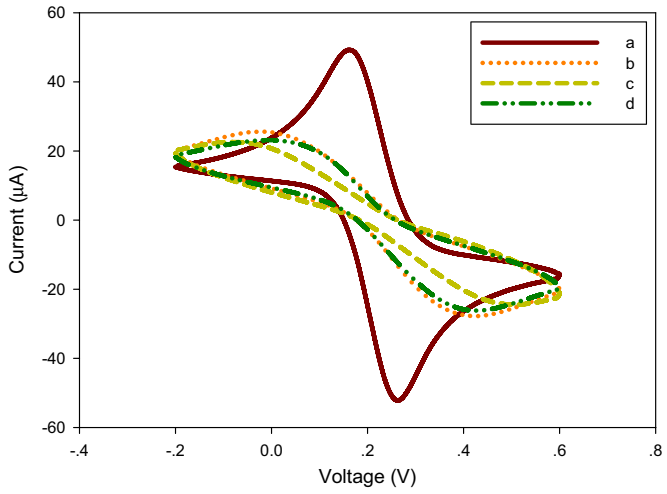


Fig. 2. Electrochemical cyclic voltammograms characterization of the same gold electrode in 10 mM PBS (pH 7.4) containing 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ and 0.1 M KCl at different stages: (a) bare gold electrode; (b) aptamer probe immobilized and mercaptohexanol blocked electrode; (c) 2.0 ng/mL PDGF-BB. exposed electrode; (d) enzyme digested electrode.

electrode surface are blocked and the diffusion of ferricyanide toward the electrode surface is hindered. Moreover, a well ordered mixed self-assembled monolayer of aptamer probe and thiol molecule can be achieved because weak adsorbed molecules can be displaced by thiol groups. Since the aptamer probe can pre-fold into a hair structure, it is expected that sufficient interstitial space between immobilized aptamer sequences can be achieved, which facilitating the binding of aptamer probe with target molecules. As shown in line c, a significant decrease of current intensity was observed when target protein specifically binds with aptamer probe. This might attribute to the fact that the target–aptamer binding event results in additional prevention of electroactive probe from reaching the electrode surface. However, enzyme digestion by endonuclease *Eco*RI brings an obvious increase of the current intensity as shown in line d. Distinctly, this experimental phenomenon agrees absolutely with the sensor designing principle that the unreacted hairpin aptamer probe can be specifically recognized and cut off by restrict endonuclease. As a result, the negative charged digest production parts away from the electrode. Therefore, the electrons transfer ability increases and the current intensity of the redox couple enhances. However, the observed peak current after digestion is a little higher than line c since part of the aptamer probe transfer into its specific structure to bind with target protein, in which the palindrome structure is disappeared and cannot be cut off by *Eco*RI. Obviously, these results further confirm the success in the design of the proposed sensing strategy.

3.3. Optimization of the experimental conditions

3.3.1. Effect of assembly time for aptamer probe

In the proposed study, aptamer probe acts as not only capture probe but also signaling probe. The assembly time of aptmaer probe directly influences the stability and surface density of the aptamer probe. Fig. S1A shows the current response of electrode for different assembly periods of aptamer probe. The detectable current intensity is relatively low when the aptamer probe is incubated for only 10 min since the aptamer probe is probably unstable under such short assembly time. The peak current increases with the augment of assembly time and tends to constant at about 120 min. When the assembly time increases continue, no apparent change in peak current is observed. Therefore, the incubation time of 120 min is used as the optimum time for self-assembly of aptamer probes in subsequent experiments.

3.3.2. Effect of endonuclease concentration

The *Eco*RI restriction endonuclease is one of the most widely used tools for recombinant DNA manipulations. Because the *Eco*RI enzyme has been extremely well characterized, *Eco*RI also serves as a paradigm for other restriction enzymes and as an important model of DNA–protein interactions (Heitman and Model, 1990). The specific recognition site for *Eco*RI endonuclease is the palindrome of GAATTC. The cleavage degree of the palindromic sequence is closely related to the endonuclease concentration. Fig. S1B depicts the current response of the aptameric sensor for different concentration of *Eco*RI. The results shows that the aptamer probes self-assembled onto the electrode surface without target protein can be efficiently cut by *Eco*RI. Compared with the original peak current of about 253 nA, a low peak current of only 14 nA is detected when a low concentration of 0.1 U μL^{-1} endonuclease is used. This result means that about 94% of the signaling aptamer probe is cut off and removed from the electrode surface, indicating the significant digest efficiency of the *Eco*RI restriction endonclease. When the endonuclease concentration further increases, the peak current response to the sensing interface decreases sharply. A zero background current is achieved when the endonuclease concentration reaches 0.9 U μL^{-1}. Concentration more than 0.9 U μL^{-1} is not recommended since exorbitant concentration is not only unnecessary but also possibly deteriorates analytical performance of the sensing interface. Therefore, 0.9 U μL^{-1} of *Eco*RI restriction endonclease is chosen in the subsequent experiments in order to eliminate the background current and to improve the detection capability.

3.3.3. Effect of digestion time

The digestion time of the restriction endonuclease also influences the cleavage degree of the recognition site for *Eco*RI. Different digest periods are investigated as shown in Fig. S1C. The peak current of signaling aptmaer probe modified electrode decreases sharply with increasing the digest time. When the digest reaction is maintained for 10 min using 0.9 U μL^{-1} of *Eco*RI restriction endonclease, the peak current for the sensing interface is only 24 nA. About 91% loos is found compared with the original peak current of 253 nA, suggesting that most of aptamer probes are cut off within several minutes. When a digest time of 120 min is involved, zero background current is achieved, indicating that the signaling aptamer probes are completely cleaved by *Eco*RI restriction endonuclease. These results also show that the digest reaction by *Eco*RI is highly efficient and specific. To achieve a high detection capability, a digest time of 120 min is used in this study.

3.4. Analytical performances of the aptameric sensor

High sensitivity and good specificity are the two most important performances for a successful biosensor. However, most electrochemical aptasensing strategies often suffer from background current, which deteriorates the detection sensitivity directly. The present study utilizes restrict endonuclease to fabricate a new kind of aptamer based biosensing strategy which can not only eliminate the background current but also provides a signal-on format detection system. To validate the utility of this aptameric sensor, different concentrations of target protein were prepared by dissolving and diluting the sample with 0.3 M buffer. The resulting target samples at different concentrations were measured using the described strategy as shown in Fig. 3. The peak current recorded for the proposed biosensor increases with the augment of the concentration of target protein. The current intensity at 0.27 V was used to obtain the calibration curve and

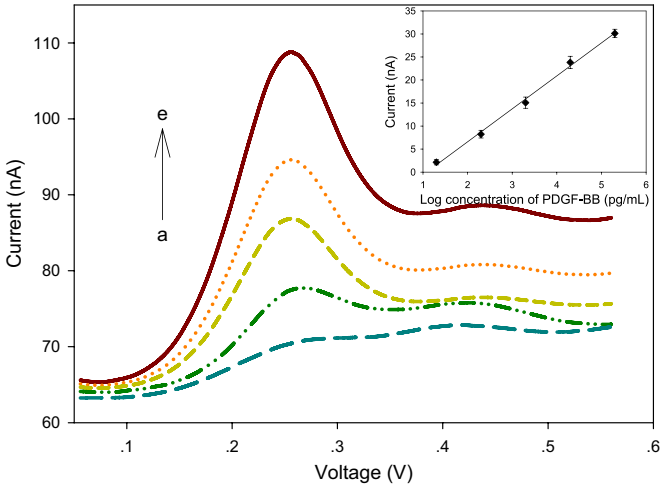


Fig. 3. Current response of the aptameric sensor to different concentration (a: 20 pg mL^{-1}; b: 200 pg mL^{-1}; c: 2 ng mL^{-1}; d: 20 ng mL^{-1}; e: 200 ng mL^{-1}) of target protein. Inset: the linear relationship between peak current intensity and Log concentration of PDGF-BB. All ac voltammogram measurements were performed in 5 mL of 1.0 M $NaClO_4$ solution.

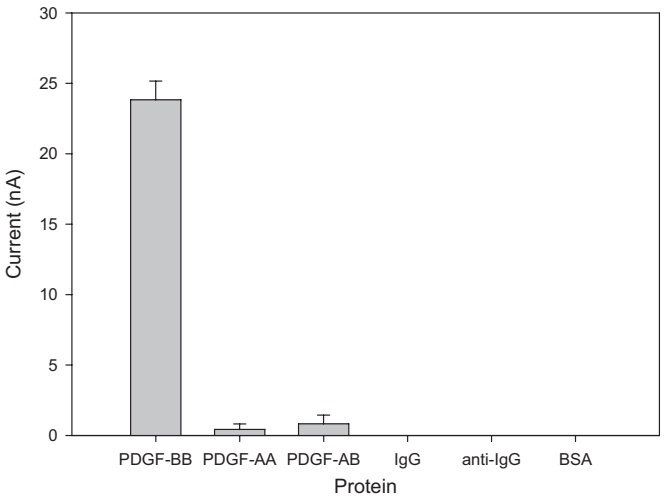


Fig. 4. Specificity investigation: the ac current response of the sensing system after being exposed to different proteins: 20 ng/mL PDGF-BB, 2 μg/mL PDGF-AA, 2 μg/mL PDGF-AB, 1 mg/mL IgG, 1 mg/mL anti-IgG and 10 mg/mL BSA. All measurements were performed in 5.0 mL of 1.0 M $NaClO_4$ solution.

evaluate the analytical performances of the aptameric sensor. As shown in inset of Fig. 3, a wide linear relationship with representative calibration curve was obtained between the peak current value and logarithm of target concentration ranging from 20 pg mL^{-1} to 200 ng mL^{-1}. The calibration equation is $I=7.157 \log C - 7.747$ with a correlation coefficient of 0.9964 ($n=5$, R.S.D=8.9%), where I and C represent the value of peak current (nA) and target concentration (pg mL^{-1}), respectively. The detection limit is estimated at 10 pg mL^{-1}, at which target protein can trigger an observable peak current slightly higher than the blank sample (see Fig. S2). To the best of our knowledge, the detection limit of 10 pg mL^{-1} (about 0.36 pM) is at least 35-fold lower than reported optical methods, such as 75 nM with a colorimetric strategy (Huang et al., 2005), 110 pM with a fluorescence quenching sensor (Fang et al., 2003), and 12.8 pM with a real-time PCR detection method (Yang and Ellington, 2008). Moreover, an improvement by at least 2800-fold in the detection limit was obtained compared with previous label-free electrochemical technique (Degefa and Kwak, 2008; Liao and Cui, 2007). For labeled electrochemical methods, the detection sensitivity achieved in the present work is also superior to or close to reported studies, such as 50 pM with a methylene blue-modified aptamer-based sensor (Lai et al., 2007), 18 pg mL^{-1} with a aptamer-primed polymerase amplification strategy (Huang et al., 2008), and 0.01 pM with a aptamer-based rolling circle amplification method (Zhou et al., 2007) as well as a gold nanoparticle based electrochemical signal amplification approach (Wang et al., 2009). A straightforward comparison of the analytical performance of this work with reported methods is shown in Table S1. However, compared with these transducing strategies mentioned above, the proposed sensing system is much more simple, inexpensive and controllable since only one oligonucleotide sequence was used to fabricate the sensor and no extra amplification means was needed. In addition, the linear dynamic range with 4 orders of magnitude was widened by more than 10-fold, demonstrating the excellent detection capability of the designed sensing scheme. The achievement of unexpected detection ability might attribute to the fact that background current was essentially eliminated by restriction endonuclease mediated digestion.

3.5. Selectivity of the sensing system

Specificity is of significant importance since a successful biosensor adapted to clinical application should not send out false positive or false negative signals. In the present study, the selectivity is mainly determined by the specific recognition ability of anti-PDGF-BB aptamer and the high fidelity of *Eco*RI restriction endonuclease. Only when the aptamer probe bond with the target protein cannot the endonuclease incise the recognition site. As shown in Fig. 4, the current responses to other different proteins were evaluated in order to validate the selectivity of the electrochemical aptameric. The intensity of peak currents upon the homologous proteins (PDGF-AA and PDGF-AB) is not more than 4% of the current intensity triggered by target protein even though their concentration is 100-fold higher than that of PDGF-BB. Moreover, for other heterogenous proteins with concentrations of 10^4-fold higher than that of target protein, no obvious peak current is observed, indicating that the aptamer probes were completely cut off by the high efficient *Eco*RI restriction endonulcease. Such high detection selectivity achieved in the present aptameric sensing system should be attributed to the recognition feature of integrated aptamer and high fidelity of *Eco*RI.

3.6. Reproducibility and reliability

Reproducibility is of significant importance in practical application for a proposed sensor. To test the intra- and inter-assay reproducibility of the proposed sensing system, three samples of different concentrations in the linear detection range were measured with the same electrode or different electrodes. The maximum value of the relative standard deviations was 9.8% ($n=5$) for intra-assay and 11.7% ($n=5$) for inter-assay, indicating that the proposed sensing system could offer an acceptable reproducibility for the protein detection. The results are partly attributed to the change of the electrode positions and/or the difference of the surface areas from electrode to electrode.

To evaluate the applicability and reliability of the proposed sensing system, the recovery experiments for PDGF-BB samples at different concentrations within the dynamic range were carried out. All the measurements were performed for three times, and the results are shown in Table 1. Recovery in the range of 96–105% with the relative standard deviation of 8.3–10.1% was achieved. In order to further assess the reliability of the present aptasensing

Table 1
Recovery of PDGF-BB assay.

Sample	Added PDGF-BB (ng/mL)	Found PDGF-BB (ng/mL)	Recovery (%)	RSD (%)
1	200.0	196.0	98	8.9
2	2.00	1.92	96	8.3
3	0.020	0.021	105	10.1

strategy, real samples were detected by adding different amount of PDGF-BB into human serum matrix as shown in Fig. S3. A wide linear range (0.16–100 ng mL^{-1}) and low detection limit (80 pg mL^{-1}) were achieved as shown in Fig. S4, indicating that the proposed restrict endonuclease based electrochemical aptasensing platform is a promising alternative for tumor maker protein analysis.

4. Conclusions

In the present work, we have demonstrated a new highly selective electrochemical aptameric sensing method based on restriction endonuclease for ultrasensitive detection of protein using PDGF-BB as the model target. The sensing interface is prepared by self-assembling ferrocene labeled aptamer probe onto gold electrode via Au-thiol affinity. Successful fabrication of the biosensor depends on the delicate design by introducing the recognition site for *Eco*RI restriction endonulease into the anti-PDGF-BB aptamer sequence. Under optimum experimental conditions, the background current for blank sample can be completely eliminated and a desirable signal-on response format is achieved. By combination of the recognition ability of aptamer with the high fidelity and efficiency of *Eco*RI, the developed aptameric system can not only provide a remarkably low detection limit with highly selectivity but also exhibit an extremely wide linear response range compared with previous reported aptameric detection systems for the same target molecule. Moreover, by skillfully designing the aptamer probe sequence, the present electrochemical sensing strategy is expected to extend to other analytes, making it universal and useful in potential application including clinical diagnostics, biomedical analysis, drug discovery, and so on.

Acknowledgments

This study is financially supported by the National Natural Science Foundation of China (Grant nos. 21205039, 21205021, 21105017, 21105124, 21405023), Natural Science Foundation of Hunan Province (Grant nos. 13JJ6071, 14JJ7073, 13C620), Open Project of State Key Laboratory of Chemo/biosensing and Chemometrics (Grant no. 2013004), Doctor's Start-up Foundation of Hunan University of Arts and Science and the Construct Program of the Key Discipline in Hunan Province (Applied Chemistry).

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.2014.11.044.

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