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Immunoreaction-triggered DNA assembly for one-step sensitive ratiometric electrochemical biosensing of protein biomarker

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ABSTRACT

A sensitive ratiometric electrochemical readout was designed with an immunoreaction-triggered DNA assembly for one-step, fast and flexible assay of protein biomarker. The sensing interface was prepared by immobilizing a ferrocene (Fc)-labeled hairpin DNA on a gold electrode. In the presence of DNA2-antibody2 (Ab2) and methylene blue (MB)-labeled DNA1-Ab1 probes, the addition of target protein could induce the sandwich immunoreaction among two probes and the protein to trigger the hybridization of DNA1 and DNA2, which subsequently unfolded the hairpin DNA to form a three-arm DNA structure on the sensing interface. The DNA assembly caused the departure of Fc from the electrode and the approach of MB to the electrode, which led to the signal decrease and increase of Fc and MB respectively for ratiometric readout. Using prostate specific antigen (PSA) as a model target, the ratiometric electrochemical assay showed a linear detection range from 0.01 to 200 ng/mL with a detection limit of 4.3 pg/mL (the mean signal of blank measures+3σ). By changing the affinity probe pairs this method could be easily expanded for other protein analytes, showing promising potential for point-of-care testing and extensive applications in bioanalysis.

1. Introduction

The flexible and controllable assembly of DNA nanostructures and nanodevices (Pei et al., 2014) has led to wide application of DNA nanotechnology in various fields including molecular computing, nanomachines, nanomedicine and biosensing (Amir et al., 2014; Wickham et al., 2012; Yuan et al., 2013; Zheng et al., 2014). Among these tremendous achievements, electrochemical DNA biosensors, which possess the characters of easy fabrication, simple signal production, and convenient miniaturization and integration (Bonham et al., 2012; Valle´e-Be´lisle et al., 2012; Zhou et al., 2014), have attracted intensive interest in the design of portable detection settings for biological analytes (Cash et al., 2009; Lubin and Plaxco, 2010).

The electrochemical DNA biosensors are often prepared by immobilizing electroactive molecule labeled hairpin DNA on electrode interface to obtain the sensing signal originated from the hybridization-induced conformational change of the hairpin structure (Fan et al., 2003; Palecek and Bartosik, 2012). In contrast to other single-strand DNA based sensors, the combination of capture and reporter probes within a single hairpin DNA makes the biosensors suitable for the detection of target in one step without the need of exogenous reagents (Cui et al., 2014; Li et al., 2010; Pei et al., 2014). These DNA biosensors have been applied in the measurements of nucleic acids (Xiao et al., 2007a, 2009; Yu and Lai, 2013), small molecules (Ferapontova et al., 2008; Zuo et al., 2007, 2009) and inorganic ions (Xiao et al., 2007b) in complex clinical samples. Particularly, by using hairpin-structural aptamer, the electrochemical DNA biosensors can be designed for protein detection (Lai et al., 2007; Xiao et al., 2005). This work used two DNA conjugated antibodies to introduce proximity hybridization into electrochemical DNA biosensing and developed a method for the detection of cancer-related protein biomarkers.

The protein biomarkers have extensively measured with sandwich immunoassay, including electrochemical immunoassay with electroactive molecule or enzyme labeled secondary antibody (Lin et al., 2014; Malhotra et al., 2012; Zhang et al., 2013). These electrochemical methods often suffer from the multi-step and time-consuming procedure, which limits their application in point-of-care testing (Rusling, 2013). The proximity immunoassay is a newly developed DNA-assisted protein assay technology (Fredriksson et al., 2007; Tavoosidana et al., 2011). By combining the proximity immunoassay with DNA-based electrochemical detection, several electrochemical proximity assays (ECPA) have been

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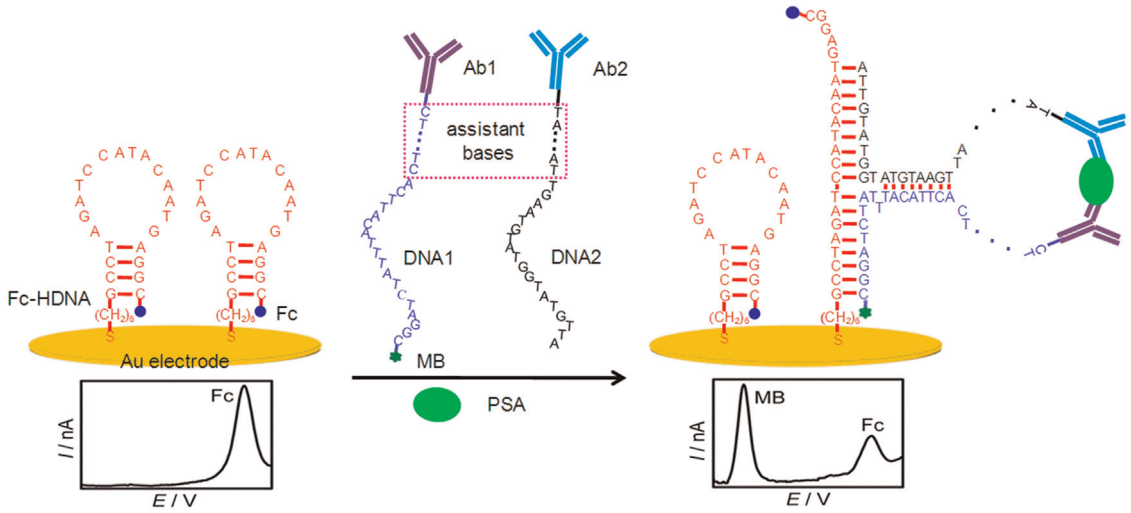

Scheme 1. Schematic illustration of immunoreaction-triggered DNA assembly for ratiometric electrochemical assay of protein biomarker.

developed for one-step and fast detection of protein biomarkers (Zhang et al., 2007). For example, an ECPA has been proposed for direct readout of protein by employing the proximity effect to regulate the hybridization of methylene blue (MB) labeled DNA on the electrode surface (Hu et al., 2012, 2014). Our previous work also reported a ratiometric ECPA by using target-triggered DNA displacement to change the distances of electrode and two electroactive molecule, ferrocene (Fc) and MB (Ren et al., 2014), in which the biosensor was constructed through the hybridization of antibody-DNA probe on hairpin DNA modified electrode, and the immunoreaction as well as the proximity hybridization happened heterogeneously on the biosensor surface. Thus, the biosensor preparation was relatively long (> 12 h), and the heterogeneous reactions increases the assay time.

Here an immunoreaction-triggered DNA assembly strategy was designed by using proximity hybridization to form a sequence for opening Fc-labeled hairpin DNA (Fc-HDNA) that was immobilized on electrode surface (Scheme 1). The proximity hybridization happened among methylene blue (MB)-labeled DNA1-antibody 1 (Ab1) probe, target protein and DNA2-Ab2 probe. Except the hybridization sequences, both DNA1 and DNA2 were designed to contain 35 assistant bases to avoid the steric hindrance caused from the sandwich complex. The formation of three-arm DNA structure brought MB to electrode surface, while Fc labeled to HDNA left from the surface, leading to the increase and decrease of the electrochemical oxidation signals of Fc and MB, respectively. The ratiometric readout of electrochemical signal led to high sensitivity of the proposed method. Since the immunoreaction and proximity hybridization happened in homogeneous solution, the reactions were more efficient and faster than those happened on sensing interface (Hu et al., 2012, 2014; Ren et al., 2014), showing a shorter assay time and a lower detection limit. In addition, the assay was performed on an electrochemical DNA sensor, and could be used to detect other protein analytes directly by using corresponding affinity pairs, showing better extensibility than the previous report (Ren et al., 2014). This assay possessed the advantages of easy operation and good flexibility, and could be used for point-of-care testing and bioanalysis.

2. Experimental

2.1. Materials and reagents

Bovine serum albumin (BSA), mercaptohexanol (MCH) and tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was supplied by Heowns Biochem LLC (Tianjin, China). PSA and mouse monoclonal anti-PSA antibodies (clone no. P27B1 as Ab1 and P27A10 as Ab2) were purchased from Shuangliu Zhenglong Biochem. Lab (Chengdu, China). The clinical serum samples from prostate cancer patients were supplied by Jiangsu Institute of Cancer Prevention and Cure (China). Ultrapure water obtained from a Millipore water purification system (≥ 18 MΩ, Milli-Q, Millipore) was used in all assays. Dithiothreitol (DTT) and oligonucleotides were obtained from Sangon Biotechnology Inc. (Shanghai, China), and their sequences were listed as follows:

Fc-HDNA: 5′-Fc-CGGAG**TAACATACC**TAGATCCG-$(CH_2)_6$-SH-3′

Thiolated DNA1: 5′-MB-CGGATCTATT**TACATTCA**CTTCTTA-TATTATATT*CTCTCTC TCTCTCTCTC*-SH-3′

Thiolated DNA2 (8-bp): 5′-SH-*TAGGAAAAGGAGGAGGGTGGT*-TATTATTATTATAT**TG AATGTA**T**GGTATGTTA**-3′

DNA2 (6-bp): 5′-*TAGGAAAAGGAGGAGGGTGG*TTATTATTATTATT-TAC**AATGTA**T **GGTATGTTA**-3′

DNA2 (8-bp): 5′-*TAGGAAAAGGAGGAGGGTGG*TTATTATTATTA-TAT**TGAATGTA**T **GGTATGTTA**-3′

DNA2 (9-bp): 5′-*TAGGAAAAGGAGGAGGGTGG*TTATTATTAT-TATT**GTGAATGTA**T **GGTATGTTA**-3′

DNA2 (10-bp): 5′-*TAGGAAAAGGAGGAGGGTGG*TTATTATTATTA-T**AGTGAATGTA**T **GGTATGTTA**-3′

Ref DNA: 5′-*CCACCCTCCTCCTTTCCTA*TTTTCTTTAATTTCT-TATTTCTCTTAATTT TAATTTGTTT*GAGAGAGAGAGAGAGAGAGA*-3′

The binding regions between Fc-HDNA and DNA1 and DNA2 were shown in underlined and bold, respectively. The binding regions between DNA1 and DNA2 were shown in both bold and underlined. The binding regions between Ref DNA and DNA1 and DNA2 were shown in italics.

2.2. Apparatus

The electrochemical measurements were performed on a CHI 660D electrochemical workstation (CH Instruments Inc., USA) at room temperature with a conventional three-electrode system composed of a platinum wire as counter, Ag/AgCl as reference and the Au electrode as working electrodes. Electrochemical impedance spectroscopic (EIS) measurements were carried out on a PGSTAT30/FRA2 system (Autolab, the Netherlands) in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$.

2.3. Preparation of MB-DNA1-Ab1 and DNA2-Ab2 probes

The probes were synthesized by chemically cross-linking the respective antibody to MB-DNA1 and DNA2 (Zong et al., 2014). The antibody (2 mg/mL) was firstly reacted with a 20-fold molar excess of SMCC in PBS (55 mM phosphate, pH 7.4, 150 mM NaCl, 20 mM EDTA) for 2 h at room temperature. In parallel, 3 μL of 100 μM MB-DNA1 or DNA2 was reduced with 4 μL of 100 mM DTT in PBS for 1 h at 37 °C. Both the products were purified by ultrafiltration (10,000 MW cut-off membrane, Millipore) for eight times and the buffer was changed to PBE (55 mM phosphate, pH 7.4, 150 mM NaCl, 5 mM EDTA). After the products were mixed to incubate over night at 4 °C and the unreacted DNA was removed by ultrafiltration (100,000 MW cut-off membrane, Millipore) for eight times, the MB-DNA1-Ab1 and DNA2-Ab2 probes were obtained.

2.4. Fabrication of electrochemical DNA biosensor

Gold electrode (~2 mm diameter, CH Instrument Inc.) was polished carefully to a mirror surface with aqueous slurry of 0.3 μm diameter alumina particles and then successively washed in an ultrasonic cleaner with water and ethanol. The electrode was then immersed into fresh piranha solution (H_2SO_4/H_2O_2, 3:1) for 10 min, rinsed with water, and dried by nitrogen. Finally, the gold electrode was electrochemically polished by scanning the potential from −0.2 to +1.6 V in 0.5 M H_2SO_4 at a scan rate of 0.1 V/s for 40 cycles. The cleaned gold electrode was thoroughly washed with water and dried under flowing nitrogen.

3 μL of Fc-HDNA (10 μM) was incubated with 3 μL of TCEP (1 mM) for 1 h to allow reduction of disulfide bonds. This solution was diluted to a total volume of 100 μL with 10 mM tris-HCl buffer (pH 7.4, 0.1 M NaCl) to obtain a final concentration of 0.3 μM reduced Fc-HDNA, then 6 μL of the above solution was dropped on the electrode to incubate at room temperature for 2 h. After rinsed with 10 mM tris-HCl buffer (pH 7.4, 0.1 M NaCl) and dried with nitrogen, 6 μL of 1 mM MCH was dropped on the electrode for 1 h to block the unmodified sites. After washing with 10 mM tris-HCl buffer (pH 7.4, 0.1 M NaCl) and drying with nitrogen, the electrochemical DNA biosensor was obtained and stored at 4 °C before use.

2.5. Measurement procedure

Prior to measurement, 9 μL of 10 mM tris-HCl buffer (pH 7.4, 0.1 M NaCl) supplemented with 0.5% BSA (w/v) and 50 nM MB-DNA1-Ab1 and DNA2-Ab2 probes were mixed with 1 μL PSA at various concentrations or serum samples obtained from patients with prostate cancer. Then, 6 μL of the mixture was dropped on the biosensor surface for 30 min incubation, followed by washing with 10 mM tris-HCl buffer (pH 7.4, 0.1 M NaCl). The biosensor was then immersed in 10 mM pH 7.4 PBS for alternating current voltammetric (ACV) detection from −0.4 to +0.6 V with a step potential of 4 mV, a frequency of 25 Hz and an amplitude of 25 mV.

3. Results and discussion

3.1. Feasibility of the ratiometric electrochemical immunoassay

EIS measurements were performed in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to monitor the biosensor preparation and measurement procedure (Fig. 1A). Compared with bare Au electrode (curve a), the Fc-HDNA modified Au electrode showed a much larger R_{et} (curve b) because the negatively charged Fc-HDNA could repel the $[Fe(CN)_6]^{3-/4-}$ to the electrode surface, which reflected the successful immobilization of Fc-HDNA. After further modification with MCH, the R_{et} increased (curve c) due to the steric repulsion of MCH. In the presence of target PSA and probes, the immunoreaction triggered the hybridization between DNA1 and DNA2 and subsequently unfolded the Fc-HDNA to form three-arm DNA structure on biosensing interface, leading to the increase of the corresponding R_{et} (curve e). In contrast, weak increase of R_{et} was observed when the biosensor was incubated with only probes (curve d), confirming the immunoreaction- triggered DNA assembly.

The feasibility of the ratiometric electrochemical assay was also confirmed by ACV detection (Fig. 1B). An obvious oxidation peak of Fc at about +0.44 V was observed on Fc-HDNA constructed biosensor (curve a). After the biosensor was incubated with the solution containing one of the DNA-Ab probes, PSA, and the mixture of PSA and one of the probes, respectively, similar ACV responses as that of the biosensor itself were observed (curves b, c, d, e, and f), indicating single probe, PSA, or the mixture of them could not induce any structure change of Fc-HDNA. When the biosensor was incubated with probes for 30 min, a weak signal of MB at −0.22 V along with a corresponding decrease of Fc was observed. This phenomenon was attributed to the slight self-hybridization between DNA1 and DNA2 to unfold small amount of Fc-HDNA (curve g), which could be served as background. However, when the biosensor was incubated with the mixture including both PSA and probes, the simultaneous recognition of the target protein by two probes triggered the hybridization of DNA1 and DNA2, which subsequently unfolded Fc-HDNA to form the three-arm DNA structure on biosensor, resulting in the departure of Fc and approach of MB to the electrode surface. This process caused the decrease and increase of oxidation signals of Fc and MB, respectively (curve h).

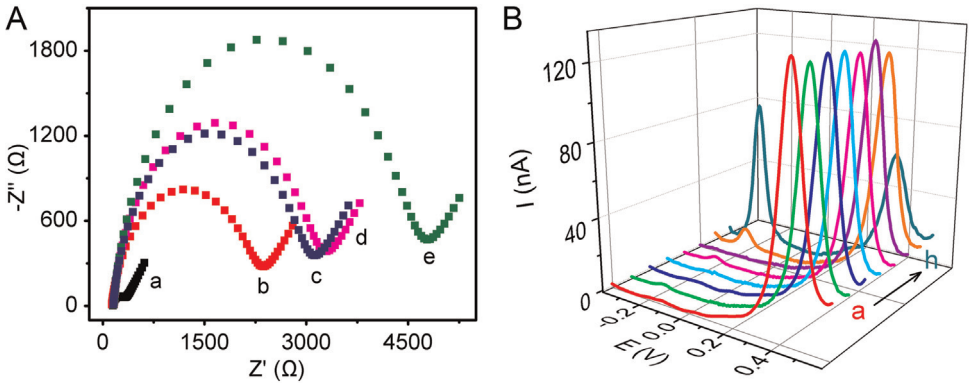

Fig. 1. (A) EIS of bare Au electrode (a), Fc-HDNA modified Au electrode (b), DNA biosensor (c), biosensor reacted with 50 nM probes in absence (d) and presence (e) of 10 ng/mL PSA. (B) ACV responses of the biosensor (a), biosensor incubated with MB-DNA1-Ab1 (b), DNA2-Ab2 (c), PSA (d), and the mixtures of MB-DNA1-Ab1 and PSA (e), DNA2-Ab2 and PSA (f), MB-DNA1-Ab1 and DNA2-Ab2 (g), and MB-DNA1-Ab1, DNA2-Ab2 and PSA (h). The concentrations of probe and PSA were 50 nM and 10 ng/mL.

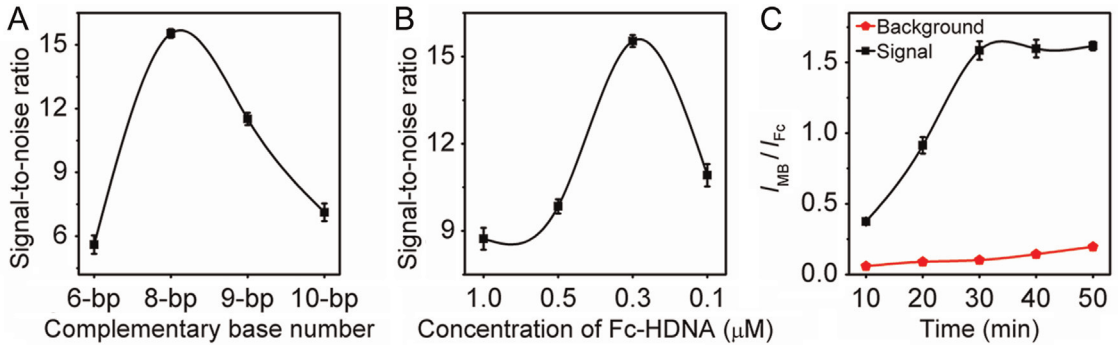

Fig. 2. Effects of (A) complementary base number between DNA1 and DNA2, (B) Fc-HDNA concentration on signal-to-noise ratio, and (C) dependence of I_{MB}/I_{Fc} on incubation time in the presence of 50 nM probes and 10 ng/mL PSA. Error bars represent standard deviations of three parallel experiments.

3.2. Optimization of detection conditions

The detection mechanism of the ratiometric electrochemical immunoassay relied on simultaneous recognition of target protein by two probes, which brought DNA1 and DNA2 in proximity to hybridization and subsequently unfold Fc-HDNA. Thus, the number of complementary bases between DNA1 and DNA2 should be firstly optimized. In order to simplify the optimization procedure, 80-nucleotide Ref DNA was used to mimic the sandwich immunocomplex (Hu et al., 2012; Kim et al., 2010). At low number of complementary bases, DNA1 and DNA2 could not hybrid even in close proximity with the help of target, while at high number of complementary bases, DNA1 and DNA2 could self-hybrid and produce a large noise. Fig. 2A shows the signal-to-noise of the system using DNA2 with 6, 8, 9 and 10 complementary bases (bp) to DNA1. In the presence of 10 nM Ref DNA, the signal-to-noise ratio quickly increased with increasing the number of complementary bases from 6 bp to 8 bp and then decreased. According to the maximum signal-to-noise ratio, DNA2 with 8-bp complementary to DNA1 was chosen for the subsequent experiments.

The density of Fc-HDNA immobilized on the gold electrode could also affect the analytical performance of the biosensor (Rant et al., 2004). After the Au electrode was incubated with Fc-HDNA for 2 h at room temperature, the maximum value of signal-to-noise ratio was achieved at the Fc-HDNA concentration of 0.3 μM (Fig. 2B). Hence, the biosensor was prepared with 6 μL of 0.3 μM Fc-HDNA.

The incubation time was another important parameter affecting the analytical performance. The value of I_{MB}/I_{Fc} was obtained by performing immunoreaction-triggered DNA assembly on the biosensor with different incubation times. As shown in Fig. 2C, the value of I_{MB}/I_{Fc} greatly increased with the increasing incubation time till 30 min, while the background was little changed. To obtain high signal-to-noise ratio, the optimum incubation time should be 30 min. The whole measuring time in this work was 30 min which was shorter than most of the previous works for PSA detection (Chen et al., 2014; Li et al., 2014; Ren et al., 2014).

3.3. Assay performance

The ratiometric electrochemical immunoassay could be conveniently used for the detection of protein biomarker under optimal experimental conditions. As shown in Fig. 3A, with the increasing PSA concentration, the Fc oxidation peak decreased and the MB oxidation peak increased correspondingly. The logarithmic value of I_{MB}/I_{Fc} increased linearly with the increasing logarithm of PSA concentration, leading to a detectable range from 0.01 to 200 ng/mL with a correlation coefficient of 0.9971 (Fig. 3B). The linear regression equation was $\log(I_{MB}/I_{Fc}) = 0.26 \log[\mathrm{PSA}] - 0.091$. Thus the detection limit could be calculated with the equation of $[\mathrm{PSA}] = 10^{(\log(I_{MB}/I_{FC})+0.091/0.26)}$ (where I_{MB}/I_{Fc} is the mean signal of blank measures $+3\sigma$, and σ is the standard deviation of three parallel blank measures. The former is 0.158, and $\sigma = 0.013$). The obtained detection limit was 4.3 pg/mL, which was much lower than that of DNA assembly-based fluorescent assays (Li et al., 2012, 2013). The pg/mL level detection limit with the detectable range of 4 orders of magnitude was comparable to other electrochemical immunoassays using nanomaterials for signal amplification (Lai et al., 2013; Yu et al., 2006) and DNA-based ECPAs (Hu et al., 2012, 2014), and lower than that reported previously (Ren et al., 2014). Compared with the calibration plots for PSA using individual MB or Fc signal (Fig. 3C), ratiometric readout showed wider detectable range and lower detection limit, which was 9.2 and 9.7 pg/mL, respectively.

The selectivity of the proposed method was evaluated by comparing the I_{MB}/I_{Fc} values toward the solutions containing either PSA or other antigen, for example carcinoembryonic antigen (CEA) (Fig. 4). As expected, the ratiometric electrochemical assay with immunoreaction-triggered DNA assembly showed obvious response to the solution containing target PSA, while negligible

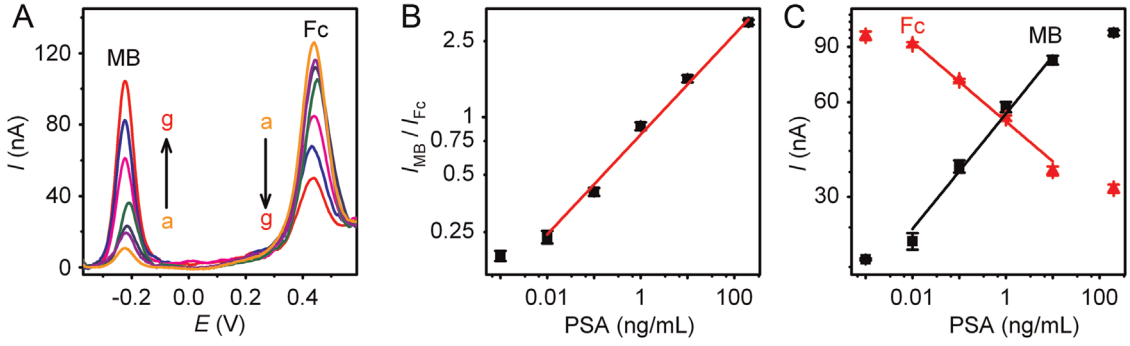

Fig. 3. (A) ACV responses for 0, 0.001, 0.01, 0.1, 1, 10 and 200 ng/mL PSA under optimal conditions (from a to g), (B) logarithmic dependence of I_{MB}/I_{Fc} on PSA concentration, and (C) logarithmic dependence of MB or Fc peak current on PSA concentration. Error bars represent standard deviations of three parallel experiments,

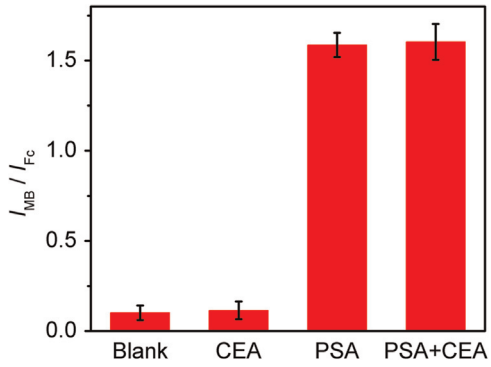

Fig. 4. I_{MB}/I_{Fc} for blank control, 10 ng/mL CEA, 10 ng/mL PSA, and the mixture of 10 ng/mL CEA and PSA. Error bars represent standard deviations of three parallel experiments.

Table 1
Assay results of clinical serum samples using the proposed and reference methods.

Sample no.	Proposed method (ng/mL)	Reference method[a] (ng/mL)	Relative error (%)
1	0.73	0.69	5.79
2	14.3	15.1	−4.69
3	6.23	6.12	1.47
4	42.6	41.3	3.14

[a] The reference levels were detected with an automated electrochemiluminescent analyzer (Elecsys 2010, Roche).

response was observed in the CEA solution. This result indicated few non-specific binding, showing excellent detection specificity for PSA.

3.4. Real sample analysis

To evaluate the practicability of the proposed method, clinical serum samples from prostate cancer patients were further analyzed, and the assay results were compared with the reference values from the commercial electrochemiluminescent testing. The results were shown in Table 1, an acceptable agreement with relative errors less than 5.79% indicated good accuracy of the proposed method for the detection of clinical samples.

4. Conclusions

This work proposed a simple, sensitive, fast and flexible electrochemical biosensing method for ratiometric detection of protein biomarker using an immunoreaction-triggered DNA assembly on DNA biosensor. The sandwich immunoreaction among target protein and two DNA-Ab probes triggered the hybridization of DNA1 and DNA2 to form a sequence for subsequently unfolding the hairpin DNA, which formed a three-arm DNA structure on sensing interface and generated measurable ratiometric electrochemical signal. Using PSA as model target, the proposed assay showed a detection range over 4 orders of magnitude with a detection limit of pg/mL level. The assay could be carried out with one step in 30 min, and easily expanded to the detection of other protein analytes by using corresponding affinity pairs.

Acknowledgments

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