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Target-Driven Triple-Binder Assembly of MNAzyme for Amplified Electrochemical Immunosensing of Protein Biomarker

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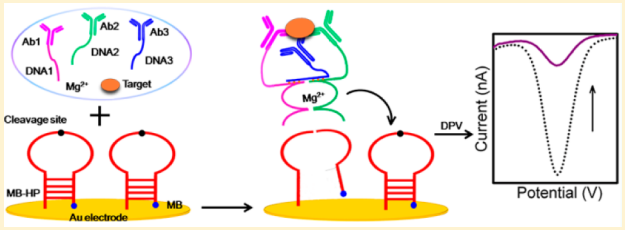
ABSTRACT: A simple electrochemical immunosensing method is presented for highly sensitive and selective detection of protein biomarker. This method uses a newly designed assembly of Mg^{2+}-dependent MNAzyme via target-driven triple-binder proximity hybridization to catalyze the cleavage of methylene blue (MB)-labeled hairpin, which leads to the departure of MB from the electrode surface and thus an amplified decrease of electrochemical signal for immunoassay of the target protein. The MNAzyme assembly is achieved by the simultaneous recognition of target protein with three DNA-labeled antibodies in the presence of Mg^{2+}, which greatly improves the detection sensitivity and selectivity. As a proof of concept, this strategy can detect carcinoembryonic antigen (CEA) ranging from 0.002 to 500 ng mL^{-1} with a detection limit of 1.5 pg mL^{-1}. The whole assay including the target-driven MNAzyme formation and subsequent cleavage of hairpin can be completed with one step in 40 min. The immunosensor, prepared with a hairpin DNA, possesses good extensibility for large protein biomarkers as CEA by using corresponding antibodies, though the protein target size dependence was not investigated in this work. The proposed immunoassay method shows the advantages of easy operation, high sensitivity, wide concentration range, good selectivity, and excellent versatility, displaying potential application for protein analysis.



Highly sensitive and selective determination of tumor markers with easy operation, low consumption, and short assay time is significant in early cancer screening and evaluation, because their levels in blood or tissue provide essential information about the stages of tumors.[1−3] Immunoassay based on the highly specific molecular recognition between antibody and antigen is the dominant analytical technology for tumor markers.[4,5] To improve the detection sensitivity, different strategies have been designed to amplify the detectable signal,[6,7] in which nanoprobe-based signal amplification is most popular. Normally, the nanoprobes can be prepared by employing nanomaterials, such as nanoparticles,[8−11] carbon nanotubes[12−15] and magnetic beads,[16−18] as nanocarriers to load a high amount of signal-related molecules including enzymes,[19−22] quantum dots,[23] and metal nanoparticles.[24−26] These nanoprobes lead to the detection limit down to pg mL^{-1} or even fg mL^{-1}. However, the nanoprobe-based methods encounter the disadvantages of denaturation and leakage of enzymes, and complicated separation and labeling steps, and therfore, they are difficult to use in practical point-of-care testing.[27]

Catalytic nucleic acids (DNAzymes) have attracted considerable interest due to their good stability, low nonspecific adsorption, and easy preparation and functionalization.[27,28] Some DNAzymes show specific ability to cleave the substrates and have been used for the design of detection methodology.[29−32] As one kind of bipartite DNAzymes, MNAzyme has also been presented for DNA detection by a target DNA-driven assembly to catalyze the cleavage of signal DNA sequence.[33] The catalyzed cleavage leads to an amplified detection signal. Due to the in situ formation, high catalytic efficiency, and gentle operation conditions, MNAzyme-based amplification has been used for convenient and highly sensitive detection of DNA.[34,35] Unfortunately, this strategy has still not been used for protein detection. In order to introduce the MNAzyme-based strategy to protein analysis, this work designed for the first time a target protein-driven proximity hybridization for the assembly of Mg^{2+}-dependent MNAzyme and following signal amplification by the MNAzyme catalyzed cleavage of methylene-blue-labeled hairpin (MB-HP).

Proximity hybridization is a DNA-assisted assembly strategy. It can use a pair of DNA-conjugated affinity probes to simultaneously recognize the target and subsequently induce the hybridization of DNA labels to trigger the detection signals.[36,37] Thus, the affinity recognition event can be encoded into a convenient DNA "signal" for readout.[38] For example, several electrochemical proximity immunoassays (EPI) have been developed for the detection of insulin and thrombin by combining the proximity hybridization with DNA sensor.[39,40]

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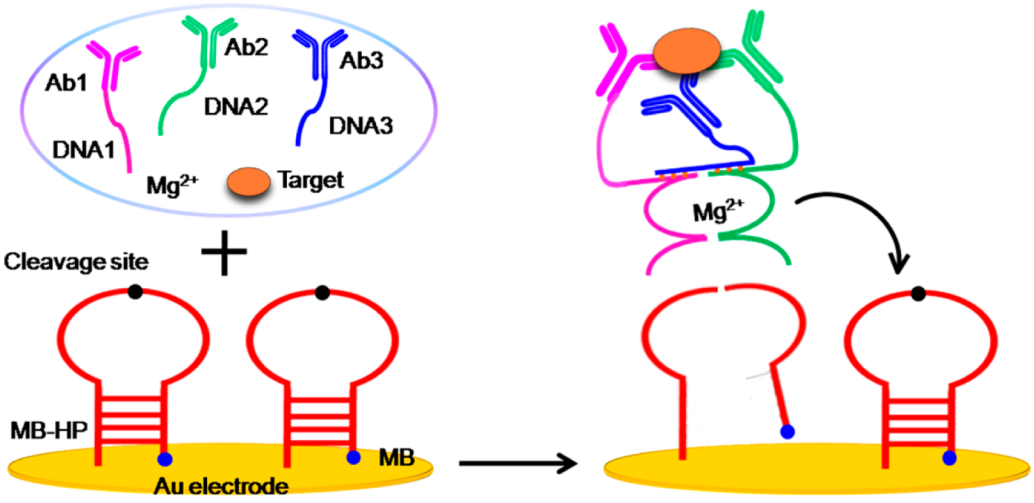
Scheme 1. Schematic Illustration of Triple-Binder Assembly of Mg^{2+}-Dependent MNAzyme along with Autocatalytic Cleavage of MB-HP on Electrochemical Biosensor



In our previous work, the dual-binder proximity hybridization was also used to design a ratiometric electrochemical readout for improving the detection sensitivity.[41] Here, a triple-binder proximity ligation[42] was introduced for target-protein-driven assembly of the MNAzyme structure, which achieved the MNAzyme-based signal amplification by cyclic cleavage of MB-HP on electrode interface to decrease the electrochemical response of MB. This strategy led to an electrochemical proximity immunosensing (defined as 3-EPI) method for sensitive detection of target protein.

As shown in Scheme 1, the assembly was composed of three DNA-conjugated antibodies (DNA1-Ab1, DNA2-Ab2, and DNA3-Ab3). In the presence of target protein and Mg^{2+}, the immunocomplex of target/Ab1,2,3 was formed to trigger the proximity hybridization of DNA3 with DNA1,2 to produce the Mg^{2+}-dependent MNAzyme. On a MB-HP-modified electrode, the MNAzyme could autocatalytically cleave the hairpin to decrease the electrochemical oxidation signal of MB. The decreased signal depended on the concentration of target protein. The proposed 3-EPI showed high sensitivity and good selectivity, and could be carried out in one step. Compared with the previous EPI,[39−41] the proximity immunocomplex was formed with a triple-binder proximity ligation in homogeneous solution, and the formed MNAzyme possessed high catalytic activity. The simultaneous recognition of target protein with three affinity probes endowed the proposed 3-EPI method with better selectivity than 2-EPI, and the cyclic cleavage of MB-HP led to a signal amplification process for obtaining higher sensitivity. As most of the natural proteins contain multi-epitopes,[43,44] this assay method could be easily extended for these large protein biomarkers with corresponding DNA-Ab probes. For small protein biomarkers, which may not give enough room for three antibodies, the assay strategy could also be extended by using the binding reagents of lower molecular weight such as Fab fragments or scaffold proteins,[42] though the protein target size dependence was not investigated in this work. The excellent performance of the proposed 3-EPI showed a promising application in protein analysis.

EXPERIMENTAL SECTION

Reagents and Materials. Bovine serum albumin (BSA), mercaptohexanol (MCH), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Carcinoembryonic antigen-(CEA) and biotinylated mouse monoclonal anti-CEA antibodies (clone no. bsm-1623 M as Ab1, bsm-1624 M as Ab2, and bsm-4792 M as Ab3) were purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China). Streptavidin from *Streptomyces avidinii* (product no. S0492) and D-biotin (product no. B0340) were purchased from Sangon Biotechnology Inc. (Shanghai, China). Oligonucleotides were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China), and their sequences were listed as following:

MB-HP: 5′-SH-$(CH_2)_6$-CGCGT**ACTCACTAT**guGGAA-GAAACACGCG-MB-3′

Fluorescent-HP: 5′-ROX-CGCGT**ACTCACTAT**guGGAA-GAAACACGCG-BHQ-2−3′

DNA1: 5′-Biotin-CGCACCGAGCTTCGACTACGACT-GACGAACCGCTTTGCCTGTTTGATCA*GGACGATG*A-*CAACGA***ATAGTGAGT-3**′

DNA1′: 5′-Biotin-CGCACCGAGCTTCGACTACGACT-GACGAACCGCTTTGCCTGTTTGATCA*GGACGATGTT-TATTT***ATAGTGAGT-3**′

DNA2: 5′-TTTCTTCCA*GGCTAGCT****CTGTAC****-C**AGTCCGTTACCTTGATTCCCCTAACCCTCTT-GAAAAATTCGGCATCGGT-Biotin-3′

DNA2′: 5′-TTTCTTCCA*TTTATTTA****TCTGTAC****-C**AGTCCGTTACCTTGATTCCCCTAACCCTCTT-GAAAAATTCGGCATCGGT-Biotin-3′

DNA2″ (7-bp):5′-TTTCTTCCA*GGCTAGCTCATCGTC-*GAGTCCGTTACCTTGATTCCCCTAACCCTCTT-GAAAAATTCGGCATCGGT-Biotin-3′

DNA2″ (8-bp):5′-TTTCTTCCA*GGCTAGCTCATCGTC-C*AGTCCGTTACCTTGATTCCCCTAACCCTCTT-GAAAAATTCGGCATCGGT-Biotin-3′

DNA2″ (9-bp):5′-TTTCTTCCA*GGCTAGCT-CATCGTCCT*CTCCGTTACCTTGATTCCCC-TAACCCTCTTGAAAAATTCGGCATCGGT-Biotin-3′

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DNA2″ (10-bp): 5′-TTTCTTCCA*GGCTAGCT*-*CATCGTCCTG*TCCGTTACCTTGATTCCCC-TAACCCTCTTGAAAAATTCGGCATCGGT-Biotin-3′

DNA3 (6-bp): 5′-Biotin-GCCAAGCAGTCAGCAT-CAGGTTCCCGCTCGTCTA***TACAGA****CATCGT*-3′

DNA3 (7-bp): 5′-Biotin-GCCAAGCAGTCAGCAT-CAGGTTCCCGCTCGTCT***GTACAGA****CATCGTC*-3′

DNA3 (8-bp): 5′-Biotin-GCCAAGCAGTCAGCAT-CAGGTTCCCGCTCGTC***GGTACAGA****CATCGTCC*-3′

DNA3 (9-bp): 5′-Biotin-GCCAAGCAGTCAGCAT-CAGGTTCCCGCTCGT***TGGTACAGA****CATCGTCCT*-3′

DNA3 (10-bp): 5′-Biotin-GCCAAGCAGTCAGCAT-CAGGTTCCCGCTCG***CTGGTACAGA****CATCGTCCTG*-3′

The binding regions between DNA3 (or DNA2″) and DNA1 (or DNA1′), DNA3 and DNA2 (or DNA2′), and MB-HB (or fluorescent-HP) and DNA1 (or DNA1′) as well as DNA2 (or DNA2′ andDNA2″) were shown in italics with underlined, italics with bold, and bold as well as underlined, respectively. The part of MNAzyme catalytic core (or variant catalytic core) was shown in italics. The cleavage site for MNAzyme was between imbedded RNA g-u.

MNAzyme buffer (pH 7.4) contained 10 mM Tris-HCl, and 30 mM $MgCl_2$. The clinical serum samples were from Jiangsu Institute of Cancer Prevention and Cure and stored at −20 °C before use. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all assays.

Apparatus. The electrochemical measurements were performed on a CHI 660D 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 electrode, respectively. 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 $K_4Fe(CN)_6$. The fluorescence spectra were obtained on a RF-5301PC spectrofluorophotometer (Shimadzu, Japan).

Preparation of DNA-Abs. The DNA-Abs were prepared through the biotin−streptavidin conjugation, which was also the most commonly used protocol for conjugation of antibodies to oligonucleotides.[42,45,46] As the three monoclonal antibodies are specific for different epitopes, they can achieve three independent recognition events on one target protein.[42] Here, 5 μL of 10 μM biotinylated oligonucleotide (DNA1, DNA2, or DNA3) was first mixed with equal volume of 10 μM streptavidin (diluted in MNAzyme buffer containing 0.5% BSA) and incubated at 37 °C for 40 min. After cooling at room temperature (RT) for 20 min, the mixture was mixed with 5 μM biotinylated monoclonal antibody (Ab1, Ab2 or Ab3) at a volume ratio of 1:1, followed with incubation at RT for 40 min. No further purification was needed after the conjugation, which could be attributed to the high-affinity interaction of biotin and streptavidin. Finally, the prepared DNA-Abs (DNA1-Ab1, DNA2-Ab2, and DNA3-Ab3) were diluted to 250 nM with MNAzyme buffer containing 0.5% BSA and 1 mM D-biotin, and stored at 4 °C before use.

Fabrication of Electrochemical 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 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^{-1} for 40 cycles. The cleaned gold electrode was thoroughly washed with water and dried under flowing nitrogen.

Five microliters of MB-HP (10 μM) was incubated with 5 μL of TCEP (1 mM) for 1 h to allow the reduction of disulfide bonds. Then, the 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 MB-HP concentration of 0.5 μM. Next, 6 μL of the MB-HP solution was dropped on the electrode to incubate at RT for 2 h. After rinsing with 10 mM Tris−HCl buffer (pH 7.4, 0.1 M NaCl) and drying with nitrogen, 6 μL of 1 mM MCH was dropped on the electrode for 1 h to block the unmodified sites. After another washing and drying operation, the MB-HP-modified electrochemical biosensor was obtained and stored at 4 °C before use.

Measurement Procedure. Prior to measurement, 9 μL of MNAzyme buffer supplemented with 0.5% BSA and 55 nM DNA1-Ab1, DNA2-Ab2, and DNA3-Ab3 was mixed with 1 μL of various concentrations of CEA or serum samples. Then, 6 μL of the mixture was dropped on the biosensor surface for a 40 min incubation, followed by washing with Tris-HCl buffer (pH 7.4). Subsequently, the biosensor was immersed in 10 mM pH 7.4 PBS with differential pulse voltammetry (DPV) from −500 to −100 mV (vs Ag/AgCl) with a pulse amplitude of 50 mV and a pulse width of 200 ms.

RESULTS AND DISCUSSION

Characterization of the3-EPI. EIS measurements were performed in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to characterize the biosensor preparation and measurement procedure (Figure 1). Compared with bare Au

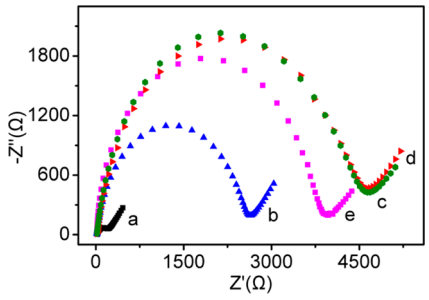

Figure 1. EIS of bare Au electrode (a), MB-HP-modified Au electrode (b), the biosensor (c), and the biosensor after incubated with three DNA-Abs at 50 nM in absence (d) and presence (e) of 10 ng mL^{-1} CEA.

electrode (curve a), the MB-HP-modified Au electrode showed a much larger electron-transfer resistance R_{et} (curve b) due to the negatively charged self-assembly layer of MB-HP. Subsequent surface blocking with MCH led to a further increase of R_{et} (curve c), indicating the successful preparation of electrochemical biosensor. In the presence of three DNA-Abs, the R_{et} did not obviously change (curve d), while the presence of both target CEA and three DNA-Abs led to the decrease of R_{et} (curve e). This appearance confirmed the release of a part of MB-HP sequence from the sensing surface, which indicated the formation of Mg^{2+}-dependent MNAzyme

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via the target-driven triple-binder proximity hybridization to autocatalyze the cleavage of MB-HP.

Feasibility of 3-EPI. The feasibility of 3-EPI was investigated by DPV detection (Figure 2A). After the biosensor

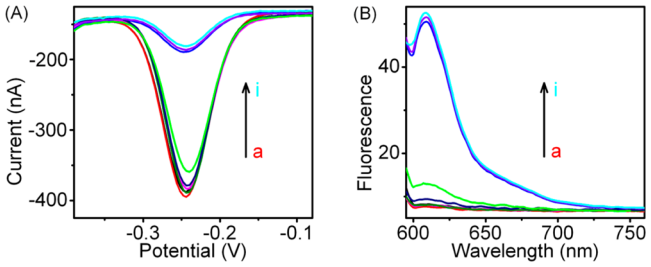

Figure 2. (A) DPV responses of biosensor and (B) fluorescence spectra of fluorescent-HP at λ_{ex} of 588 nm after incubated with (a) MNAzyme buffer, (b-i) MNAzyme buffer containing (b) CEA, DNA2-Ab2 and DNA3-Ab3, (c) CEA, DNA1-Ab1 and DNA3-Ab3, (d) CEA, DNA1-Ab1 and DNA2-Ab2, (e) three DNA-Abs, (f) CEA, DNA1′-Ab1, DNA2′-Ab2 andDNA3-Ab3, (g) CEA and three DNA-Abs, and (h) non heat-treated or (i) heat-treated blood serum samples from healthy control supplemented with CEA and three DNA-Abs for 40 min at RT. The concentrations of CEA and three DNA-Abs were 10 ng mL^{-1} and 50 nM, respectively.

was incubated with MNAzyme buffer, a large oxidation peak of MB was observed at −0.25 V (curve a), indicating the successful construction of the biosensor according to Scheme 1. After the biosensor was incubated with MNAzyme buffer containing CEA and two DNA-Abs (curves b, c, and d) or only three DNA-Abs (curve e), negligible change of DPV response was observed. Thus, no reaction happened at the biosensor in the absence of one DNA-Ab or target protein CEA. As control, DNA1′ and DNA2′ containing some variant bases within catalytic core domain were used to assemble a mutated Mg^{2+}MNAzyme. In the presence of CEA, DNA1′-Ab1, DNA2′-Ab2 and DNA3-Ab3, the DPV peak current decreased slightly (curve f), suggesting the mutated MNAzyme could not cleave the MB-HP. After the biosensor was incubated with the mixture of target protein and three DNA-Abs, the DPV response corresponding to the electrochemical oxidation of MB greatly decreased (curve g). The decrease could be attributed to the cleavage of the immobilized MB-HP due to the formation of the Mg^{2+}-dependent MNAzyme via the proximity hybridization of DNA1,2,3. The formation of MNAzyme relied on the simultaneous recognition of the target protein with three DNA-Abs. At the same CEA concentration, both non heat-treated and heat-treated blood serum samples showed a similar decrease of DPV signal (curve h and i), suggesting the good stability of DNA-Abs against enzymatic digestion, and the complex components in serum did not interfere with the assembly of MNAzyme structure and subsequent catalytic cleavage.

The assay feasibility was further confirmed using a fluorescent-HP, in which the HP was modified with fluorescein (ROX) and Black Hole Quencher (BHQ-2) at its 5 and 3 termini, respectively. As shown in Figure 2B, the fluorescence spectra of the incubated mixtures showed very low signal, similar to that of fluorescent-HP(curve a), in the absence of either DNA-Ab (curves b, c, and d) or target protein (curve e) or in the mixture of DNA1′-Ab1, DNA2′-Ab2, DNA3-Ab3 and CEA (curve f). In contrast, the fluorescence spectra of the mixtures containing both three DNA-Abs and CEA or the CEA-spiked non heat-treated or heat-treated blood serum samples showed similar strong fluorescent intensity (curves g, h, and (i), thus confirming the good stability of DNA-Abs, the formation of MNAzyme and the subsequent catalytic cleavage of the HP to separate BHQ-2 from ROX.

Optimization of Detection Conditions. The designed 3-EPI relied on not only the simultaneous recognition of target protein by three DNA-Abs to trigger the proximity hybridization of DNA3 with DNA1 and DNA2 for forming Mg^{2+}-dependent MNAzyme, but also the subsequent cyclic cleavage of the MB-HP on sensing surface. Thus, the number of complementary bases between both DNA3 and DNA1 and DNA3 and DNA2 was first optimized. Here the suppression percentage of DPV peak current of MB served as the signal for detection. The current suppressions using DNA3 with 6, 7, 8, 9, and 10 complementary bases to both DNA1 and DNA2 are shown in Figure 3A. In the presence of 10 ng mL^{-1} CEA, the

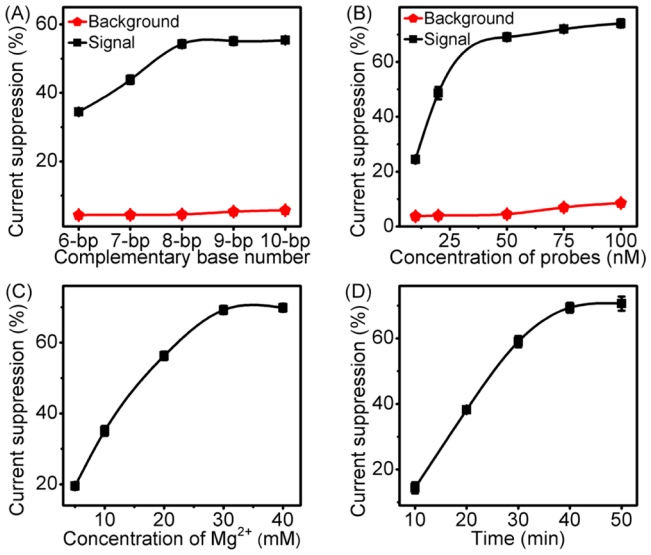

Figure 3. Effects of (A) complementary base number among DNA3, DNA1, and DNA2, (B) concentration of DNA-Abs, (C) Mg^{2+} concentration, and (D) reaction time on current suppression. Error bars represent standard deviations of three parallel experiments.

current suppression increased with the increasing number of complementary base and trended to level off at 8 bp, indicating the proximity hybridization of DNA3 with DNA1 and DNA2 could not efficiently occur at low complementary base number. However, the background suppression was increased at high number of complementary bases due to the self-hybridization process. According to the maximum signal-to-noise ratio (S/N), DNA3 with 8 complementary bases to both DNA1 and DNA2 was chosen for the subsequent experiments. Here the hybridization sequences, DNA1, DNA2 and DNA3, were designed to contain 49, 49, and 32 assistant bases (~11 nm) for avoiding the steric hindrance caused from the sandwich complex.

The concentration of DNA-Abs was optimized at 100 ng mL^{-1} CEA. It was clear that the current suppression increased with increasing the DNA-Ab concentration and tended to a maximum value at 50 nM, whereas the background showed little changed from 10 to 50 nM and became larger from 50 to 100 nM (Figure 3B). To obtain the high S/N, 50 nM was selected as the optimum concentration of the DNA-Abs.

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The effect of Mg^{2+} concentration was also examined at 100 ng mL^{-1} CEA. As shown in Figure 3C, the current suppression increased with the increasing Mg^{2+} concentration from 5 to 30 mM and then reached the platform, indicating the saturated formation of the Mg^{2+}-dependent MNAzyme. Hence, 30 mM Mg^{2+} was used for whole experiments.

The incubation time was an important parameter to affect the amounts of both formed MNAzyme and cleaved MB-HP. The suppression percentage greatly increased with the increasing incubation time and tended to level off until 40 min (Figure 3D). Thus, 40 min was chosen as the optimum reaction time.

Assay Performance. The analytical performance of the 3-EPI was characterized under optimal experimental conditions. The oxidation peak current of MB decreased with the increasing CEA concentration in the incubation mixture (Figure 4A). The current suppression was linearly depended

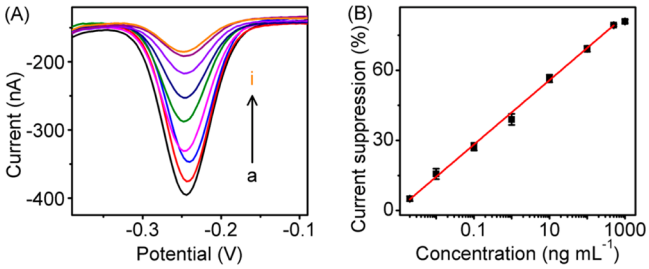

Figure 4. (A) DPV responses of 3-EPI at 0, 0.002, 0.01, 0.1, 1.0, 10, 100, 500, and 1000 ng mL^{-1} CEA (from a to (i) under optimal conditions, and (B) calibration curve for CEA detection. Error bars represent standard deviations of three parallel experiments.

on the logarithm of CEA concentration in the range of 0.002 to 500 ng mL^{-1} with a correlation coefficient of 0.9998 (Figure 4B). The detection limit corresponding to a S/N of 3σ was 1.5 pg mL^{-1}, which was much lower than those of fluorescent immunoassays using binding-induced DNA strand displacement strategy,[47,48] and 133-fold lower than that of commercially available elecsys reagent kit (0.2 ng mL^{-1}, cat. no. 11731629160). The detection limit of pg mL^{-1} level along with a detectable concentration range of 5 orders of magnitude was comparable to previous EPI,[41−43] and the electrochemical immunoassays using nanoprobes for signal amplification.[49,50]

To substantiate the high sensitivity and selectively of the triple-binder assembled MNAzyme strategy, the S/N of the proposed 3-EPI for 10 ng mL^{-1} CEA in both buffer and blood serum were compared with those of 2-EPI. The 2-EPI was designed to use two DNA-Abs (DNA1-Ab1 and DNA2″-Ab2) to recognize target protein. The hybridization of DNA1 with DNA2″ produced the Mg^{2+}-dependent MNAzyme for catalytic cleavage of MB-HP. Similar to that in 3-EPI, the number of complementary bases between DNA2″ and DNA1 was also optimized (Figure 5A). The current suppression increased with the increasing number of complementary base from 7 to 10. However, the large number of complementary bases also caused a large background. Thus, according to the maximum signal-to-background ratio, DNA2″ with 8 complementary base to DNA1 was chosen for 2-EPI. Under their respective optimum conditions, 3-EPI obviously offered higher S/N ratio than 2-EPI for both CEA standard solution and CEA-spiked blood serum sample (Figure 5B), indicating higher detection sensitivity. Moreover, 2-EPI showed obvious interference of components contained in serum, indicating the better selectivity of 3-EPI. The better results of 3-EPI were attributed to the recognition of three independent epitopes on one target protein which could reduce the risk of self-assembly of MNAzyme and the cross-reactivity of closely related proteins.[42,45]

The selectivity of 3-EPI was further evaluated by comparing the current suppression in the presence of different antigen, for example prostate specific antigen (PSA) (Figure 5C). As expected, the 3-EPI showed obvious current suppression to the solutions containing target CEA, although the suppression similar to background was observed in the solution containing only PSA, showing excellent detection specificity for CEA.

Reproducibility and Precision. Both the intra-assay and interassay precisions of the 3-EPI were examined with 10 ng mL^{-1} CEA for 5 times. The relative standard deviations (RSD) were 3.3% and 5.8%, respectively, showing good precision and acceptable fabrication reproducibility of the biosensor. In addition, when the biosensor was stored in dry at 4 °C, over 93% of the initial response of MB was remained after a storage period of 2 weeks, indicating the proposed biosensor possessed acceptable stability.

Real Sample Analysis. To evaluate the analytical reliability and application potential of the proposed method, clinical serum samples from carcinoembryonic cancer patients were further analyzed. The assay results were compared with the reference values from the commercial electrochemiluminescent testing. As shown in Table 1, the results with relative errors less than 6.81% indicated good accuracy of the proposed method for the detection of clinical samples.

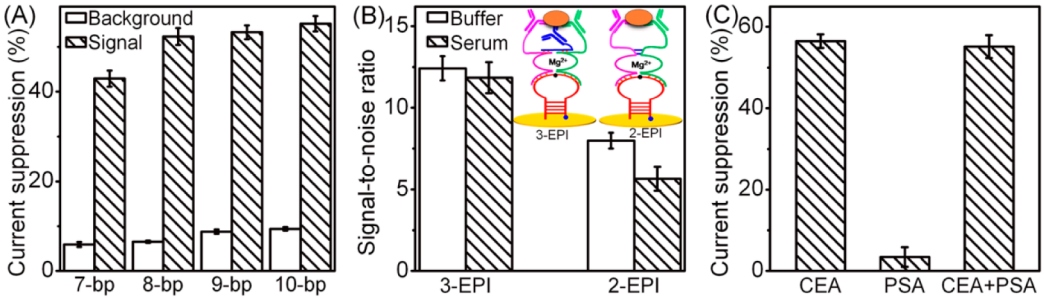

Figure 5. (A) Effect of complementary base number between DNA1 and DNA2″ on the response of 2-EPI, (B) comparison of signal-to-noise ratios from 3-EPI and 2-EPI for 10 ng mL^{-1} CEA in buffer and blood serum from healthy control, and (C) current suppression for 3-EPI of 10 ng mL^{-1} CEA, 10 ng mL^{-1} PSA, and the mixture of 10 ng mL^{-1} CEA and PSA. Error bars represent standard deviations of three parallel experiments.

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Table 1. Assay Results of Clinical Serum Samples Using the Proposed and Reference Methods

sample no.	proposed method (ng mL^{-1})	reference method[a] (ng mL^{-1})	relative error (%)
1	244	232	5.1
2	5.54	5.57	−3.6
3	52.1	49.9	4.4
4	20.4	19.1	6.8

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

CONCLUSION

This work proposed an amplified electrochemical proximity immunoassay for simple, sensitive and selective detection of protein biomarker. This strategy used three DNA-Abs to recognize target protein for the assembly of Mg^{2+}-dependent MNAzyme via target-driven triple-binder proximity hybridization. The formed MNAzyme structure subsequently autocatalyzed the cleavage of MB-HP and thus produced amplified detectable signal. The triple-binder assembly strategy along with the MNAzyme-based catalytic amplification greatly improved the detection sensitivity and selectivity. The proposed 3-EPI method could be carried out with one step in 40 min on an easily prepared DNA biosensor and thus possessed good extensibility for other large protein biomarkers with corresponding DNA-antibody probes.

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Notes

The authors declare no competing financial interest.

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