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Combining Peptide and DNA for Protein Assay: CRIP1 Detection for Breast Cancer Staging

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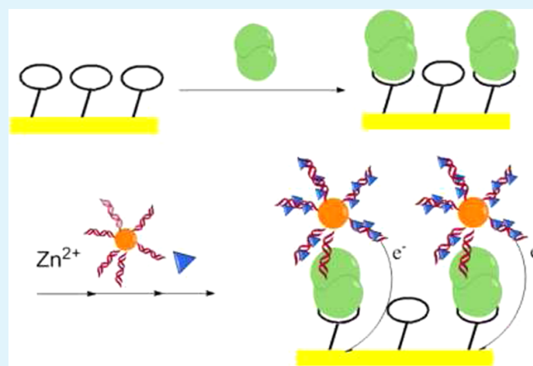
Supporting Information

ABSTRACT: In this work, a novel method for a protein assay is proposed which uses the specific protein-binding peptide of the target protein and sequence-specific DNA to interact with the target as the capture and detection probe, respectively. Meanwhile, since the DNA sequence can be coupled with gold nanoparticles to amplify the signal readout, a sensitive and easily operated method for protein assay is developed. We have also employed a transcription factor named as cysteine-rich intestinal protein 1 (CRIP1), which has been identified as an ideal biomarker for staging of breast cancer, as the model protein for this study. With the proposed method, CRIP1 can be determined in a linear range from 1.25 to 10.13 ng/mL, with a detection limit of 1.25 ng/mL. Furthermore, the proposed method can be directly used to assay CRIP1 in tissue samples. Owing to its desirable sensitivity, excellent reproducibility, and high selectivity, the proposed method may hold great potential in clinical practice in the future.



KEYWORDS: *gold nanoparticles, breast cancer, cysteine-rich intestinal protein 1, cyclic peptide, electrochemistry*

■ INTRODUCTION

Development of a rapid, sensitive, selective, and reproducible method for the assay of proteins is of great importance for both biological studies and clinical diagnostics. Therefore, the study of new analytical protocols for the development of new methods for a protein assay is receiving more and more interest. For instance, detection of transcription factors (TFs) is highly required. TFs are sequence-specific DNA-binding proteins that control gene expression by binding to DNA and subsequently recruiting downstream effector proteins.[1,2] TFs play pivotal roles in the pathways and networks of gene expression regulation, and their expression levels sensitively reflect cellular development and disease state.[3,4] The common methods to detect TFs primarily rely on the traditional immunoassay,[5,6] which mainly involves antibodies. However, antibodies have some fundamental shortcomings for protein analysis, such as high cost, high time consumption, and complicated operation, among others.

On the basis of the molecular recognitions among TFs, their target DNA and effector proteins, we propose a novel and universal method in this work to detect TFs by using the specific protein-binding peptide of the target protein and sequence-specific DNA, which may also bind with the target protein. Recently, due to the study on the original TF−effector protein interaction, a short peptide has been developed as a more compelling choice than an antibody in TF recognition,[7−9] while a peptide may also have several significant advantages. For instance, the following are advantages of peptides: peptides have definite chemical structure; peptide synthesis is straightforward and cost-effective; and peptides can be modified easily in a site-specific manner to offer an even larger chemical versatility than antibodies. However, the property of a peptide cannot provide much opportunity for the use of the currently employed enzyme/nanoparticle-based sensing strategies to develop new methods for the assay of target proteins. In this context, we propose that protein-specific-binding DNA is a helpful complement of protein-binding peptides. On the one hand, DNA has the same advantages as a peptide in terms of synthesis and stability. On the other hand, DNA may play an important part in signal amplification of the detections,[10−12] and actually, there have been many strategies to utilize a DNA sequence to realize signal amplification,[13,14] such as DNA rolling circle amplification (RCA), DNA sequence extension via click chemistry, coupling with gold nanoparticle (GNPs), and so forth.[15,16] On the basis of the above reasoning, we propose that protein-binding peptides may serve as the capture probe to provide intrinsic recognition, while protein-binding DNA sequences may act as the detection probe that can provide

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extrinsic signal readout. So, the multidomained and complicatedly glycosylated anti-TFs antibodies can be substituted by the simple functional equivalents, and the assay of TFs can be greatly simplified.

The above strategy has also been demonstrated in this work by using cysteine-rich intestinal protein 1 (CRIP1), a TF present in abnormally high amounts in 90% of breast cancer cases.[17−19] CRIP1 has been identified as an ideal biomarker for detecting breast cancer at earlier and more treatable stages, and it has significant prognostic impact on breast cancer.[20,21] In this study for CRIP1 detection, a cyclic peptide and an AT DNA are selected as the capture probe and the detection probe, respectively. The cyclic peptide can bind with the N-terminal one of the two zinc finger domains of CRIP1,[21] while the AT DNA recognizes the C-terminal zinc finger in the presence of zinc ion.[22,23] Moreover, the cyclic peptide may exhibit enhanced affinity toward CRIP1 due to the constrained conformation, while the AT DNA can be coupled with GNPs to amplify readout signal for the detection.[24−31] This is the first report to make use of the synergistic effects of protein-binding peptides with DNA sequences to present a simple and convenient method for the assay of TFs or even all kinds of proteins. Moreover, the feasibility of our method has been tested in the quantification of CRIP1 in tissue samples from breast cancer victims of different stage, and the detected signal readout is in parallel with the progression of breast cancer; thus, the proposed strategy for the protein assay may have great potential application in the future.[32,33]

EXPERIMENTAL SECTION

Reagents and Chemicals. The cyclic peptide ([K-11-mercaptoundecanoic acid (MUA)-SRHNDKLG]c, received as powder, with the purity higher than 95%) was custom-synthesized by Shanghai Science Peptide Biological Technology Co., Ltd. Bovine serum albumin (BSA) and human CRIP1 (recombinant, purity >98%,) were from TechnoGene. HPLC-purified oligonucleotides were obtained from Takara (Dalian) cooperation, with sequences given as follows: 3′-thiolated single-stranded DNA (SH-ssDNA): 5′-TTTTTTTTTTCCTCTCAACTCGTA(SH)-3′. Complementary DNA (cDNA): 5′-TACGAGTTGAGAGGAAAAAAAAAA-3′. Other reagents employed in this work were all of analytical-grade and were used as received. The powder of the peptide was dissolved and diluted with phosphate-buffered saline (PBS, pH 7.4) to 5 μM as the stock solution. The recombinant CRIP 1 (received as powder) was dissolved using 10 mM PBS (pH 7.4) to various concentrations as the standard samples. The ultrapure water employed for the dilution of all the solutions described above and below was produced by a water purification system (Milli-Q), the purity of which was warranted by purification until the gold standard resistance of 18 MΩ·cm. Human tissue samples from breast cancer victims of different stages were collected from the Department of Oncology, the First Affiliated Hospital of Nanjing Medical University, which were approved by the local hospital ethical committees. Pretreatment of the fresh tissue samples was conducted using a kit according to the manual (Active Motif). According to the manufacturer's instructions, the tissue samples were diced and homogenized, followed by fractionation, and then the cytoplasmic fraction of protein sample was collected for CRIP1 detection.

Preparation of Functionalized GNPs. Synthesis of GNP and the fabrication of AT DNA modified GNPs all followed well-established protocols.[34] Briefly, 100 mL 0.01% $HAuCl_4$ was reduced by a 1%, 3.5 mL sodium citrate solution. The reaction took place in a boiled water bath, with the reductant being rapidly added followed by vigorous stirring. The synthesized GNPs were allowed to cool overnight and were filtered through a 8 μm filter via centrifugation (13 000 rpm, 20 min). For immobilization of AT ssDNA on the GNPs, thiol-tethered AT ssDNA activated by Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was incubated with the as-prepared GNPs for 16 h at 4 °C. After this step, salting treatments (1 M NaCl) were repeated six times, followed by an overnight incubation for the preparation of AT ssDNA modified GNPs to mature. After another centrifugation step (13 000 rpm, 20 min), precipitates were collected and resuspended. Complementary DNA was hybridized with the AT ssDNA-immobilized on the GNPs through 95 °C incubation for 2 min and a subsequent gradual cooling. The AT dsDNA modified GNP had been examined by UV−vis spectra (Figure S4).

Gold Electrode Treatment and Modification. First, residual impurity, especially organic impurity, on the gold disk electrode (3 mm diameter) was thoroughly removed by piranha rinsing buffer. This buffer was formulated by 70% H_2SO_4 (concentrated) and 30% hydro peroxide (H_2O_2); therefore, precautions should be followed when using this solution. Meanwhile, since this rinsing buffer could react with organic material very violently, a droplet of it should be dripped onto the electrode surface with extreme caution, so that the droplet could be held by surface tension to stay within the periphery of gold surface, without contacting with the surround insulating epoxy resin. This cleaning step lasted for 5 min, followed by thoroughly rinsing with redistilled water. The electrode was then polished to a mirror-like smooth surface with the slurry prepared with alumina. Alumina powder with an average diameter of particle of 1 and 0.3 μm were separately employed for polishing. After a water rinse, the remaining powder of alumina on the electrode was removed by water-bath sonicating. This step was repeated in different media including water and pure alcohol, each for 5 min. Sulfuric acid (0.5 M) was then adopted to electrochemically clean the electrode. For the modification of the electrode, the freshly cleaned electrode was first dried under a mild stream of nitrogen with high purity. The electrode was immediately subjected to a solution containing 2.5 μM substrate cyclic peptide and 5 mM TCEP in 10 mM PBS, pH 7.4. This step lasted for 16 h at 4 °C. After self-assembly of the peptide, the electrode was passivated by 9-mercapto-nonal (1 μM in 5 mM TCEP in 10 mM PBS, pH 7.4) treatment for 3 h.

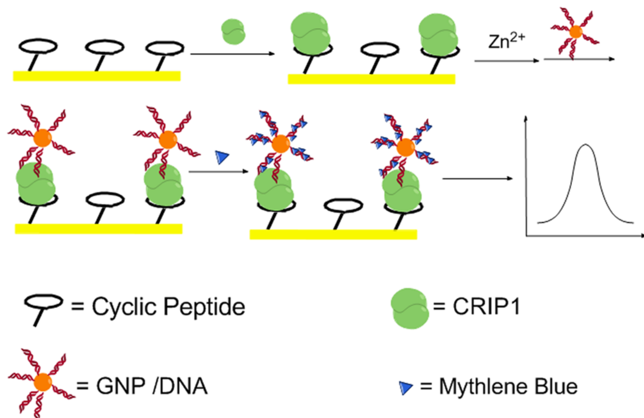
CRIP1 Detection. The sample, either standard sample prepared from recombinant protein, or the biologically complicated sample fractioned from the tissue samples of cancer victims, was incubated with the as-prepared electrode for 1 h at 37 °C. To lower the interference rising from the adsorption of irrelevant protein species, after the above incubation step, the electrode was washed repeatedly with redistilled water. To further reduce such types of interference, 5% Tween-20 was incubated with the electrode for 30 min. To facilitate subsequent binding of GNP/DNA conjugates to the target proteins immobilized, the electrode was first treated with 50 mM Tris-HCl 20 μM zinc chloride solution at room temperature for 1 h to induce CRIP1 conformational change. AT dsDNA modified GNPs (10 mM PBS, pH 7.4) was then incubated to the electrode surface for a 75 min treatment at room temperature. Finally, 100 μM MB (10 mM PBS, pH 7.4) was incubated with the electrode for 1 h.

Electrochemical and UV−Vis Measurements. Electrochemical assays were carried out on a CHI660D potentiostat. A commonly employed electrode system consisting of three types of electrode was used to construct the electrolysis cell, namely, a saturated calomel electrode (SCE), a platinum rod, and the gold electrode modified cyclic peptide. SWVs were recorded in 10 mM PBS (pH 7.4), and 5 mM $[Fe(CN)_6]^{3-/4-}$ with 1 M KNO_3 was used to obtain EIS. Both solutions were deoxygenated by purging with nitrogen gas and maintained under this inert atmosphere during the above electrochemical scans. Experimental parameters for the amplified response of MB are as follows. SWV: scan range, −0.6−0 V; step potential, 5 mV; frequency, 15 Hz; amplitude, 25 mV. EIS: biasing potential, 0.224 V; amplitude, 5 mV; frequency range, 0.1 Hz to 10 kHz. The data are obtained from at least three repetitions of independent experiments, and error bars are shown in the figures.

■ RESULTS AND DISCUSSION

The principle of the proposed method for the CRIP1 assay has been illustrated in Scheme 1. First, the CRIP1-binding cyclic peptides are self-assembled onto an electrode surface. After incubation of the electrode with the solution containing CRIP1, a fraction of the peptides on the electrode surface become protein-bound. Subsequently, the protein-bound electrode is treated in sequence with zinc chloride and GNPs modified by AT DNA. Zinc ion can coordinate the captured CRIP1 molecules to trigger their conformational change favorable to subsequent AT DNA binding. Consequently, some of the AT DNA molecules modified onto the surface of GNPs are bound with the captured CRIP1 molecules, while most of them are then employed to recruit methylene blue (MB) to produce signal readout. Because the AT DNA molecules are tethered with GNPs, and one strand out of the many on the surface of one nanoparticle can bind with the captured CRIP1 while all the other strands can serve to generate signal, the GNPs modified with AT DNA molecules will result in vast signal amplification. With the increase of CRIP1 concentration, the readout signal shows the same growth tendency in a greater scale, which forms the basis of the proposed novel method to assay CRIP1.

Scheme 1. Principle of the CRIP1 Assay[a]



[a]Not drawn to scale.

In order to further validate the proposed method described in Scheme 1, electrochemical impedance spectra (EIS) are used to demonstrate the stepwise treatment and modification of the electrode (Figure 1). EIS of the bare electrode (curve a) is similar to a straight line. Nevertheless, after the immobilization of the cyclic peptides, as is shown in curve b, a semicircle can be observed, indicating an increase of interfacial electron transfer resistance because the self-assembly monolayer of peptides may hinder the ET between electroactive species $[Fe(CN)_6]^{3-/4-}$ and the underlying electrode. Moreover, when the electrode is further treated with CRIP1 protein, the diameter of the semicircle dramatically increases (curve c), manifesting binding of CRIP1 with the cyclic peptides. Nevertheless, after interaction with zinc ion, the diameter of the semicircle decreases slightly (curve d), implying the CRIP1−Zn^{2+} coordination. Moreover, as is shown in curve e, when the electrode is subsequently treated with AT DNA modified GNPs, the resistance further decreases, which resulted from the good conductivity of GNPs and their proximity to the electrode surface.

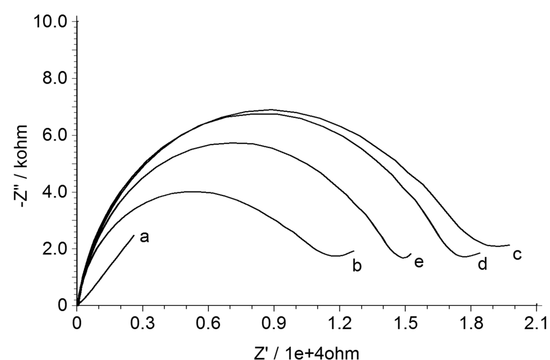


Figure 1. Nyquist diagrams corresponding to each step of the modification of the electrode: (a) the bare gold electrode, (b) the electrode after peptide modification, (c) after treatment with 10.13 ng/mL CRIP1, (d) after treatment with 20 μM Zn^{2+}, (e) after incubation with 14 nM GNPs modified by AT dsDNA. Electrochemical species: 5 mM $[Fe(CN)_6]^{3-/4-}$. Biasing potential: 0.224 V. Amplitude: 5 mV. Frequency range: 0.1 Hz to 10 kHz.

To improve the performance of the proposed assay, some important aspects for our method have been optimized. First, the incubation time of the cyclic peptide modified electrode with CRIP1 is investigated. As is shown in Figure S1, the peak current increases along with the reaction time until 60 min, and then it remains nearly unchanged; therefore, 60 min is chosen as the optimal reaction time. Second, the effect of Zn^{2+} concentration is investigated. It can be observed in Figure S2 that the peak current is positively related to Zn^{2+} concentration, and it reaches a plateau at 20 μM. Therefore, 20 μM Zn^{2+} has been used in the subsequent experiments. Finally, incubation time for the binding of CRIP1 with AT DNA immobilized onto the surface of GNPs has been optimized as well. As is shown in Figure S3, a 75 min incubation time at room temperature is sufficient.

Figure 2a depicts square wave voltammograms (SWVs) recorded by analyzing CRIP1 at different concentrations. It can be observed in Figure 2 that the readout signal increases along with the concentration of CRIP1. A plot of the peak currents versus the CRIP1 concentrations may reveal a positive correlation in the range from 1.25 to 10.13 ng/mL with a detection limit of 1.25 ng/mL, defined as signal-to-noise ratio of 3:1. Thus, the obtained limit of detection in this work can be comparable to the current antibody-based method. The average coefficient of variation shows a desirable reproducibility of our data, which is below 5%.

To examine the specificity of our method, a series of control experiments have been conducted. As is shown in Figure 3, all control proteins result in signal readout nearly the same as that for the blank control. This result is reasonable because the cyclic peptide probes cannot bind to the other nonspecific proteins. These experimental results have also confirmed that CRIP1 can bind with the cyclic peptides in a sequence-specific fashion, and our method for protein assay may have satisfactory target specificity.

Performance of the proposed new assay method in clinical application has also been tested by using tissue samples from breast cancer victims of different stages. CRIP1 is not only an ideal marker for early stage breast cancer detection but also its expression increases with cancer development, the status of which correlates with adverse clinical prognostic indicators. Tissue samples from ductal carcinoma in situ (DCIS) (stage 0), low stage (stage 1−2), and high stage (stage 3−4) together

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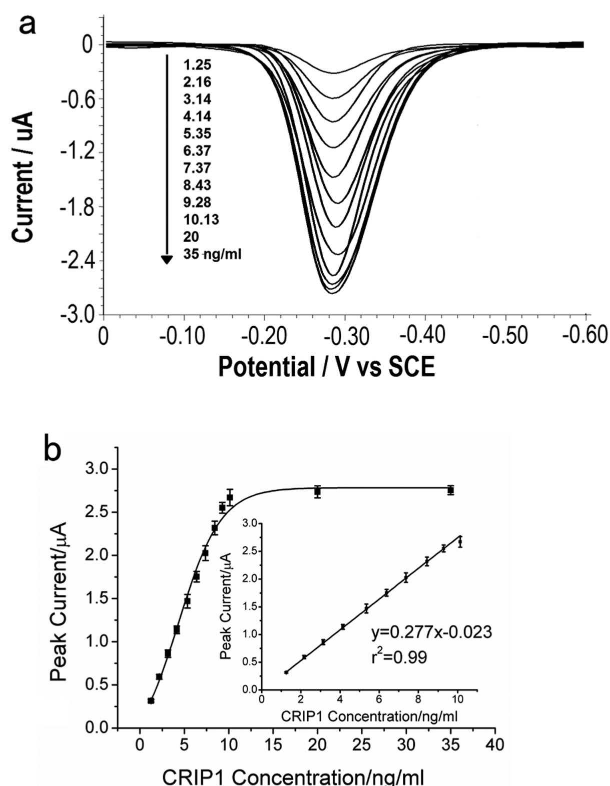

Figure 2. SWVs obtained at the cyclic peptide modified electrode for the measurements of CRIP1 at different concentrations. The electrode has undergone the same treatment as described in Figure 1. Electrochemical parameters: scan range, −0.6–0 V; step potential, 5 mV; frequency, 15 Hz; amplitude, 25 mV. (b) Relationship between the peak current and the CRIP1 concentration. Inset shows the linear range. Error bars represent standard deviations of measurements (n = 3).

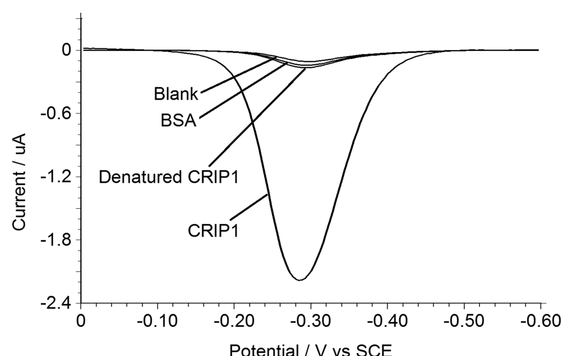

Figure 3. SWVs obtained at the cyclic-peptide-modified electrode to show the interference of control species. All targets are at 10.13 ng/mL. Control experiments are obtained after the same procedure as Figure 2, except that a standard CRIP1 sample and several control samples are incubated, respectively, with the cyclic peptide-modified electrode. Blank control: 10 mM PBS (pH 7.4).

with pericancerous tissue as reference are separately collected and fractionated for CRIP1 assays. Figure 4 shows the comparison of the expression level of CRIP1 in normal tissue, DCIS, low stage, and high stage of breast cancer, which can clearly show the difference. This analysis has revealed a significant correlation between CRIP1 and tumorigenesis. With the deepening of the degree of tumor progression, the expression of CRIP1 can also be observed to increase

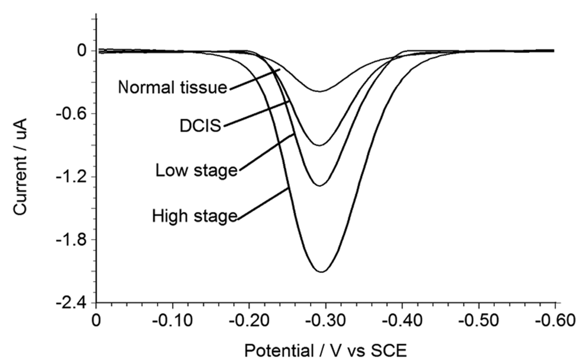

Figure 4. SWVs obtained at the cyclic peptide modified electrode for the analysis of CRIP1 from the tissue lysate of breast cancer samples with different stages. All the other conditions are the same as Figure 2.

constantly. Therefore, this new method proposed in this work may enable efficient analysis of CRIP1 expression in clinical applications.

■ CONCLUSIONS

In this work, we have proposed a novel method for a TF assay by using its specific-binding peptide and sequence-specific DNA separately as the capture probe and the detection probe.[35,36] Highly sensitive detection of CRIP1 down to 1.25 ng/mL is attained by coupling the CRIP1-binding AT DNA with GNPs. Moreover, the proposed method can perform well in the CRIP1 assay using biopsy samples from breast cancer victims, and the detected CRIP1 expression can serve as a reliable reference for the advancement of cancer. On the other hand, the proposed method in this study can be developed as a universal approach for the assay of other TFs or even other kinds of proteins if the peptide and DNA used in this work are separately replaced by the corresponding protein-binding peptide and protein-binding DNA. So, this work may not only have great potential clinical use in the future due to the high sensitivity and selectivity as well as excellent results for the test of tissue samples from breast cancer victims of different stages, but this study has also extended the peptide-based protein assay approaches, which may open more opportunities for the use of peptides as recognition element for protein assays.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary data on the optimization of various experimental conditions as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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