Supplementary Information

A new electrochemical aptasensor for sensitive assay of a protein based on the dual-signaling electrochemical ratiometric method and DNA walker strategy

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1. Materials and reagents

Human α-thrombin, 6-mercaptohexanol (MCH) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Exo III was obtained from New England Biolabs Co., Ltd (Beijing, China). The normal human serum was purchased from Anyan Inc. (Shanghai, China). Tris-(hydroxymethyl) aminomethane (Tris) was purchased from Solarbio Science & technology Co., Ltd. (Beijing, China). NaCl, KCl, KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, $HAuCl_4 \cdot 4H_2O$ and H_2SO_4 were purchased from Sinopharm Chemical Reagent Co., Ltd (China). All chemicals were of analytical grade. Aqueous solutions were prepared by ultrapure water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA). The TBA were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). The other sequences of the oligonucleotides used in this work were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China).

Table S1 Sequences of oligonucleotides used in this paper[a]

Name	**Sequence (5′→3′)**
TBA	GGTTGGTGTGGTTGGAAA AAATTT TTT
DWs	SH-$(CH_2)_6$-$(T)_{40}$-*CCA ACCACAC*CAACCTTTTTT
Fc-DNA	Fc-TTGGCAATGCGGTGGTCGAGGTTTGCCA*GGTTGGT GTG*
MB-DNA	MB-TTGGCAAACCTCGACCACCGCATTGCC-$(CH_2)_6$-SH

[a]The sequences in the same color in different oligonucleotides are complementary and the underlined sequences in the same oligonucleotide are complementary. The DWs in italics and the Fc-DNA in italics are complementary.

2. Apparatus

The morphology of the electrode was characterized by scanning electron microscopy (SEM, JSM-6700, JEOL Ltd. Japan). All electrochemical measurements, including cyclic voltammetry (CV) and square wave voltammetry (SWV), were carried out on a CHI 660A Electrochemical Workstation (Chenhua Instrument Company of Shanghai, China). Electrochemical impedance spectroscopy (EIS) results were obtained on CHI 660B Electrochemical Workstation (Chenhua Instrument Company of Shanghai, China). The gel electrophoresis images were obtained on a Gel Images System (Azure C300). A conventional three-electrode system was used with a glassy carbon electrode (GCE, 3 mm) as the working electrode, a platinum wire as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode. All potentials in this paper were referred to SCE.

3. Preparation of the basal solution

First, DWs (2 μL, 1 μM) and TBA (10 μL, 1 μM) were mixed, incubated at 95 ^{o}C for 10 min and slowly cooled down to room temperature to obtain the DWs/TBA double-stranded DNA. Then, the obtained complex and 10 μL of 10 μM MB-DNA were blended in 78 μL of 34 mM Tris-HCl buffer (containing 233 mM NaCl, 8.5 mM KCl, 1.7 mM $CaCl_2$, 1.7 mM $MgCl_2$, pH = 7.4) to acquire the basal solution.

4. Fabrication of the electrochemical biosensor

Before modification, the GCE was polished carefully to a mirror-like surface with 0.5 and 0.05 μm alumina slurries, followed by ultrasonication in ultra pure water and ethanol. The electrode was further treated by electrochemical oxidation and reduction in 0.5 M H_2SO_4 aqueous solution by potential cycling in the potential range from -0.2 to 1.2 V with a scan rate of 100 mVs^{-1}. The purpose of this kind of treatment for GCE surface is to improve the hydrophilicity of the electrode surface, which is beneficial to the deposition of AuNPs on the GCE surface. Then, the GCE was washed with copious amounts of ultra pure water and dried under nitrogen gas. After that, the pretreated GCE was immersed in a 24.3 mM chloroauric acid solution and kept at -0.2 V for 60 s to electrodeposit AuNPs.[1] After washed with copious amounts of ultra pure water, the AuNPs/GCE was incubated with 10 μL basal solution for 16 h at room temperature. Then, the electrode was incubated with 10 μL of 10 mM Tris-HCl buffer (pH = 7.4) with 1 mM MCH for 1 h to block the uncovered AuNPs/GCE surface. Finally, the above electrode was incubated with 2 μM Fc-DNA solution at 37 ^oC for 2 h. The obtained electrode was labeled as the Fc-DNA/MCH/MB-DNA/DWs/TBA/AuNPs/GCE.

5. Electrochemical measurements

The Fc-DNA/MCH/MB-DNA/DWs/TBA/AuNPs/GCE was incubated with 0.1 M PBS buffer (pH = 7.0, containing 0.1M KCl) containing different concentrations of TB at 37 ^oC for 60 min. After rinsed with Tris-HCl buffer (pH = 7.0), the electrode was incubated with 1 unit/μL EXO III (supplied with 1×NEBuffer 1, pH = 7.0) at

37 ^{o}C for 80 min. It was further immersed in a solution with high ionic strength (10 mM PBS containing 140 mM NaCl and 5 mM $MgCl_2$, pH = 7.4) for 30 min at room temperature, inducing the formation of stem-loop structure of MB-DNA on the electrode surface. Finally, the electrochemical performance of the above electrode was investigated by SWV in 0.1 M PBS (pH = 7.0, containing 0.1 M KCl) in the potential range from -0.5 to 0.5 V with amplitude 0.025 V, a frequency of 25 Hz, and a step potential of 4 mV.

6. Characterization of GCE and Au NPs/GCE

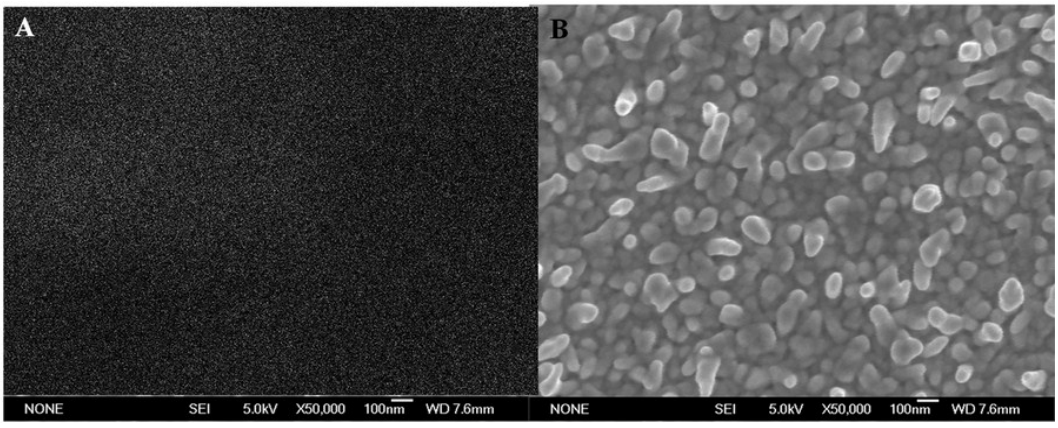

Fig. S1. SEM image of GCE (A) and AuNPs-modified GCE (B) .

7. Polyacrylamide gel electrophoresis

The cleavage results of Exo III were analyzed by (15%, w/w) polyacrylamide gel electrophoresis (PAGE) at 84 V for 2.5 h (Fig. S2). It is noted that the DWs/TBA double-stranded DNA can be unzipped due to the specific interaction between TBA and TB (lane 2 vs. lane 4). From the results shown in lane 5 and lane 6, it is clear that

MB-DNA/Fc-DNA can not be cleaved by Exo Ⅲ. However, for the MB-DNA/Fc-DNA + DWs/TBA + TB + Exo III sample (lane 1), the band corresponding to the MB-DNA/Fc-DNA disappears (lane 1 vs. lane 5), meanwhile, a new band with higher electrophoretic mobility appears in lane 1, corresponding to that of MB-DNA. This indicates that the hybrid products of DWs and MB-DNA/Fc-DNA can be cleaved by Exo Ⅲ. Furthermore, for the MB-DNA/Fc-DNA + DWs/TBA + Exo III sample, MB-DNA/Fc-DNA remains clear (lane 3). It confirms the cleavage process described in Scheme 1. Note that all of nucleic acids move faster after mixing with Exo Ⅲ (lane 3 vs. lane 4; lane 5 vs. lane 6), as reported in the previous published papers.[2, 3, 4, 5, 6]

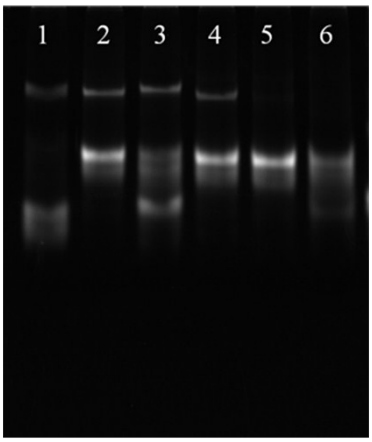

Fig. S2. The selective cleavage results of Exo III. MB-DNA/Fc-DNA+ DWs/TBA + TB + Exo III (Lane 1), MB-DNA/Fc-DNA+ DWs/TBA (Lane 2), MB-DNA/Fc-DNA + DWs/TBA + Exo III (Lane 3), MB-DNA/Fc-DNA+ DWs/TBA + TB (Lane 4), MB-DNA/Fc-DNA (Lane 5), MB-DNA/Fc-DNA + Exo III (Lane 6).

8. Feasibility of the developed biosensor

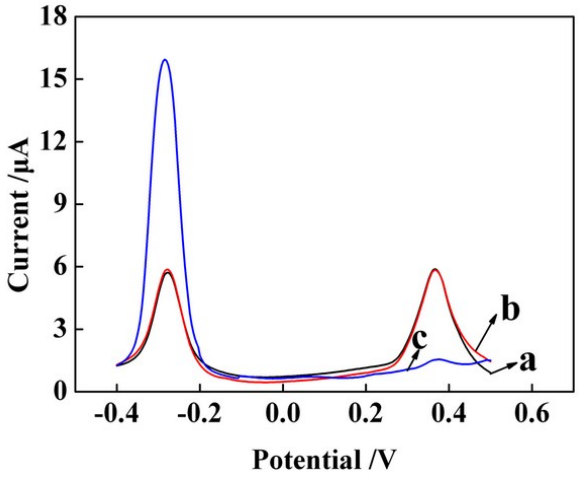

Fig. S3. SWV responses in the absence of Exo III and TB (a), in the absence of TB (b), in the presence of both Exo III and TB (c).

9. Optimization of experimental conditions

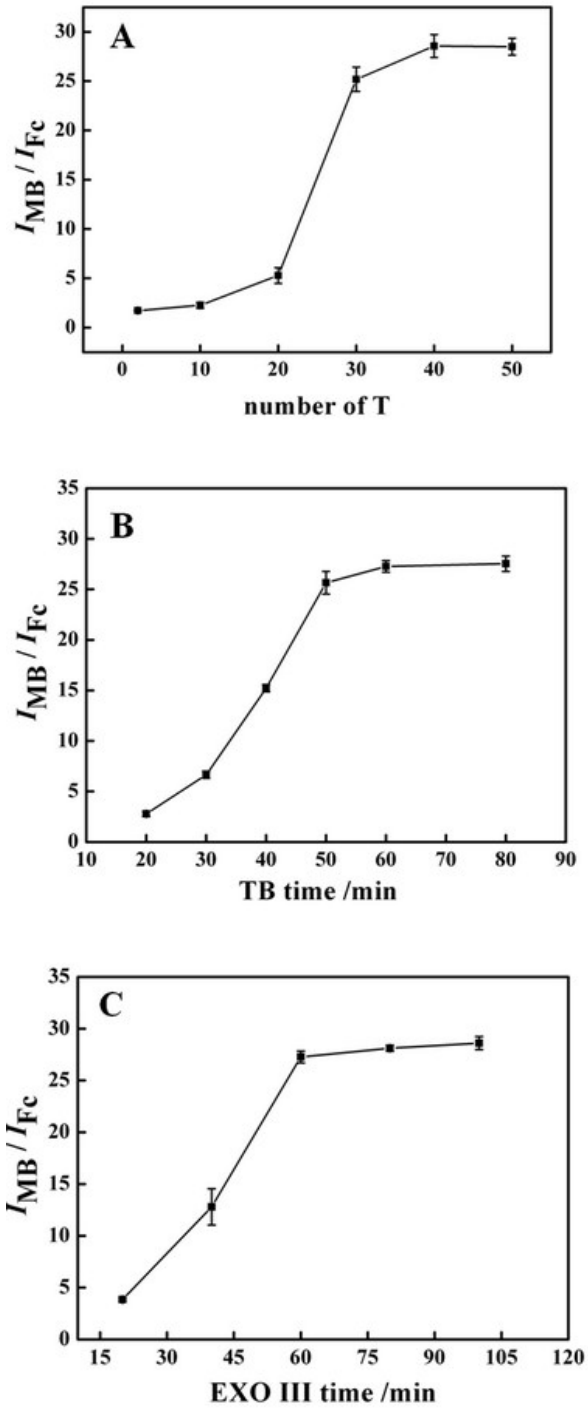

Fig. S4. The effects of experimental conditions on the I_{MB}/I_{Fc} values of the developed electrochemical aptamer-based biosensor. (A) number of T bases in the DWs; (B) reaction times of TB; (C) Exo III cleavage time.

10. Comparison of different electrochemical methods for TB assay

Table S2. Comparison of different electrochemical methods for TB assay.

Method	Linear range	Detection limit	Ref.
SWV	1-600 nM	170 pM	7
DPV	0.1-10 nM	70 pM	8
DPV	0.1-20 nM	20 pM	9
ACV	0.01-100 nM	2.5 pM	10
SWV	0.005-1 nM	1.7 pM	11
DPV	0.001-30 nM	0.32 pM	12
SWV	0.1-10 pM	0.056 pM	This work

11. Recovery test

Table S3. Recovery assay of TB in normal human serum samples.

Sample number	**Added (pM)**	**Found (pM)**	**Recovery (%)**
1	**0.1**	**0.09**	**90**
2	**1**	**0.96**	**96**
3	**2**	**2.1**	**105**

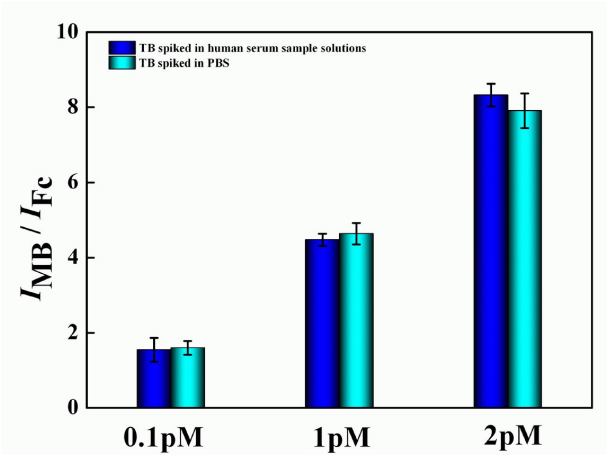

Fig. S5. Comparison assay of TB in 10 mM PBS and normal human serum samples.

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