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

Kewei Ren,† Jie Wu,† Huangxian Ju,*† and Feng Yan*†

1State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P.R. China
2Department of Clinical Laboratory, Nanjing Medical University Cancer Hospital & Jiangsu Cancer Hospital, 42 Baiziting Road, Nanjing 210009, P.R. China

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 Mg²⁺-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 Mg²⁺, 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 nanoprobe can be prepared by employing nanomaterials, such as nanoparticles,⁸⁻¹¹ carbon nanotubes¹²⁻¹⁵ and magnetic beads,¹⁶⁻¹⁸ as nanocarriers to load a high amount of signal-related molecules including enzymes,¹⁹⁻²⁶ quantum dots,²⁷ and metal nanoparticles.²⁴⁻²⁶ These nanoprobe 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 therefore, 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 Mg²⁺-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 immunosassays (EPI) have been developed for the detection of insulin and thrombin by combining the proximity hybridization with DNA sensors.³⁹,⁴⁰

Received: September 15, 2014
Accepted: January 4, 2015
Published: January 4, 2015
In our previous work, the dual-binder proximity hybridization was also used to design a ratiometric electrochemical readout for improving the detection sensitivity. Here, a triple-binder proximity ligation 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	extsuperscript{2+}, the immunocomplex of target/Ab1,2,3 was formed to trigger the proximity hybridization of DNA3 with DNA1,2 to produce the Mg	extsuperscript{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, 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, 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, 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.

**Scheme 1. Schematic Illustration of Triple-Binder Assembly of Mg	extsuperscript{2+}-Dependent MNAzyme along with Autocatalytic Cleavage of MB-HP on Electrochemical Biosensor**
DNA2 (10-bp): 5′-TTTCTTCCAGGCTAGCTCATCGTCCTTGCTGGTACCTTGACCCCACTCGTCCTG-3′
DNA3 (6-bp): 5′-Biotin-GCCACGAGTCGAGCATCAGGTTCGGCTGCTTATACACAATGCTG-3′
DNA3 (7-bp): 5′-Biotin-GCCACGAGTCGAGCATCAGGTTCGGCTGCTTGATACACAATGCTG-3′
DNA3 (8-bp): 5′-Biotin-GCCACGAGTCGAGCATCAGGTTCGGCTGCTTGATACACAATGCT-3′
DNA3 (9-bp): 5′-Biotin-GCCACGAGTCGAGCATCAGGTTCGGCTGCTTGATACAC-3′
DNA3 (10-bp): 5′-Biotin-GCCACGAGTCGAGCATCAGGTTCGGCTGCTTGATAC-3′

The binding regions between DNA3 (or DNA2) and DNA1 (or DNA1′), DNA3 and DNA2 (or DNA2′), and MB-HP (or fluorescent-HP) and DNA1 (or DNA1′) as well as DNA2 (or DNA2′ and DNA2″) 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 MgCl2. 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 (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 mM 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 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 mV 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 K3Fe(CN)6 and K4Fe(CN)6 to characterize the biosensor preparation and measurement procedure (Figure 1). Compared with bare Au 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 Mg2+-dependent MNAzyme 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 H2SO4 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.
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 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 Mg2+-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 Mg2+-dependent MNAzyme via the proximity hybridization of DNA1, DNA2, and DNA3. 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 fluorescence 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 Mg2+-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 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.
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 incubation time and tended to level off until 40 min (Figure 3D). Thus, 40 min was chosen as the optimum reaction time.

### 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.

(B) Calibration curve for CEA detection.

The selectivity of 3-EPI was further evaluated by comparing the current suppression in the presence of different antigens, 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.

### Figure 5

(A) Effect of complementary base number between DNA1 and DNA2\(^{2+}\) 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.

### 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 comparable to previous EPI,\(^{41}\) with a detectable concentration range of 5 orders of magnitude.

### 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.

### 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.
amplificaiton. The formed MNAzyme structure subsequently autocatalyzed the cleavage of MB-HP and thus produced the desired products.

REFERENCES

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge National Natural Science Foundation of China (21135002, 21105046, and 21475063), the Leading Medical Talents Program from Department of Health of Jiangsu Province, and Science Foundation of Jiangsu (BL2013036).

REFERENCES

(20) Zhuo, Y.; Yuan, P. X.; Yuan, R.; Chai, Y. Q.; Hong, C. L. Biomaterials 2009, 30, 2284–2290.

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

<table>
<thead>
<tr>
<th>sample no.</th>
<th>proposed method (ng mL⁻¹)</th>
<th>reference method (ng mL⁻¹)</th>
<th>relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>244</td>
<td>232</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>5.54</td>
<td>5.57</td>
<td>−3.6</td>
</tr>
<tr>
<td>3</td>
<td>52.1</td>
<td>49.9</td>
<td>4.4</td>
</tr>
<tr>
<td>4</td>
<td>20.4</td>
<td>19.1</td>
<td>6.8</td>
</tr>
</tbody>
</table>

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


