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Electrochemical biosensor based on base-stacking-dependent DNA hybridization assay for protein detection

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ABSTRACT

An antibody-based electrochemical biosensing platform has been developed and used for the detection of protein. In the presence of the target, an antibody pair binds to the protein simultaneously, which causes two oligo-DNAs conjugated with the antibody pair to hybridize to each other and become a big "stem–loop" structure. Subsequently, the longer oligo-DNA of the stem, with a methylene blue (MB) label at the terminal, hybridizes stably with capture DNA owing to the enhancement of base stacking. The strong redox current signal of MB is used for protein quantification. Using α-fetoprotein (AFP) as a model, the proposed method could detect AFP at a concentration as low as 2 pg ml^{-1} with a dynamic range of 4 orders of magnitude, which approaches traditional assays such as enzyme-linked immunosorbent assay.

Proteins are the building blocks of life. Changes in the expression level of some proteins are often related to a specific disease. In fact, many proteins are used as biomarkers to diagnose a disease, including cancer. So, protein detection plays an important role in clinical diagnosis and biomedical research. Many powerful techniques based on antibodies have been developed for protein detection, such as the enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay [1–3]. ELISA is a standard protein detection method because of its sensitivity and specificity [1,4–7]. Despite its outstanding utility, many aspects still need to be improved, such as long detection time, high cost, and complexity [3].

To overcome the shortcomings of ELISA, various biosensors have been designed for protein detection. These biosensors can be classified into two types: one is based on aptamers [8–11], the other on antibodies [3,12]. Aptamers are single-stranded oligonucleotides. They bind targets with high affinity and selectivity. Theoretically, they have more versatility in immobilization, labeling, and amplification. They also can be easily generated from SELEX (systematic evolution of ligands by exponential enrichment) [13,14]. In fact, only a few matching aptamers for proteins have been found. But, thousands of antibodies for antibody-based immunoassay have been developed [12]. Antibodies for most proteins associated with growth and development, metabolism, or cancer biomarkers can be obtained conveniently. So, an antibody was selected as the recognition molecule in this work.

The thermal stability of the DNA double helix is affected by several factors, including temperature, base composition, strand length, secondary structure, ionic strength, etc. [15–18]. Base pairing between complementary strands and stacking between adjacent bases are the main factors responsible for the stability of the DNA duplex [19,20]. Base stacking is the dipole-induced dipole interaction between the planar aromatic bases in two contiguous nucleotides [21]. When two contiguous tandem sequences are annealed to a longer strand, coaxial base stacking at the nick site brings additional stability to the hybridization [20,22]. The principle has been successfully applied to protein assay by chemiluminescence [23].

Electrochemical detection is a promising technique because of its simplicity, high sensitivity, low detection limit, low cost, and capacity for miniaturization [24–26]. Therefore, many electrochemical sensors for proteins have emerged [8,9,12,27]. Thus, we combined the base-stacking-dependent DNA hybridization assay with the electrochemical technique. Three oligo-DNAs were designed: the first was thiolated and immobilized on a gold electrode, the second was modified by methylene blue at the terminal, the second and the third were respectively conjugated with an antibody pair, and the first and the third had short sequences complementary to the second. In addition, a pair of antibodies used to recognize different sites of the target protein was included in the design. Here, two oligo-DNAs conjugated to antibody would hybridize each other only if their conjugated antibody pairs simultaneously bound to the target protein. Then, capture DNA immobilized on an electrode hybridized with the oligo-DNA-tethered

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methylene blue (MB) efficiently because the coaxial base stacking brought additional stability. When MB-tethered oligo-DNA hybridized with capture DNA, MB molecules were pulled close to the electrode surface and caused an electrochemical readout signal.

In this work, α-fetoprotein (AFP), a serum biomarker of hepatocellular carcinoma, was used as a model molecule. The elevated level of AFP is an indication of some cancerous diseases, such as gastric cancer, nasopharyngeal cancer, and testicular cancer [28,29]. Thus, the detection of AFP is necessary in patient diagnosis and clinical research. Based on the experimental results, the proposed design shows high sensitivity, low detection limit, and high reliability. Moreover, this strategy exhibits good selectivity and has been successfully used to quantitate AFP in human blood serum.

Materials and methods

Apparatus and reagents

Ultraviolet (UV) detection was performed with a UV-3600 spectrophotometer (Shimadzu, Kyoto, Japan). All electrochemical measurements were carried out using a CHI 760b electrochemical workstation (Chenhua Corp., Shanghai, China). A three-electrode system consisted of a modified gold electrode as working electrode, a saturated calomel electrode (SCE) as reference electrode, and a platinum wire auxiliary electrode. All potentials were respective to the SCE.

Oligonucleotides were synthesized by Sango Biotechnology Co., Ltd (Shanghai, China). The design of sequences was aided by the mfold Web server (http://mfold.rna.albany.edu) and is shown in Table 1. Bovine serum albumin (BSA), human immunoglobin G (IgG), and thrombin were bought from Sigma–Aldrich (St. Louis, MO, USA). Prostate-specific antigen (PSA), interleukin-6 (IL-6), and carcinoembryonic antigen (CEA) were obtained from Shanghai Linc-Bio Science Co., Ltd. (Shanghai, China). Recombinant human AFP was from Biocell Biotechnology Co., Ltd. (Zhengzhou, China). Mouse anti-human AFP monoclonal antibodies AFP-Y1 and AFP-Y2 were purchased from YES Biotech Laboratories Ltd (Mississauga, ON, Canada). All chemicals used in this work were of analytical reagent grade. All aqueous solutions were prepared with ultrapure water ($\geqslant$18.3 MΩ; Milli-Q, Millipore, Billerica, MA, USA).

Preparation of antibody–oligonucleotide conjugates

The antibody–oligonucleotide conjugates, AFP-Y1:DNA1 and AFP-Y2:DNA2, were prepared by conjugating DNA1 to mouse anti-human AFP monoclonal antibody AFP-Y1 and DNA2 to mouse anti-human AFP monoclonal antibody AFP-Y2, respectively. Conjugation reactions and purification were performed using an Antibody–Oligonucleotide All-In-One Conjugation Kit (Solulink), according to the technical manual. Briefly, the amino-modified oligonucleotides were first activated with sulfo-S-4FB, and their quantities and qualities were confirmed by UV detection, specifically, A_{260nm} of unmodified activated oligonucleotides and the A_{260nm} to A_{360nm} ratio after the modification of activated oligonucleotides. Antibodies were also activated with S-HyNic. HyNic-modified protein and 4FB-modified oligonucleotide were then mixed and incubated at room temperature for 2 h. Conjugates were further purified using the supplied Zeba spin column after the conjugation reaction. The final concentrations of the conjugates were determined with a Coomassie (Bradford) Protein Assay Kit. AFP-Y1:DNA1 and AFP-Y2:DNA2 were synthesized with 75% and 78% recovery from the initial amount of antibodies (100 μg).

Table 1
Oligonucleotides synthesized in the experiments.

Name	Sequence
Capture probe	5′-CTCTCTGT-$(CH_2)_6$-SH-3′
DNA1	5′-NH_3-$(CH_2)_6$-TTT-T_{35}-TTCTACGC-3′
DNA2	5′-MB-ACAGAGAGGCGTAGTT-T_{35}-TTT-$(CH_2)_6$-NH_3-3′
DNA3	5′-ACAGAGAGGCGTAGTT-T_{35}-TTT-$(CH_2)_6$-NH_3-3′

MB, methylene blue at the 5′ end.

Electrode preparation

The gold electrode (d = 2 mm, CH Instruments) was thoroughly polished to a mirror surface with 0.05 μm alumina slurry and then successively sonicated in water and ethanol for 10 min. The electrode was then etched for 5 min in a warm fresh Piranha solution (H_2SO_4/H_2O_2, 3/1) and rinsed with deionized water. Finally, the electrode was cleaned by cycling the potential scans from −0.6 to +1.2 V in 0.1 M H_2SO_4 at a scan rate of 0.1 V s^{-1} for 20 cycles. The cleaned gold electrode was rinsed thoroughly with deionized water and ethanol and then dried under nitrogen gas.

Two microliters of 200 μM thiolated capture DNA was treated with 0.10 M dithiothreitol for 60 min at room temperature (24 °C) and then purified using a NAP-10 column (GE Healthcare) [30]. Then the solution was diluted to a total volume of 400 μl in 10 mM phosphate-buffered saline (PBS; pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) [8,9] with a final concentration of 1 μM. For immobilization, the reduced thiolated capture DNA solution (10 μl) was dropped on the previously cleaned gold electrode surface and incubated for 16 h at 4 °C. Following the formation of a self-assembled monolayer, the resulting sensor was rinsed with deionized water to remove the excess of thiolated DNA. The surface coverage was estimated by using $Ru(NH_3)_6Cl_3$ [31], and it was in the range of 7.8–9.6 pmol/cm^2.

Electrochemical detection of AFP

All electrochemical measurements were performed using cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The reported DPV curves were background-subtracted through extrapolation to the baseline in the regions far from the peaks [32]. Electrochemical impedance spectra (EIS) were implemented in the frequency range from 0.1 Hz to 100 kHz with 5 mV as the frequency modulation at a bias potential of 0.19 V.

The incubation and detection were carried out in 10 mM PBS (pH 7.4) containing 0.1 M $KClO_4$. PBS/$KClO_4$ buffer was supplemented with 0.5% BSA (w/v) to minimize antibody adsorption. After AFP was incubated in 10 nM AFP-Y1:DNA1 and AFP-Y2:DNA2 for 1 h at room temperature, the incubations were added into the glass electrochemical cell. Before voltammetric measurements were conducted, the sensor surface was allowed to react with the incubations for 30 min at 35 °C. Selectivity tests containing other proteins (IgG, PSA, CEA, or IL-6) were done under the same conditions.

Results and discussion

The design of electrochemical sensor

The overall design of the sensor is shown in Fig. 1. We present a new strategy for protein detection based on molecular pincers and base-stacking-dependent DNA hybridization. Thiolated capture probes were immobilized on gold electrodes by the alkanethiol moiety at the 5′ terminus. A pair of antibodies recognizing different epitopes of the target protein (AFP) was labeled with a pair of short complementary oligo-DNAs using a poly(T) oligo linker; one of the DNAs was complementary to the capture probes and encompassed a MB-labeled 3′ terminus. As shown in Fig. 1, in the presence of

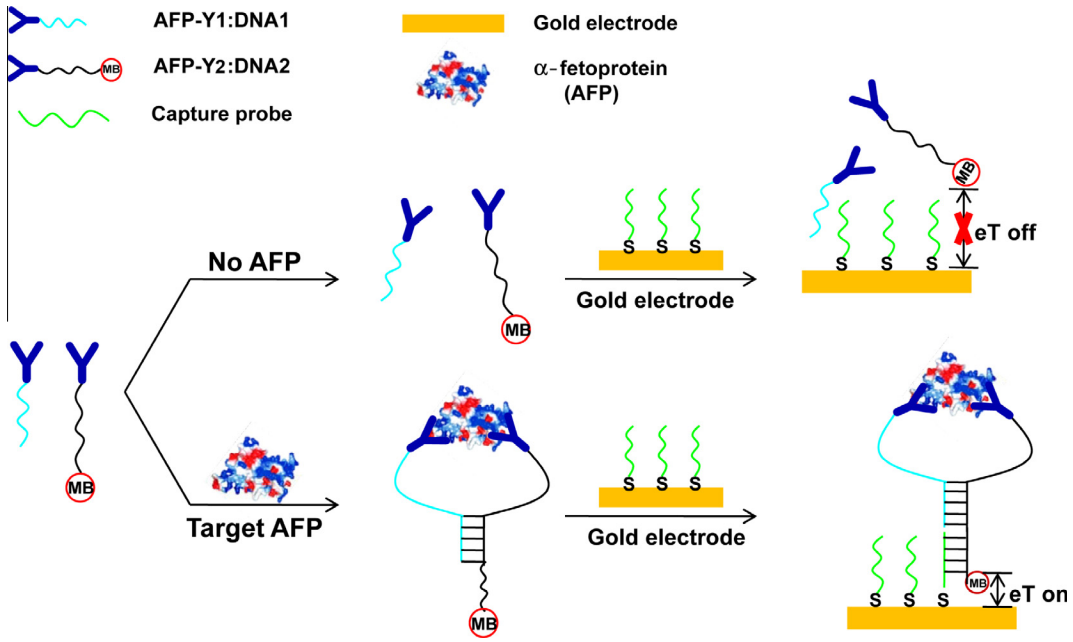

Fig.1. The protein detection strategy based on base-stacking surface hybridization assay.

AFP, the antibody pairs (AFP-Y1 and AFP-Y2) would simultaneously bind to AFP at different epitopes, and the antibody-coupled oligo-DNAs (DNA1 and DNA2) would hybridize to form a hairpin [3], then the remainder of the 3′ terminus sequence of DNA2 could anneal to the capture probes because the base stacking brings additional stability [20–22]. This leads to the MB labels tagged on the 3′ terminus of DNA2 being drawn close to the electrode surface and thus producing a readily detectable redox current. In the absence of AFP, the complementary antibody-conjugated DNA pairs could not hybridize stably because of the predesigned low melting temperature (<20 °C) [3], and the MB-labeled DNA could not hybridize with capture probes for the same reason. As a result, the MB labels were away from the electrode surface, the impeding electron transfer was shut off, and only background current was observed.

Electrochemical characterization

Fig. 2A shows CVs of the electrochemical sensor before and after reaction with AFP. No redox peaks were observed when AFP was absent (curve a), indicating that the hybridization of MB-labeled DNA–antibody chimeras with surface-immobilized capture DNA was extremely low efficiency. In the presence of 20 ng ml^{-1} AFP, the CVs of the electrode exhibited a pair of well-defined redox peaks located at −0.23 and −0.30 V (curve b), which was attributed to the redox of tagged MB. DPV has a much higher current sensitivity than cyclic voltammetry. As shown in Fig. 2B, before reaction with target protein AFP, only a very small oxidation peak appeared around −0.25 V (curve a). It was attributed to the nonspecific adsorption of MB-labeled DNA–antibody chimeras to the electrode surface. After reacting with 20 ng ml^{-1} AFP, the current peak increased dramatically (curve b). The increase was almost 10-fold over the background current. This indicates that the complementary antibody-conjugated DNA pairs (DNA1 and DNA2) could hybridize stably to form the hairpin structure in the presence of AFP, which substantially improved the hybridization efficiency of MB-tagged DNA2 with capture probes.

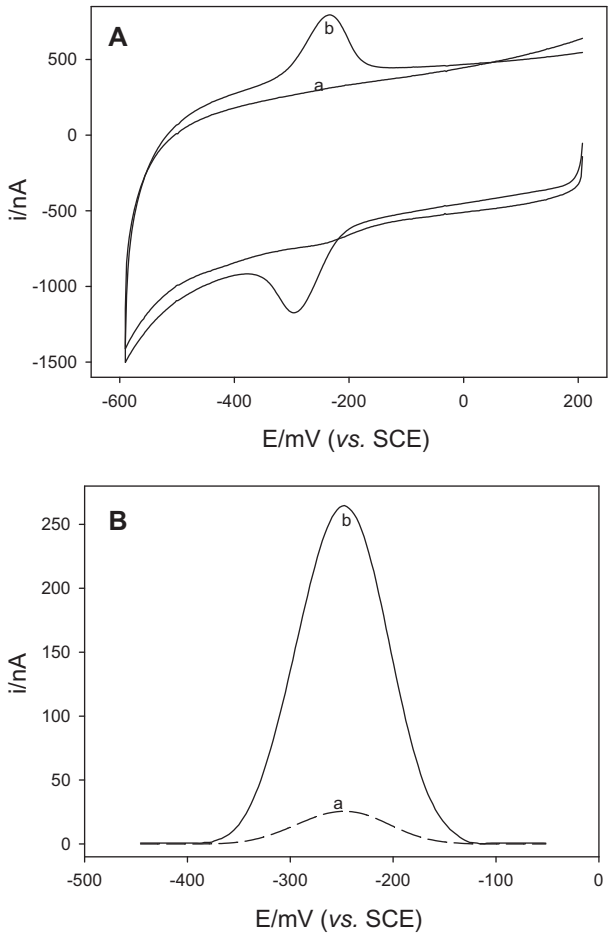

Fig.2. (A) CVs of sensor (curve a) before and (curve b) after reaction with 20 ng ml^{-1} AFP at a scan rate of 100 mV s^{-1}. (B) DPVs of sensor (curve a) before and (curve b) after reaction with 20 ng ml^{-1} AFP.

Fig. 3 shows the CVs of the sensor after reaction with 10 ng ml^{-1} AFP at various scan rates. A good linear relationship could be found between the redox peak current and the scan rate (ν) in the range of 10–150 mV s^{-1}. The linear regression equations were $i_{p,a}$ (nA) = 2.372 + 3.761ν (mV s^{-1}) (n = 6), with a correlation coefficient of 0.999, and $i_{p,c}$ (nA) = −3.017−3.984ν (mV s^{-1}) (γ = 0.999). This suggests that the electrochemical behavior of MB is a typical adsorption-controlled process, confirming that the redox species of MB was confined to the electrode surface.

To demonstrate that the sensing interface was constructed successfully, we utilized EIS to monitor the impedance change in each process. The results are shown in Fig. 4. Here, DNA3, the same sequence as DNA2 but without redox tags, was used to confirm its hybridization with capture probes. It was observed that the bare electrode showed a very small Faraday impedance (curve a). After the electrode was assembled with capture probes, a large semicircular domain was found (curve b). Subsequently, after capture probes hybridized with the mixture of AFP-Y2:DNA3, AFP-Y1:DNA1, and AFP, the Faraday impedance was further increased (curve d), which indicated the surface binding events. But, when the AFP-Y2:DNA3 was replaced with the MB-labeled AFP-Y2:DNA2, the electrochemical impedance was dramatically decreased (curve c). This confirmed that the surface confinement of the MB labels facilitated electron transfer kinetics. The mono-electron transfer rate constants were estimated to be increased from 0.32 to 5.12 s^{-1} based on the EIS data [33].

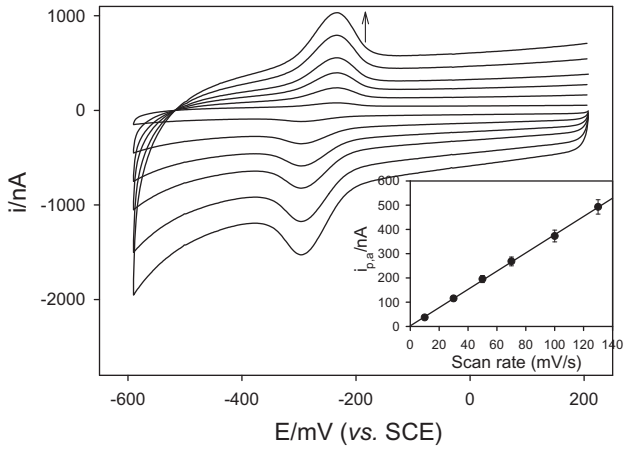

Fig.3. CVs obtained in the presence of 5 ng ml^{-1} AFP at various scan rates. From bottom to up: 10, 30, 50, 70, 100, 130 mV s^{-1}. Inset: the relationship between peak current and scan rate (n = 6).

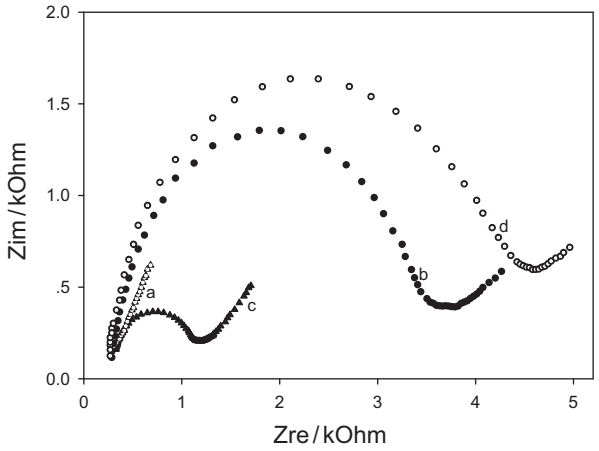

Fig.4. Nyquist diagram of the faradic impedance measurements in the presence of 5.0 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ at the open circuit potential 0.24 V. (Curve a) Bare Au electrode; (curve b) thiolated DNA-modified Au electrode; (curve c) modified electrode after hybridization with 10 nM AFP-Y1:DNA1, AFP-Y2:DNA2, and 10 ng ml^{-1} AFP; (curve d) modified electrode after hybridization with 10 nM AFP-Y1:DNA1 (without MB tag), AFP-Y2:DNA3, and 10 ng ml^{-1} AFP. Frequency range, 0.1 Hz–100 kHz; AC amplitude, 5 mV.

Optimization of experimental conditions

Temperature has a great effect on hybridization. At lower temperatures, AFP-Y1:DNA1 and AFP-Y2:DNA2 could hybridize with the capture DNA stably even in the absence of AFP, thus increasing the background current and degrading sensitivity. On the other side, AFP-Y1:DNA1 and AFP-Y2:DNA2 could not hybridize with the capture DNA stably even in the presence of AFP at higher temperature, thus decreasing the response current and degrading sensitivity. The influence of temperature was investigated over the range 20 to 45 °C in 4 ng ml^{-1} AFP. Fig. 5 shows that the current responses changed only a little when increasing temperature up to 35 °C. But the background current decreased significantly. When the temperature was over 35 °C, the current responses exhibited a fast decline, but the background current decreased slowly. Considering the high detection response and low background current, 35 °C was adopted as the optimum reaction temperature.

The appropriate concentrations of AFP-Y2:DNA2 and AFP-Y1:DNA1 were also studied. The fixed ratio of DNA2: antibody and DNA1:antibody was 1:1. Fig. 6 indicates that the response gain (Δi) increased when 0.1–10.0 nM AFP-Y2:DNA2 and AFP-Y1:DNA1 was added to the incubation solution, whereas more led to a slight decrease in Δi, suggesting that 10.0 nM was the maximum amount for the reaction. It may be that a high concentration of AFP-Y2:DNA2 and AFP-Y1:DNA1 increases their association population, but also augments the sensor's background current and degrades sensitivity. However, a low concentration destabilizes the hybridization and reduces the affinity to capture probes. It was observed that 10 nM produced optimal current increasing. Thus the optimal amount of AFP-Y2:DNA2 and AFP-Y1:DNA1 was chosen at 10 nM.

The performance of the determination of AFP

The typical DPVs of various concentrations of AFP are shown in Fig. 7. The peak current increased as the concentration of AFP increased. A linear dose–response curve was obtained in the range

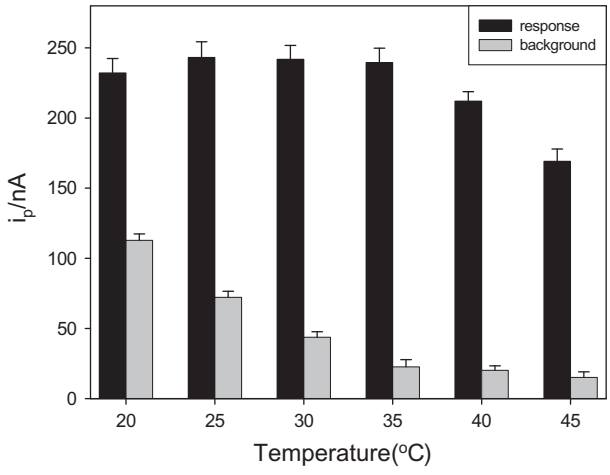

Fig.5. The effects of temperature on response current and background current. The modified electrode was incubated in the presence of 4 ng ml^{-1} AFP.

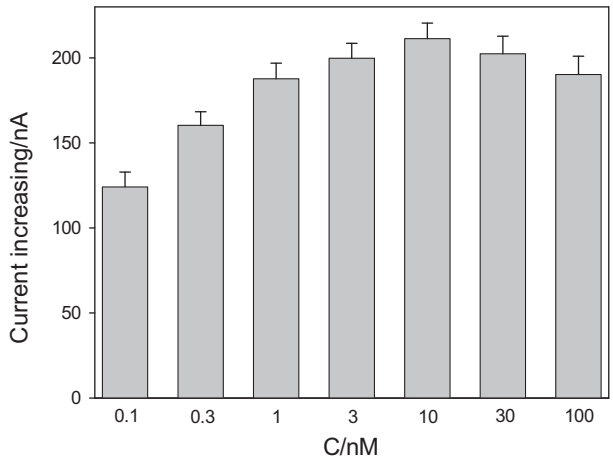

Fig.6. The effects of the amount of DNA2:antibody and DNA1:antibody on the response current increase (Δi). The modified electrode was incubated in the presence of 4 ng ml^{-1} AFP.

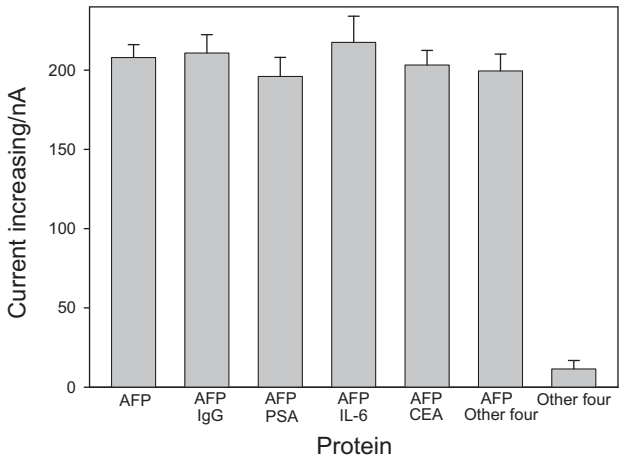

Fig.8. Selectivity of the presented electrochemical biosensor in 4 ng ml^{-1} AFP solutions without and with four proteins (IgG, PSA, IL-6, CEA), separately and together, and in a solution of the four proteins without AFP (Other four). The error bars show the SD of five repetitive experiments.

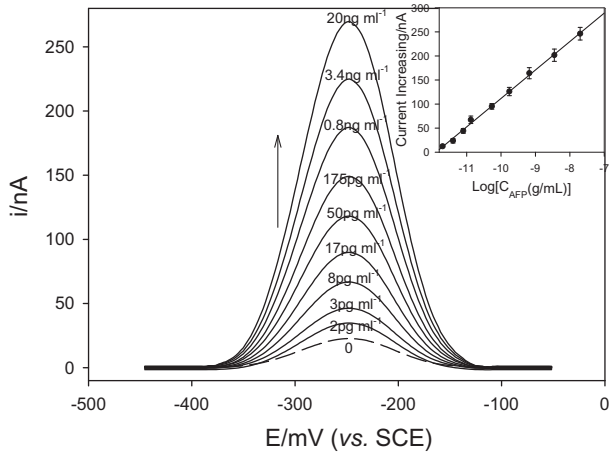

Fig.7. DPVs of various concentrations of AFP. Inset: the relationship between peak current increasing and concentration of AFP ranging from 2 pg ml^{-1} to 20 ng ml^{-1}. The error bars represent SD (n = 5 experiments).

Table 2
Comparison of AFP levels detected using the presented method and ELISA.

	Serum samples					
	1	2	3	4	5	6
ELISA (ng ml^{-1})	33.5	16.3	42.8	140.9	106.2	153.8
Presented sensor (ng ml^{-1})	35.2	18.4	45.9	147.3	113.5	162.6
Standard deviation (presented sensor)	2.83	1.77	4.32	10.75	6.14	10.19

n = 5 serum samples.

from 2.0 pg ml^{-1} to 20.0 ng ml^{-1} with a detection limit of 1.0 pg ml^{-1} (signal/noise = 3). The detection sensitivity was about 1000-fold higher than that obtained by amperometric immunosensor [34,35]. Compared with EIS assay [36], the present strategy afforded a wider linear range with 1 order of magnitude improvement, demonstrating that the design offered a more sensitive platform for protein assay.

The effects of possible interfering proteins on the determination of AFP were investigated. The current responses of 4 ng ml^{-1} AFP without or with 200 ng ml^{-1} PSA, CEA, IgG, or IL-6 are shown in Fig. 8. Compared with the current response obtained in the presence of only AFP, no significant current differences were observed in the mixed protein solution, although the concentration of interfering proteins is 50-fold that of AFP. The specificity of the proposed strategy was further evaluated by measuring the mixture containing 4 ng ml^{-1} AFP and 200 ng ml^{-1} PSA, CEA, IgG, and IL-6. No obvious change appeared even containing four different proteins and each concentration was 50 times that of AFP. All results revealed that the observed response was mainly caused by specific binding, and the interference from nonspecific adsorption could be neglected.

Six human serum samples collected from three patients and three healthy people (serum samples used in this work were collected after informed consent) at the Department of Hepatobiliary Surgery of Hunan Provincial People's Hospital were assayed by the electrochemical sensor and ELISA as the reference [37]. The results are listed in Table 2. They are in good agreement, indicating that the proposed strategy holds great promise as a feasible technique for the determination of AFP in real serum samples.

Reproducibility and stability

Similar to the previous design for the surface-tethered monolayer oligonucleotides hybridization assay [38], the proposed sensor could be reused over 11 times without significant loss of sensitivity; the relative standard deviation was 8.93%. The sensor interface could be conveniently regenerated by washing the electrode with 1.0 M NaOH at 50 °C, because a high concentration of alkaline solution disassembled the DNA duplex structure formed on the electrode surface [8,9]. In addition, the present sensor was also fairly robust under cold storage conditions. It could keep 91.27% of its initial response after storage at 4 °C for a week. The excellent reproducibility and stability can be attributed to its compact design and strong S–Au covalent bond [39].

Conclusion

We report on an electrochemical protein-sensing platform based on base-stacking-dependent DNA hybridization. The sensor depends on simultaneous recognition of the target protein by two DNA-tagged antibodies to promote the annealing of their tagged DNA and then stable hybridization to the surface-immobilized DNA capture probes with the help of base-stacking

interactions. The presented method can be applied to detect other proteins just by using corresponding antibodies with different binding sites to substitute for AFP antibodies. The sensor can detect as low as 2.0 pg ml^{-1} protein with a linear dynamic range span of 4 orders of magnitude. Furthermore, it performed well in a real serum sample assay.

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