Ratiometric electrochemical proximity assay for sensitive one-step protein detection

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This work proposes the concept of ratiometric electrochemical proximity assay (REPA), which can be used for one-step, highly sensitive and selective detection of protein. The assay strategy was achieved on a sensing interface that was formed by hybridization of methylene blue (MB)-labeled antibody-DNA probe (MB-DNA1-Ab1) with ferrocene (Fc)-labeled DNA capture probe (Fc-P) modified gold electrode. On the interface the target protein could trigger the formation of immunocomplex between MB-DNA1-Ab1 and detection antibody-DNA probe (Ab2-DNA2) and subsequently the proximity hybridization of DNA1-DNA2, which led to the departure of MB-DNA1-Ab1 from the interface. The remained Fc-P could form a hairpin structure to take Fc group to electrode surface. Therefore, the recognition of target protein to Ab1 and Ab2 resulted in both the "signal-off" of MB and the "signal-on" of Fc for dual-signal electrochemical ratiometric readout. The proposed REPA could be carried out in one-step with 40-min duration and showed a wide detection range from 0.05 to 100 ng/mL with pg/mL limit of detection, displaying great potential for convenient point-of-care testing and commercial application.

A s the levels of tumor biomarkers in serum/tissue are positively correlated with the stages of tumors, accurate tumor marker detection with a simple operation is very important in early cancer screening and evaluation[1–4]. The development of rapid, easy-to-use, low-cost point-of-care methods for the quantitative detection of tumor markers has attracted the great efforts of the scientific organizations[5]. Immunoassay is one of the dominant methods for detection of tumor protein marker due to its highly specific molecular recognition between antibody and antigen. However, conventional immunoassays such as enzyme-linked immunosorbent assay (ELISA) are difficult to realize point-of-care detection[6]. Currently, DNA-assisted protein detections have been developed for clinical diagnosis, food and environmental analyses[7–11]. In these techniques, powerful nucleic acid detection is used for protein analysis by equipping protein-binding reagent, typically antibody, with DNA strand to improve the analytical performance[12]. Immuno-PCR is one of the most successful examples[13]. Similar to ELISA, it is performed on a capture antibody immobilized solid-support. After a sandwich immunoreaction, the DNA sequence labeled on the immunocomplex is exponentially amplified by PCR to sensitively signal the immune-recognition event[14–18]. The sensitivity of immuno-PCR can be further improved by introducing a biobarcode probe[19]. Other isothermal DNA amplification strategies, such as rolling circle amplification and hybridization chain reaction, have also been employed for sensitive protein detection by in situ DNA amplification[20–25]. Unfortunately, these assays require multi-step procedures, which limits their application in accurate and quick point-of-care testing.

The proximity ligation assay (PLA) is a newly developed homogenous DNA-assisted immunoassay. Its detection mechanism relies on the simultaneous recognition of target protein by a pair of PLA probes and the replication of the ligation product with PCR[26–29]. Compared with ELISA and immuno-PCR, PLA is much simpler and faster[30–33]. This work combined the ratiometric detection technology with the proximity ligation to propose a novel concept, named as ratiometric electrochemical proximity assay (REPA), for one-step sensitive detection of protein.

Electrochemical devices are attractive in point-of-care testing because of their high sensitivity with relatively simple and low-cost measurement systems[2,34]. By designing artful target-induced DNA assemblies on the electrode surface, various electrochemical bioanalysis strategies have been proposed for the detection of DNA and proteins[35–38]. For example, by combining the proximity ligation with DNA-based electrochemical detection, different electrochemical proximity assays have been presented[39–41]. In these assays, the target protein can be

measured directly by using the proximity effect to regulate the electrochemical active label on electrode surface. However, these methods generally show relatively narrow detection range. The introduction of ratiometric detection technology in this work greatly extended the detectable concentration range.

Ratiometric detection has extensively been developed in fluorescence and electrochemiluminescence analysis of biomolecules[42–44]. Recently this technology has been employed in voltammetric detection of metal ion using a two-channel biosensor array[45]. Similar to a recently reported sensing interface, which was structured using a methylene blue (MB)-labeled DNA modified gold electrode to hybridize ferrocene (Fc)-labeled aptamer probe for detection of ATP with the sum of peak current changes[46], this work immobilized Fc-labeled DNA probe (Fc-P) and MB-labeled antibody-DNA (MB-DNA1-Ab1) at the same electrode. Upon the proximity hybridization with another antibody-DNA triggered by target protein, MB released from the electrode surface and Fc approached to the electrode due to the formation of a hairpin structure, which led to "signal-off" and "signal-on" elements for dual-signal electrochemical ratiometric readout (Figure 1). The dual-peak current ratiometry provided precise and sensitive measurement. The excellent performance of the proposed REPA for detection of prostate specific antigen (PSA) showed potential application of this strategy in convenient point-of-care testing.

Results

Characterization of REPA. Electrochemical impedance spectroscopic (EIS) measurements were performed in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to provide information on the surface conductivity of the REPA sensor (Figure 2A)[47]. Compared with the bare Au electrode (curve a), the Fc-P modified Au electrode showed a much larger R_{et} (curve b) because the self-assembly layer of negatively charged Fc-P could repel the $[Fe(CN)_6]^{3-/4-}$ to the electrode surface. Subsequent surface blocking with mercaptohexanol (MCH) led to an increase of R_{et} (curve c). After MB-DNA1-Ab1 was captured on the Au electrode through its hybridization with Fc-P, the R_{et} further increased due to the insulativity of protein (curve d). Similarly, target protein, PSA, could also resist the electron transfer, resulting in the increasing impedance (curve e), which reflected the successful recognition between MB-DNA1-Ab1 and PSA. However, in the presence of both target PSA and detection antibody-DNA probe
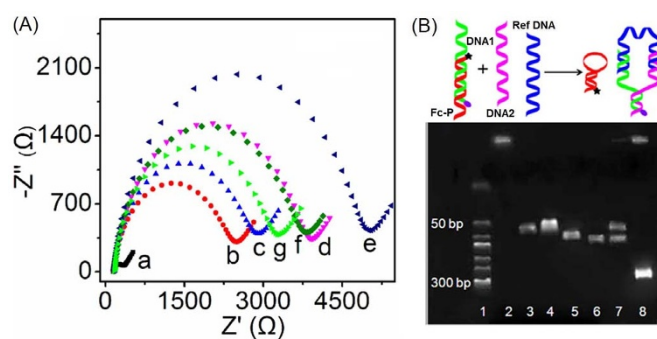

Figure 2 | Characterization of REPA. (A) EIS of bare Au electrode (a), Fc-P modified Au electrode (b), (b) blocked with MCH (c), REPA sensor (d), (d) reacted with 10 ng/mL PSA (e), (d) reacted with 50 nM Ab2-DNA2 (f), and (d) reacted with the mixture of 10 ng/mL PSA and 50 nM Ab2-DNA2 (g). (B) PAGE analysis of DNA-based REPA. Lanes 1–8 represent the DNA ladder marker, Fc-P, DNA1, DNA2, Ref DNA, the mixture of Fc-P and DNA1, the mixture of Fc-P, DNA1 and DNA2, and the mixture of Fc-P, DNA1, DNA2 and Ref DNA at 10 μM, respectively. DNA2 (L13) was used in PAGE.

(Ab2-DNA2), the immunoreaction of Ab1-target-Ab2 triggered the proximity hybridization of DNA1-DNA2 and the formation of a cooperative complex between MB-DNA1-Ab1 and Ab2-DNA2, leading to the dissociation of MB-DNA1-Ab1 from the sensor surface, and thus the decrease of the corresponding R_{et} (curve g). In contrast, no change of R_{et} was observed after the REPA sensor was incubated only with Ab2-DNA2 (curve f). Overall, all these experimental results demonstrated the successful fabrication of the sensor and the REPA of PSA according to Figure 1.

Furthermore, this work utilized a Ref DNA to mimic the Ab1-target-Ab2 complex in REPA for polyacrylamide gel electrophoresis (PAGE) analysis (Figure 2B). The Ref DNA could bring the DNA1 and DNA2 in proximity to form a cooperative complex. Fc-P, DNA1, DNA2 and Ref DNA exhibited individual clear band, respectively (lanes 2–5). As Fc-P had 12 bases complementary to DNA1, the mixture of Fc-P and DNA1 produced dsDNA, which moved slower than both Fc-P and DNA1 (lane 6). In the absence of Ref DNA, the mixture of Fc-P, DNA1 and DNA2 displayed two bands located in
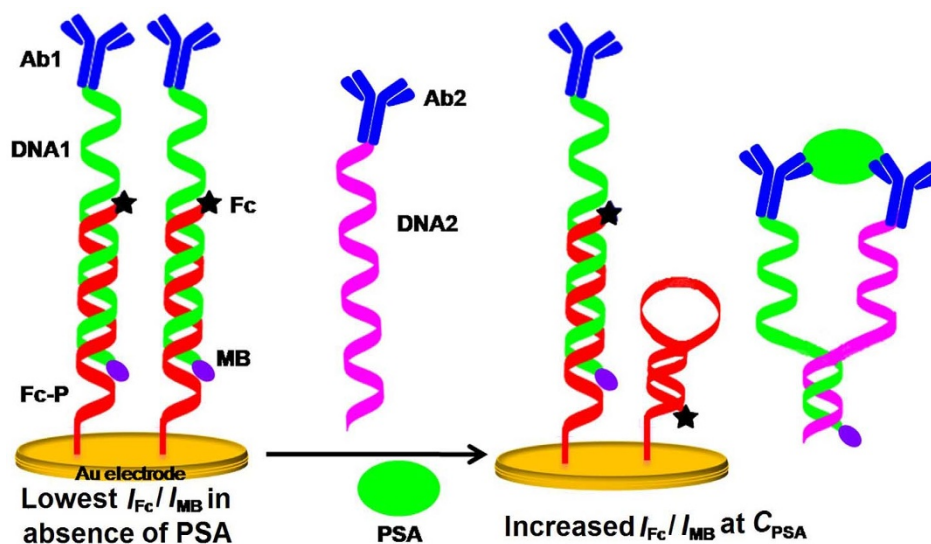

Figure 1 | Schematic illustration of REPA. The assay uses Fc-P modified gold electrode to hybridize MB-DNA1-Ab1 for preparation of sensing interface. The target protein triggers the immunoreaction of Ab1-target-Ab2 and subsequently the proximity hybridization of DNA1 and DNA2, which leads to the departure of MB-DNA1-Ab1 from the sensing surface and the formation of a hairpin structure of Fc-P to produce "signal-off" of MB and "signal-on" of Fc for dual-signal electrochemical ratiometric readout.
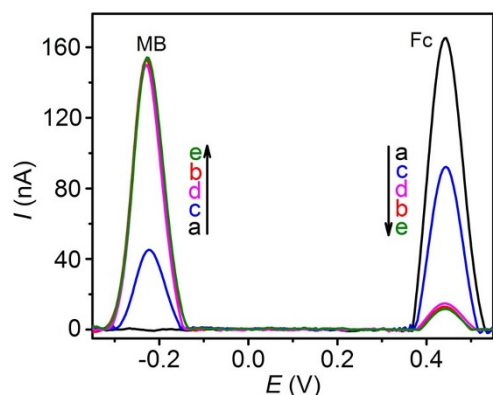
Figure 3 | Feasibility of REPA. ACV responses of Fc-P modified Au electrode (a), REPA sensor (b), (b) incubated with Ab2-DNA2 in presence (c) and absence (d) of 10 ng/mL PSA, and (b) incubated with 10 ng/mL PSA without Ab2-DNA2 (e).

the same position as the dsDNA and DNA2 (lane 7), indicating no hybridization occurred between dsDNA and DNA2. Upon the addition of Ref DNA to the mixture of Fc-P, DNA1 and DNA2, a new band with a slow migration speed appeared, along with the appearance of Fc-P band (lane 8). This result should be contributed to the formation of proximate complex product of DNA1-Ref DNA-DNA2 and the displacement of Fc-P from dsDNA, verifying the proximity hybridization induced DNA displacement strategy.

Feasibility of REPA. The feasibility of REPA was investigated by measuring alternating current voltammetric (ACV) responses of different modified electrodes (Figure 3 and Supplemental Data Figure s1). The Au electrode modified with hairpin structured Fc-P showed only large oxidation peak of Fc at about +0.44 V (curve a). After hybridization with MB-DNA1-Ab1, a sharp MB peak appeared at −0.22 V, accompanied by a substantial decrease of the Fc response (curve b), suggesting the successful construction of the REPA sensor according to Figure 1. Here, although MB and Fc underwent a 2-electron and 1-electron transfer upon the electrochemical oxidation, respectively, they showed similar change of absolute current, indicating their different electron-transfer rate constants due to the different distances from the electrode[48]. When the REPA sensor was incubated both with PSA and Ab2-DNA2, the simultaneous recognition of the target protein by the two Ab-DNA probes along with the hybridization of complementary bases in DNA1 and DNA2 led to the formation of proximate cooperative complex between MB-DNA1-Ab1 and Ab2-DNA2, resulting in the dissociation of MB-DNA1-Ab1 from the electrode, meanwhile, the complementary base pairs in left Fc-P formed a hairpin structure on the electrode surface. As a result, the Fc group approached electrode surface to increase its peak current, and the peak current corresponding to the oxidation of MB significantly decreased (curve c). In contrast,

in the absence of target protein, no current change was observed for both MB and Fc (curve d), indicating that the hybridization between DNA1 and DNA2 did not happen without the help of proximity effect. Therefore, the proximity ligation induced DNA displacement and the following peak current changes depended on the presence of target protein. Interestingly, in the absence of DNA2, although the target protein could be recognized by MB-DNA1-Ab1 to be bound on electrode surface, the ACV signal changes of both MB and Fc were also very slight (curve e), indicating the bound protein did not affect the probe dynamic position on surface. This could be attributed to the controllable surface structure of the sensor, which led to the stable distance between the electrode and electroactive groups, thus their electron-transfer constants did not obviously change.

Optimization of detection conditions. The detection mechanism of REPA relied on simultaneous recognition of target protein by a pair of antibody-DNA affinity probes which brought DNA1 and DNA2 in proximity to process the DNA displacement. Thus, the number of complementary bases between DNA1 and DNA2 should be firstly optimized. This was because at low number of complementary bases DNA2 could not displace the DNA1 from the sensor surface even with the help of proximate effect, while at high number of complementary bases DNA2 could hybridize with DNA1 without the help of proximate effect, producing a large background. Figure 4A shows the signals and backgrounds of the REPA when using Ref DNA and DNA2 with 11, 13 and 15 complementary bases (L11, L13, and L15) to DNA1 for proximity hybridization. In the presence of 10 nM Ref DNA, the ratiometric peak current (I_{Fc}/I_{MB}) increased with the increasing number of complementary bases from 11 to 15. However, the large number of complementary bases also caused a larger background. Thus, according to the maximum signal-to-background ratio, the DNA2 (L13) was chosen for the subsequent experiments.

The incubation time was another important parameter affecting the analytical performance of REPA. At room temperature, the value of I_{Fc}/I_{MB} greatly increased with the increasing incubation time till 40 min, while the background was little changed (Figure 4B). To obtain high sensitivity, the optimum incubation time should be 40 min.

The density of immobilized Fc-P onto the gold electrode could affect the analytical performance of the sensor. At an assembly time of 2 h at room temperature, the maximum value of I_{Fc}/I_{MB} occurred at the Fc-P concentration of 0.5 μM (Figure 4C). Higher density when higher Fc-P concentration was used would induce steric hindrances, which decreased the ability of Fc-P to form the hairpin structure on the electrode surface, while lower density would affect the detection efficiency of the sensor to target protein. Hence, the sensor was prepared with 6 μL of 0.5 μM Fc-P.

Assay performance. The analytical performance of REPA was characterized under optimal experimental conditions. As shown in Figure 5A and Supplemental Data Figure s2, with the increasing PSA concentration, the MB oxidation peak decreased and the Fc
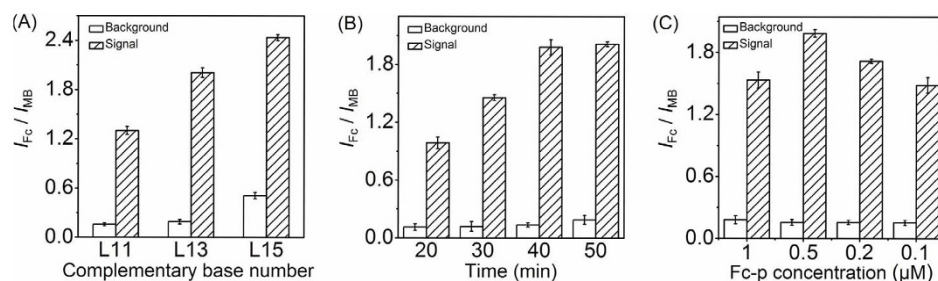

Figure 4 | Optimization of detection conditions. Effects of (A) complementary base number between DNA1 and DNA2, (B) incubation time and (C) Fc-P concentration on I_{Fc}/I_{MB}. Error bars represent standard deviations of three parallel experiments.
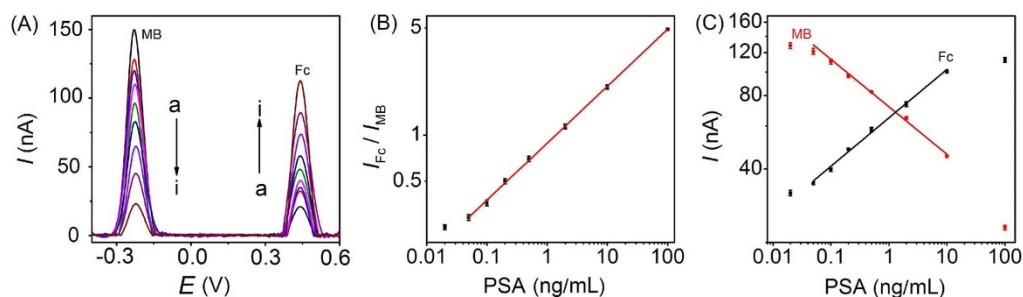
Figure 5 | Sensitive detection of PSA. (A) ACV responses of REPA sensor to 0, 0.02, 0.05, 0.1, 0.2, 0.5, 2, 10, and 100 (from a to i), (B) logarithmic dependence of I_{Fc}/I_{MB} on PSA concentration, and (C) logarithmic dependence of MB (a) or Fc (b) peak current on PSA concentration. Error bars represent standard deviations of three parallel experiments.

oxidation peak increased correspondingly. The logarithmic value of I_{Fc}/I_{MB} linearly depended on the logarithm of PSA concentration in the range of 0.05 to 100 ng/mL with a correlation coefficient of 0.9999 (Figure 5B). The linear regression equation was $\log(I_{Fc}/I_{MB}) = 0.37 \log[C] - 0.053$. The detection limit corresponding to a signal-to-noise ratio of 3 was 16 pg/mL. It was worthy to note that the electrochemical ratiometry displayed a more sensitive and effective detection than that using single redox label. Figure 5C shows the calibration plots of the proximity assay using individual Fc or MB signal for PSA detection. Both of them exhibit good linear logarithmic relationship between the oxidation peak current and PSA concentration in the range of 0.05 to 10 ng/mL, along with detection limits of 43 and 48 pg/mL, respectively. Obviously dual-signal ratiometric readout extends the linear range for 1 order of magnitude, which is beneficial to real clinical application. The detection limit of the proposed REPA was also lower than other electrochemical immunoassays using complex amplification strategies[49,50].

The selectivity of the REPA was evaluated by comparing the I_{Fc}/I_{MB} values toward the solutions containing either PSA or other antigen only, for example carcinoembryonic antigen (CEA), and the mixture of PSA and CEA (Figure 6). As expected, the REPA showed obvious responses to the solutions containing target PSA, while negligible response was observed in the CEA solution, indicating few non-specific binding was observed. Overall, the high sensitivity, wide detection range, easy operation and good selectivity of the REPA indicated its great potential in point-of-care testing.

Real sample analysis. To evaluate the analytical reliability and application potential of the proposed method, REPA was used to detect PSA in clinical serum samples (Supplemental Data Figure s3) and the assay results were compared with the reference values from the commercial electrochemiluminescent testing. The results
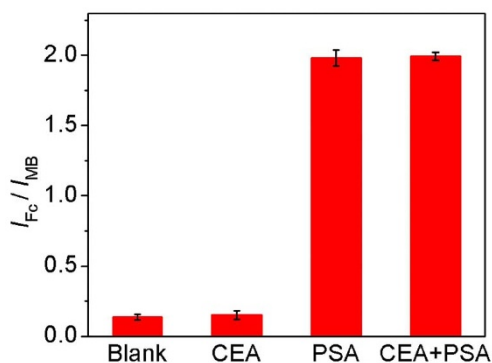

Figure 6 | Selectivity of REPA. I_{Fc}/I_{MB} on REPA sensor incubated with blank control, 10 ng/mL CEA, 10 ng/mL PSA, and the mixture of 10 ng/mL CEA and PSA. Error bars represent standard deviations of three parallel experiments.

were shown in Table 1, an acceptable agreement with relative errors less than 10.3% indicated good accuracy of the proposed method for the detection of clinical samples.

Discussion

This work has proposed a concept of ratiometric electrochemical proximity assay for one-step, highly sensitive and selective detection of protein. The sensing interface can be structured by self-assembling a Fc-labeled DNA probe on gold electrode and then hybridizing with a MB-labeled DNA1-Ab1 affinity probe. The simultaneous recognition of the target protein by DNA1-Ab1 and DNA2-Ab2 as detection probe triggers the hybridization of proximity complementary bases in DNA1 and DNA2 to form a proximate cooperative complex, which leads to the dissociation of MB-DNA1-Ab1 from the electrode and the approach of Fc group to electrode surface by forming a hairpin structure of Fc-labeled DNA probe. As a result, the oxidation peak current ratio of Fc to MB significantly increases. Using PSA as a protein model, the electrochemical ratiometric readout showed wide detection range, high sensitivity, low detection limit, good selectivity and acceptable accuracy. The REPA is simple, convenient and fast, and possesses favorable expansibility for detection of other proteins, showing great potential for point-of-care testing and commercial application.

Methods

Reagents and materials. 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 were from Jiangsu Institute of Cancer Prevention and Cure. Ultrapure water obtained from a Millipore water purification system ($\geq$18 MΩ, Milli-Q, Millipore) was used in all assays. 6 × DNA loading buffer was bought from Solarbio Co. Ltd (Beijing, China). Low molecular DNA ladder was purchased from Fermentas. Dithiothreitol (DTT) and oligonucleotides were obtained from Sangon Biotechnology Inc. (Shanghai, China), and their sequences were listed as following:

Fc-P: 5′-Fc-CGCGTTAAC**CATACAATAGAT**CGCG-$(CH_2)_6$-SH-3′

MB-labeled DNA1: 5′-MB-GCG**GATCTATTGTAT**CACATATTT̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲ *CACCGTATGCTACTGTGTAGAT*-SH-3′

Thiolated DNA2 (L13): 5′-SH-*TAGGAAAAGGAGGAGGGTGG*T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲ T̲T̲T̲T̲T̲T̲T̲**AGATACAATAGATC**-3′

DNA2 (L11): 5′-*TAGGAAAAGGAGGAGGGTGG*T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲TACT **TACAATAGATC**-3′

DNA2 (L13): 5′-*TAGGAAAAGGAGGAGGGTGG*T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲AGA **TACAATAGATC**-3′

DNA2 (L15): 5′-*TAGGAAAAGGAGGAGGGTGG*T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲T̲AGTGA **TACAATAGATC**-3′

Ref DNA: 5′-*CCACCCTCCTCCTTTTCCT*ATTTTCTTTAATTTCTTATTTCTCTTAATTT TAATTTGTTTA*TCTACAGTAGCATACGGTG*-3′

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	10.95	11.61	−5.68
2	6.83	6.19	10.34
3	0.359	0.332	8.13
4	8.39	8.01	4.74

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

The bold and italics parts represented the binding regions of DNA1 to Fc-P (or DNA2), and Ref DNA to DNA1 and DNA2, respectively.

Apparatus. The electrochemical measurements were performed on a CHI 630D electrochemical workstation (CH Instruments Inc., U.S.A.) 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 measurements were carried out on a PGSTAT30/FRA2 system (Autolab, the Netherlands) in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$. PAGE analysis was performed on an Electrophoresis Analyser (Liuyi Instrument Company, China) and imaged on the Bio-rad ChemDoc XRS (Bio-Rad, USA).

Preparation of Ab-DNA probes. The Ab-DNA probes were synthesized by chemically cross-linking the respective antibody to thiolated ssDNA with and without MB label[51]. 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 thiolated ssDNA 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 Ab-DNA probe was obtained.

Fabrication of REPA sensor. 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 under a stream of nitrogen gas. 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 scan rate of 0.1 V/s for 40 cycles. The cleaned gold electrode was thoroughly washed with water and dried under flowing nitrogen.

6 μL of 0.5 μM Fc-P was dropped on the electrode to incubate at room temperature for 2 h. After rinsed with 10 mM pH 7.4 Tris−HCl buffer 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 Tris−HCl buffer and drying with nitrogen, 6 μL of 1 μM MB-DNA1-Ab1 containing 0.5% BSA (w/v) was coated on the electrode for overnight incubation. After another washing step, the REPA sensor was obtained and stored at 4°C before use.

Measurement procedure. Prior to measurement, Tris-HCl buffer (pH 7.4, 0.5 M NaCl) was supplemented with 0.5% BSA (w/v), 50 nM Ab2-DNA2, and various concentrations of PSA or serum samples. 6 μL of above solution was dropped on the sensor surface for 40-min incubation, followed by washing with Tris-HCl buffer (pH 7.4)[52]. Then, the sensor was immersed in 10 mM pH 7.4 PBS for 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.

PAGE analysis. The 12% native PAGE was prepared using 1 × Tris-Borate-EDTA (TBE) buffer. The loading sample was mixed with 7 μL DNA sample, 1.5 μL 6 × loading buffer and 1.5 μL UltraPower dye, and kept for 3 min so that the dye could integrate with DNA completely. Then the loading sample was applied onto the lane. The gel was run at 90 V for 90 min in 1 × TBE buffer, and scanned using a Molecular Imager Gel Doc XR (BIO-RAD, USA).

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Author contributions

K.R., J.W. and H.J. conceived and designed the experiments. K.R., J.W. and F.Y. performed the experiments. K.R., J.W. and H.J. analysed the data and wrote the paper. All authors discussed and commented on the manuscript.

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