Article

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Peptide Cleavage-Based Ultrasensitive Electrochemical Biosensor with an Ingenious Two-Stage DNA Template for Highly Efficient DNA Exponential Amplification

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Abstract

The direct transduction of peptide cleavage event into DNA detection always produced output DNA with some amino acid residues, which influenced the DNA amplification efficiency in view of their steric hindrance effect. Here, an ingenious two-stage DNA template was designed to achieve the highly efficient DNA amplification by utilizing DNA exponential amplification reaction (EXPAR) as model. The usage of two-stage DNA template not only accomplished the traditionally inefficient EXPAR triggered by output DNA with some amino acid residues, but also simultaneously produced a newly identical DNA trigger without any amino acid residues to induce an extra efficient EXPAR, which significantly improved the DNA amplification efficiency, realizing the ultrasensitive detection of target. On the basis of proposed highly efficient DNA amplification strategy, a novel peptide cleavage-based electrochemical biosensor was constructed to ultra-sensitively detect matrix metalloproteinases-7 (MMP-7). As a result, this developed assay demonstrated excellent sensitivity with a linear range from 0.1 $pg \cdot mL^{-1}$ to 50 $ng \cdot mL^{-1}$ and detection limit down to 0.02 $pg \cdot mL^{-1}$, which paved a novel avenue for constructing ultrasensitive peptide cleavage-based biosensor.

Introduction

Sensitive quantification of protein biomarkers is of particular importance in early clinical diagnosis, disease prevention and treatment.[1-3] Typically, protein recognition elements include antibody,[4,5] peptide[6,7] and aptamer.[8,9] Among them, peptide is a more compelling choice for its prominent advantages, such as accessibility, simplicity, cost effectiveness and so on.[10-12] Various peptide-based methods have been developed for protein analysis, including fluorescence[13], electrochemiluminescence,[14] and electrochemical assays,[15] in which electrochemical assays have gained particular attention owing to its inherent advantages in terms of convenience, sensitivity, and speed[16-18]. Revzin[19] and Kelley[20] groups have built some electrochemical methods on the basis of target specifically cleaved substrate peptides for various proteins detection. These strategies enable the monitoring of target in an effective and simple fashion, however, these biosensors are signal-off assays suffering from drawbacks of "false positive" results and limited signal output. Thus, it is particularly valuable to explore more reliable and sensitive strategy for trace protein.

The direct transduction of peptide cleavage event into DNA detection may be a fascinating strategy in enhancing detection sensitivity for proteins because it enables further employment of many DNA-based amplification technologies, such as hybridization chain reaction,[21,22] rolling circle amplification[23,24] and enzyme-assisted recycling amplification.[25,26] However, this transduction strategy has rarely been applied in peptide biosensor, since the obtained output DNA *via* peptide cleavage

always had some amino acid residues with steric hindrance effect suffering from limited DNA amplification efficiency. If the output DNA with amino acid residues can be quickly translated into a new DNA without any amino acid residues to induce DNA amplification, it may greatly improve the DNA amplification efficiency and have potential to construct an ultrasensitive peptide cleavage-based biosensor.

In response, here, we took exponential amplification reaction (EXPAR)[27-29] as model to design an ingenious two-stage DNA template, which quickly converted the output DNA with amino acid residues to new DNA without amino acid residues to induce another highly efficient EXPAR in creating massive of product DNA (S1). On the basis of proposed amplification strategy, a sensitive peptide cleavage-based electrochemical biosensor was constructed to detect matrix metalloproteinases-7 (MMP-7). The target MMP-7 specifically cleaved peptide in a magnetic peptide-DNA probe and released a DNA with some amino acid residues (trigger 1) to initially induce EXPAR by using the designed two-stage DNA template. Surprisingly, this process generated few products DNA (S1) as well as DNA fragments (without any amino acid residues, termed as trigger 2) that had same sequence with trigger 1. This generated trigger 2 could initiate effectively cyclic EXPAR in creating abundant S1 for producing amplified signal with the aid of catalyzed hairpin assembly (CHA) recycling. With the cascade amplification reactions of EXPAR and CHA, the sensitivity of the proposed method had been significantly enhanced, which also provided an attractive strategy for other proteins detection in peptide biosensor.

Experimental Section

Materials and Reagents

Streptavidin-coated magnetic microbeads (STV-MB), D,L-dithiothreitol (DTT) and 6-mercapto-1-hexanol (MCH) and N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP) were provided by Sigma Chemical Co. (St. Louis, MO, USA). The Nt.BtsNBI nicking enzyme and Vent (exo-) polymerase were obtained from New England Biolabs Ltd. (Beijing, China). Matrix metalloproteinases-7 (MMP-7) and matrix metalloproteinases-2 (MMP-2) were supplied by Sino Biological Inc. (Beijing, China). The biotinylated peptide (biotin-RPLALWRSCCC-SH) was obtained from Shanghai GL Biochem Ltd. (Shanghai, China). 0.1 M PBS (pH 7.0) and 20 mM Tris-HCl (pH 7.4) was respectively served as working buffer and binding buffer. All DNA oligonucleotides were provided by Sangon Inc. (Shanghai, China) and listed in Table S1 (see the Supporting Information)

Apparatus

Cyclic voltammetric (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements were conducted on a CHI 660E electrochemistry workstation (Shanghai Chenhua instrument Co., Ltd., China) with traditional three-electrode system.[30] Gels image was obtained by the Bio-Rad imaging system (Hercules, CA, U.S.A.).

Preparation of Magnetic Peptide-DNA Probe

First, 20 μL streptavidin-coated magnetic microbeads (STV-MB) was mixed with 20 μL biotinylated peptide biotin-RPLALWRSCCC-SH (10 μM) for 40 min under shaking to obtain peptide-conjugated magnetic microbeads (MB-peptide) through the specific interaction between streptavidin and biotin. After being rinsed twice with PBS to remove uncoupled peptide, the MB-peptide conjunction was re-suspended in 100 μL PBS solution. Subsequently, amine-modified DNA was covalently coupled to resulting MB-peptide with a heterobifunctional cross-linking reagent SPDP by linking amino and thiol groups. Details were listed as follows: amine-modified DNA was first activated by SPDP for 1 h. Then, activated DNA was added into prepared MB-peptide and incubated for another 1 h. The finally obtained MB-peptide-DNA magnetic probes were washed with PBS to remove uncoupled DNA, and re-suspended in 0.1 M PBS solution for the following experiment.

Preparation of the MCH/H1/depAu/GCE

First, a cleaned GCE was electrodeposited a thin layer of AuNPs (depAu) at -0.2 V for 30 s.[31-33] Next, 10 μL of 2 μM H1 was modified on the electrode for 12 h to obtain H1/depAu/GCE. Finally, the H1/depAu/GCE was incubated with MCH (10 μL, 1.0 mM) for 30 min to block the nonspecific binding sites.

Peptide Cleavage Triggered EXPAR Reaction

20 μL different concentrations of target MMP-7 was mixed with the above 20 μL prepared magnetic nanoprobe MB-Peptide-DNA in a plastic tube and incubated at 37 ℃ for 40 min. In this process, MMP-7 selectively cleaved the peptide in the probe

and released DNA, which could be acted as a trigger for EXPAR amplification. The detailed operation process of EXPAR was listed: first, the released DNA from magnetic probe was mixed with 200 nM model template. The mixture was heated for 10 min at 88℃ and then cooled to room temperature slowly to ensure the hybridization between DNA and template. After the introduction of 0.05 U μL^{-1} Vent (exo-) DNA polymerase, 250 μM dNTPs and 0.4 U μL^{-1} Nt·BstNBI nicking enzyme, the mixture reacted at 53 ℃ for 50 min. Then, the solution was heated at 90 ℃ for 10 min to terminate reaction, and obtained products would be served as catalysts to further induce CHA recycling on the modified electrode surface and output electrochemical signal.

Experimental Measurements

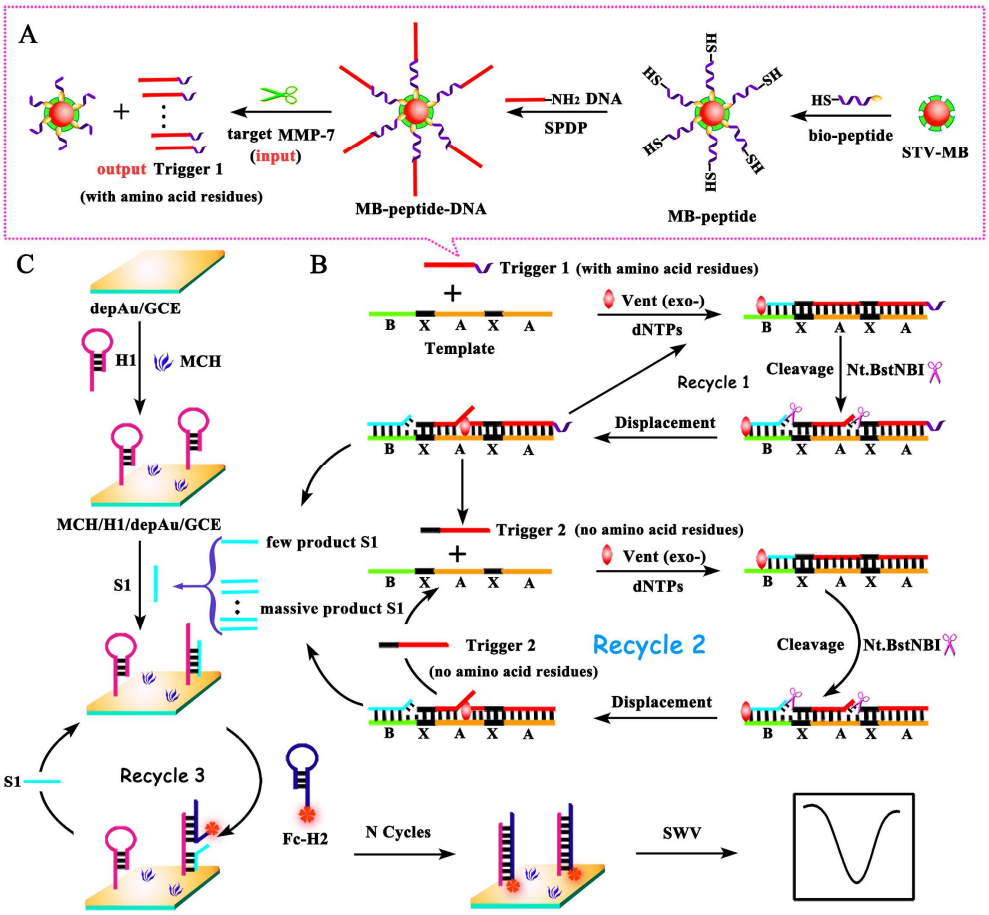
Before electrochemical measurements, 5 μL EXPAR products and 5 μL ferrocene-labeled H2 (Fc-H2) were dropped onto the surface of H1/depAu/GCE and incubated for 2 h at 37 ℃. The obtained electrode then was rinsed to remove the unbound reagents and investigated by SWV in 2 mL PBS buffer. The SWV parameters for measurement were as follows: potential scan range, 0.6~0 V; amplitude, 25 mV; frequency, 25 Hz; step potential, 4 mV.

Results and Discussion

The Assay Principle of Developed Strategy

The assay principle of peptide-cleavage triggered cascade amplification strategy for MMP-7 detection was illustrated in Scheme 1. The section A depicted the

preparation procedure of magnetic nanoprobe (MB-peptide-DNA). With the introduction of target MMP-7, peptide was specifically cleaved and DNA was released from the magnetic probe, which was acted as trigger 1 to hybridize with a template to initiate EXPAR amplification. Here, the designed template involved in five domains (AXAXB). Two repeated A domains were complementary with the DNA sequences of trigger 1. For domain X, its complementary strand contained the recognition site of nicking enzyme Nt·BstNBI. And domain B was complementary to the product DNA (S1). Upon the formation of partial duplex based on base complementary pairing between trigger 1 and designed template, the Vent (exo-) polymerase then catalyzed the extension of trigger 1 (with the assistance of dNTPs) to obtain a new DNA strand. Meanwhile, two recognition sites for Nt·BstNBI were produced, which could induce Nt·BstNBI to cleave two strand nicks in extended DNA. Afterwards, the cleaved DNA strand would extend again, therefore preceding extended DNA fragment complementary with domain A and product S1 would be displaced owing to the strand-displacement activity of Vent (exo-) DNA polymerase. Here, it should pay attention to that, the displaced DNA fragment complementary with domain A had identical oligonucleotides sequences with trigger 1 and did not have any amino acid residues, which can be served as a new trigger 2 to induce efficiently cyclic exponential amplification and generate amounts of S1. Then these S1 as catalysts can further induced a CHA recycling on electrode surface to immobilize Fc-H2 and obtain an amplified electrochemical SWV response for indirect determination of MMP-7.



Scheme 1. The schematic illustration of proposed electrochemical sensing system for MMP-7 determination: (A) target induced cleavage of magnetic nanoprobe (MB-peptide-DNA) to release trigger 1; (B) the highly efficient EXPAR amplification process based on an ingenious two-stage DNA template; (C) the electrode modification and signal output process on electrode with CHA recycling amplification.

Investigation of the EXPAR and CHA *via* Polyacrylamide Gel Electrophoresis (PAGE)

The feasibility of EXPAR and CHA were verified by PAGE. As depicted in

Figure 1, the single strand trigger 1 DNA with the lowest molecular weight ran fastest on the agarose gel (lane 1). After trigger 1 hybridized with template, a bright band was observed (lane 2). When the EXPAR reaction was conducted in the introduction of Vent (exo-) polymerase and Nt•BstNBI, dual-band appeared (lane 3) including a bright band matching the hybridization product of DNA and template and a blurred band matching the product DNA (S1), suggesting the successful completion of EXPAR. Lane 4 showed the band of control sample S1. Hairpin H1 and hairpin H2 exhibited single band in Lane 5 and lane 6, respectively. The lane 7 loaded with the mixture of H1 and H2, there were no reaction occurred in absence of EXPAR products. Instead, when the EXPAR products were introduced into the mixture of H1 and H2, a bright lane in accordance with the hybridization product of DNA and template was obtained at the top of lane 8. And there was another bright lane matching with the H1-H2 duplex in lane 9 that produced by S1 inducing CHA reaction directly. Comparing the results of line 8 and line 9, we also could inferred that the EXPAR products had initiated CHA reaction successfully.

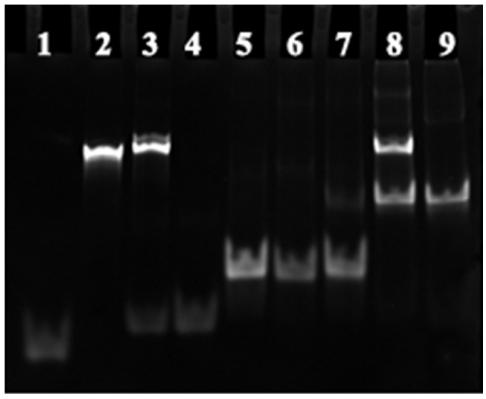


Figure 1 PAGE result for different samples: Lane 1, single-stranded DNA trigger 1; lane 2, the mixture of trigger 1 and designed template; lane 3, amplification product of

EXPAR; lane 4, single-stranded DNA (S1); lane 5, hairpin H1; lane 6, hairpin H2; lane 7, a mixture of H1 and H2; lane 8, the mixture of EXPAR product, H1 and H2; lane 9, the amplification product by CHA (S1, H1 and H2).

The Stepwise Electrochemical Characterization of Modified Electrode

The interface properties of modified electrode were firstly characterized by CV experiments. Clearly seen in Figure 2A, a pair of well-defined $[Fe(CN)_6]^{3-/4-}$redox peak (curve a) was exhibited on bare GCE. After AuNPs were electrodeposited onto electrode, the peak current (curve b) significantly increased owing to a fact that the electron transfer kinetics associated with $Fe(CN)_6^{3-/4-}$ process at Au surface is faster than bare GCE electrode. When H1 was modified onto the electrode, peak current signal (curve c) decreased, amounts of literatures[34-37] had reported that this can be primarily ascribed to the electrostatic repulsion between $[Fe(CN)_6]^{3-/4-}$and the negative charges on the phosphate backbones of the immobilized H1. With the introduction of blocking reagent MCH, a further decreased peak current (curve d) was obtained. These characterizations revealed that the sensing interface was successfully constructed.

The fabrication procedure of modified electrodes also was characterized with EIS. Figure 2B showed the impedance spectra of modified electrode at different stages. Compared with bare GCE (curve a), a smaller semicircle (curve b) was obtained at depAu/GCE. When H1 was fabricated on electrode surface, the semicircle diameter (curve c) increased. Upon blocking with MCH, a further increased semicircle diameter (curve d) was obtained. These results were consistent with that

observed in CV, indicating the modified electrode interface had been successfully fabricated.

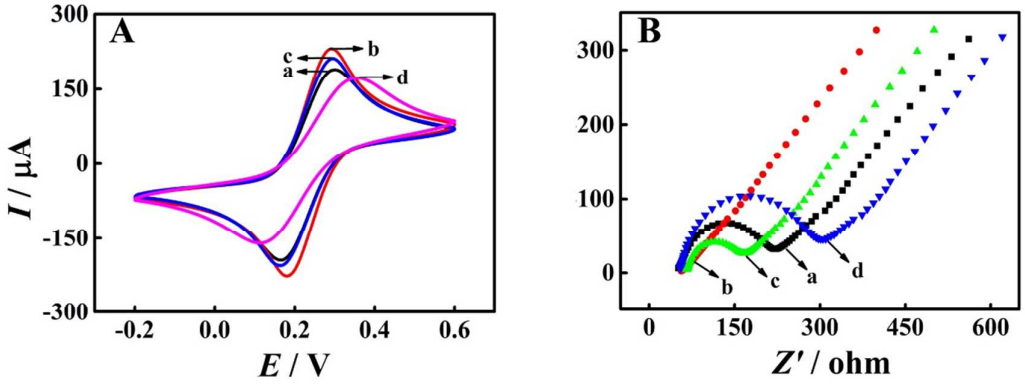


Figure 2. CV (A) and EIS (B) characterizations of modified electrode at different stages in $[Fe(CN)_6]^{3-/4-}$ (5.0 mM) solution: (a) bare GCE, (b) depAu/GCE, (c) H1/depAu/GCE, (d) MCH/H1/depAu/GCE.

Optimization of the Experimental Condition

The electrochemical response signal of fabricated biosensor was depended on the amount of immobilized H2, which was affected by the products of EXPAR. Thus, a time-course experiment for EXPAR was carried out by using the 1 ng·mL^{-1} target MMP-7 in conditions of 0.05 U μL^{-1} Vent (exo-), 250 μM dNTPs and 0.4 U μL^{-1} Nt·BstNBI. As depicted in Figure 3, the electrochemical signal increased gradually and reached a stable status at 50 min. This can be attributed to that with longer EXPAR time, more products DNA (S1) were generated to induce the hybridization between H2 and H1 until to reach saturation. Therefore, 50 min has been selected as the optimal EXPAR time.

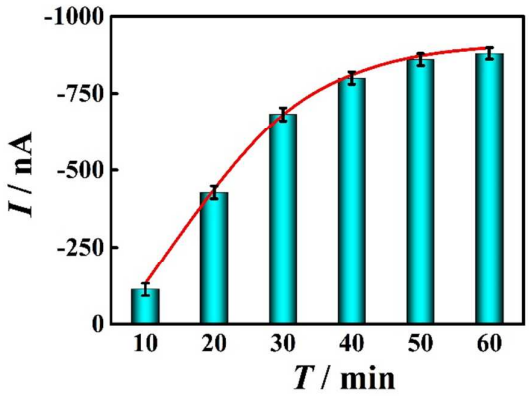


Figure 3. The effect of EXPAR time on electrochemical response (0.05 U μL^{-1} Vent (exo-), 250 μM dNTPs and 0.4 U μL^{-1} Nt·BstNBI).

Comparison of Different Signal Amplification Strategies

To clarify this EXPAR signal amplification, a control experiment had been conducted and the results was listed as followed in Figure 4. First, a magnetic peptide-DNA (S) probe was prepared to act as a recognition probe. Since the target MMP-7 can specifically cleave the peptide in recognition probe, the DNA (S) was quantitatively released from magnetic microbeads in the presence of MMP-7, which could be as a trigger to initiate the CHA reaction directly and produced a measurable electrochemical signal (curve a, 390 nA). Comparatively, there appeared an obvious increase in current response (curve b, 1125 nA) when DNA exponential amplification reaction (EXPAR) and catalyzed hairpin assembly (CHA) recycling were combined. The reason might be ascribed to the fact that EXPAR amplification could generate amounts of product DNA (S1) to induce continuous CHA recycle, resulting in remarkable electrochemical signal amplification.

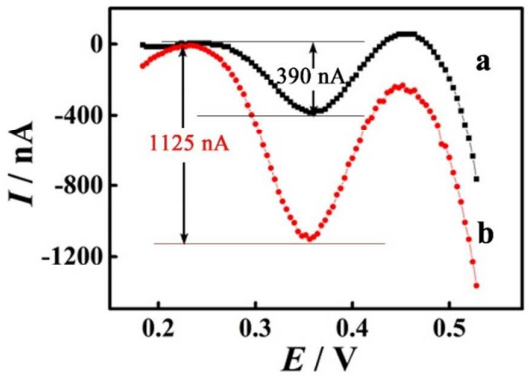


Figure 4 The electrochemical response of modified electrode using S to initiate the CHA reaction directly (curve a) and the electrochemical response of modified electrode combining the EXPAR and CHA recycling (curve b).

Analytical Performance of the Biosensor

The SWV responses of proposed biosensor for MMP-7 with various concentrations were investigated to evaluate the sensitivity of the method. As depicted in Figure 5A, the SWV response current increased gradually with the increase of MMP-7 concentration from 0.1 pg·mL^{-1} to 50 ng·mL^{-1} (curve a-h). Figure. 5B displayed relevant calibration curve of developed biosensor for quantitative analysis of MMP-7, where the response current had a desirable linear relationship on the logarithm of MMP-7 concentration ranged from 0.1 pg·mL^{-1} to 50 ng·mL^{-1}. And the obtained regression equation was I = -255.11 lg$c_{\mathrm{MMP\text{-}7}}$ - 1072.8 with a correlation coefficient (r) of 0.9979. The detection limit could be obtained down to 0.02 pg·mL^{-1}. In addition, compared with other reported methods, the proposed method exhibited a relatively lower detection limit (Table S2, see the Supporting Information). These results demonstrated that cascade signal amplification strategies of EXPAR and CHA

had improved the sensitivity of biosensor effectively.

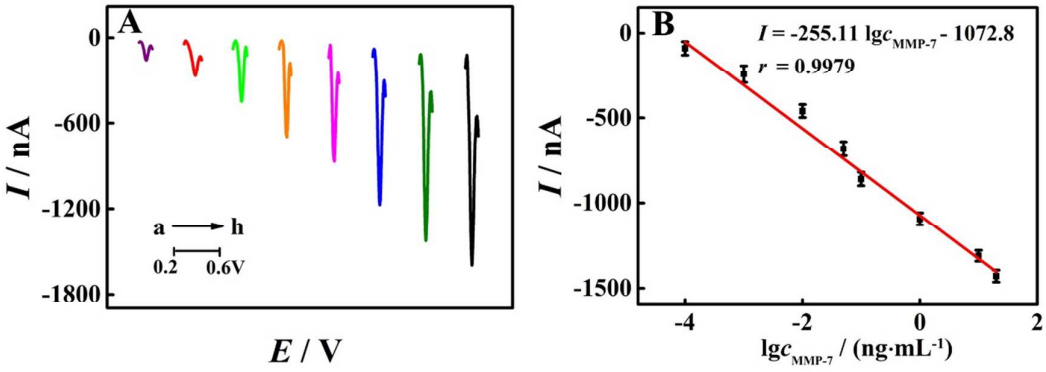


Figure 5 (A) SWV response of proposed electrochemical biosensor incubated with various MMP-7 concentrations (from a to h: 0.0001, 0.001, 0.01, 0.05, 0.1, 1, 10, 50 ng·mL^{-1}). (B) The calibration plot of SWV response current versus the logarithm of $c_{MMP\text{-}7}$.

Reproducibility, Stability and Specificity of Proposed Biosensor

Herein, the reproducibility experiments had been performed with four electrodes toward 1 ng·mL^{-1} target MMP-7 (termed as inter-assay) and the same electrode repeated for four measurements (termed as intra-assay). The relative standard deviation (RSD) of inter-assay and intra-assay were 5.6% and 4.9%, respectively, which revealed acceptable reproducibility of the method.

The stability of proposed biosensor was judged *via* storing the biosensor at 4 ℃ and measuring every 5 days. After 15 days, the electrochemical response of the biosensor retained about 91.2% of its initial response, indicating the satisfactory stability of prepared biosensor.

To further explore the specificity, biosensor was exposed to four interfering proteins, including matrix metalloproteinase-2 (MMP-2), hemoglobin (Hb), thrombin

(TB) and prostate-specific antigen (PSA). As shown in Figure 6, relatively low electrochemical signals were obtained in the detection of MMP-2 (10 ng·mL^{-1}), Hb (10 ng·mL^{-1}), TB (10 ng·mL^{-1}) and PSA (10 ng·mL^{-1}). In contrast, a high SWV signal was obtained when biosensor had been incubated with target MMP-7 (1 ng·mL^{-1}). In addition, when 1 ng·mL^{-1} MMP-7 mixed with aforementioned four interferences, the peak current was almost the same as the value obtained from MMP-7 only. This result revealed that the proposed biosensor had good specificity for MMP-7 detection.

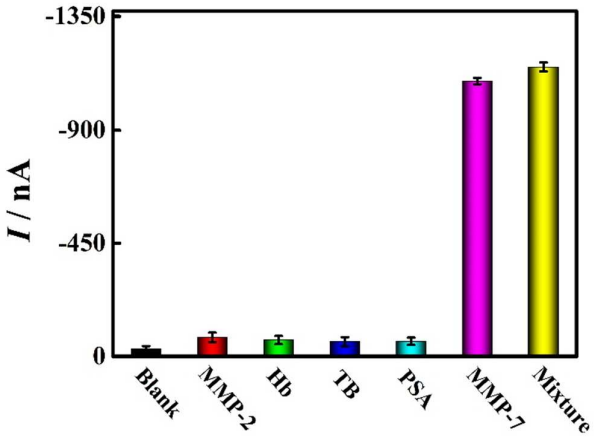


Figure 6. The specificity of proposed electrochemical biosensor toward MMP-7 (1 ng·mL^{-1}) against other nonspecific proteins: MMP-2, Hb, TB, and PSA at 10 ng·mL^{-1}.

Recovery Test

Recovery experiments had been carried out to evaluate the reliability and applicability of proposed electrochemical biosensor for MMP-7 assay. Briefly, different concentrations of MMP-7 were added into 10-fold-diluted human serum respectively, and the recoveries of MMP-7 then were monitored with developed biosensor. Experimental results were shown in Table 1, the recoveries ranged from 93.2%

to 102.4% and the RSD varied from 2.7% to 6.3%. These results demonstrated that the developed biosensor was promising in monitoring MMP-7 in human serum.

Table 1 MMP-7 assay in human serum (n=3) with proposed biosensor.

Sample number	Added/(ng·mL^{-1})	Found/(ng·mL^{-1})	Recovery/%	RSD/%
1	0.0010	0.000972	97.2	6.3
2	0.010	0.0102	102.4	4.9
3	1.0	0.932	93.2	5.2
4	10	9.65	96.5	2.7

Conclusion

In this work, using EXPAR as model, we designed an ingenious two-stage DNA template to quickly converted output DNA with amino acid residues into a new DNA trigger without any amino acid residues for inducing highly efficient DNA amplification and realizing ultrasensitive electrochemical detection of MMP-7. Compared with other protocols, the method displayed several fascinating features: First, it allowed the conversion of input target MMP-7 into output DNA in solution, which diminished complicated electrode fabrication process and improved the cleavage efficiency of target toward peptide substrate. Second, the translation of output DNA with amino acid residues into a new DNA without any amino acid residues, which reduced steric hindrance effect and thus effectively improved DNA amplification efficiency. Additionally, this strategy could be readily expanded to detect other proteins that displayed enzymatic cleavage activity, especially provided a novel avenue for constructing ultrasensitive peptide cleavage-based biosensor.

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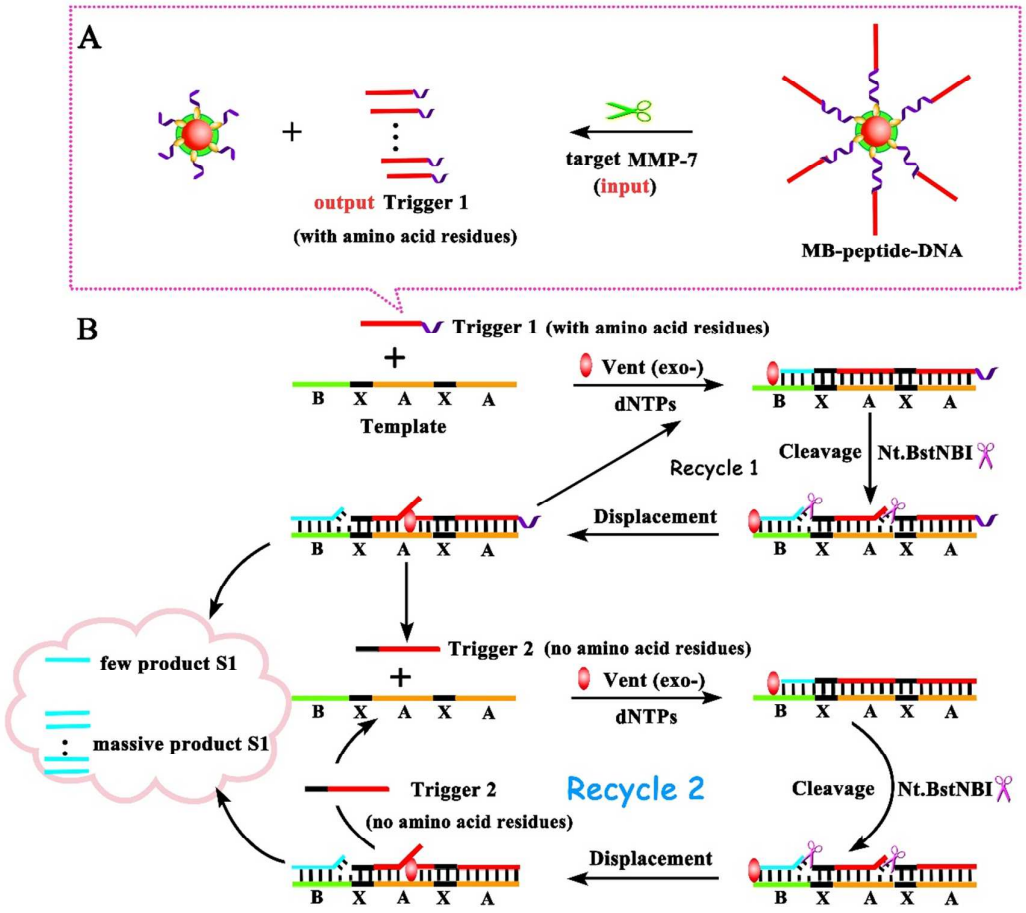
For TOC Only


A
output Trigger 1
(with amino acid residues)
target MMP-7
(input)
MB-peptide-DNA
B
Trigger 1 (with amino acid residues)
Template
Vent (exo-)
dNTPs
Cleavage
Nt.BstNBI
Recycle 1
Displacement
Trigger 2 (no amino acid residues)
few product S1
massive product S1
Recycle 2