Supporting Information

Hemin/G-quadruplex simultaneously acts as NADH oxidase and HRP-mimicking DNAzyme for simple, sensitive pseudobienzyme electrochemical detection of thrombin

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Table 1
Analytical properties of hemin/G-quadruplex assembled aptasensors for thrombin detection.

Ref.	Reproducibility	Detection limit	Detection time	Linear range	Analytical method
1	5.2%	0.39 pM	40 min	0.001~30 nM	DPV
2	5.1%	2 pM	40 min	0.01~50 nM	DPV
3	-----	20.5 nM	40 min	50~5000 nM	UV-vis
4	3.4%	1 pM	40 min	0.01~0.12 nM	fluorescence
5	2.43%	0.5 nM	90 min	0.5~20 nM	UV-vis
Our work	5.1%	0.15 pM	40 min	0.0005~20 nM	DPV

Abbreviation: differential pulse voltammetry (DPV); UV-vis absorbance measurements (UV-vis).

EXPERIMENTAL SECTION

Reagents and Materials

Tris-hydroxymethylaminomethane hydrochloride (tris) was obtained from Roche (Switzerland). Thrombin (enzymatic activity of 40-300 NIH units/mg, lyophilized power), lysozyme, IgG, L-cysteine, thionine (TH), hemin, gold chloride ($HAuCl_4$) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). $NaBH_4$ was obtained from Kelong Chemical Company (Chengdu, China).

Thiol-terminated thrombin binding aptamer (TBA): 5-SH-$(CH_2)_6$-GGT TGG TGT GGT TGG-3; Amido-terminated thrombin binding aptamer (TBA'): 5-NH_2-$(CH_2)_6$-GGT TGG TGT GGT TGG-3 were obtained from TaKaRa (Dalian. China). All other chemicals were of reagent grade and used as received.

0.10 M sodium phosphate buffer (PBS) containing 10 mM KCl and 2 mM $MgCl_2$ (pH 7.00) was used as DPV working buffer solution. 20 mM Tris-HCl buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1mM $MgCl_2$ was used as a binding buffer. 0.10 M PBS containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (pH 7.0) was employed for EIS investigation. Double distilled water was used throughout this study.

Apparatus

All electrochemical measurements, including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and differential pulse voltammograms (DPV) were performed with a CHI 660D electrochemical workstation (Shanghai Chenhua Instrument, China). The pH measurements were finished with a pH meter (MP 230, Mettler-Toledo, Switzerland). A three-electrode system contained a modified glassy carbon electrode (GCE, ϕ = 4 mm) as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as a reference electrode.

Preparation of Au-TH-Au nanocomposites

AuNPs was firstly prepared according to the literature[6] and the size was 13 nm. Then, 300 μL of 3 mM thionine (TH) was added into 2 mL prepared AuNPs solution and stirring for 12 h. After centrifugation at 12 000 rpm for 30 min to remove unbounded TH, the sediment of the Au-TH nanocomposites were resuspended in 2 mL PBS solution (pH 7.0). Subsequently, 200 μL of $HAuCl_4$ (1%) was put into the solution under stirring condition. Then, fresh $NaBH_4$ solution was slowly added

and an extra reaction time of 10 h was needed. Lastly, the obtained solution was subject to centrifugation at 12 000 rpm for 30 min and the sediment (Au-TH-Au nanocomposite) was again resuspended in 2 mL PBS solution.

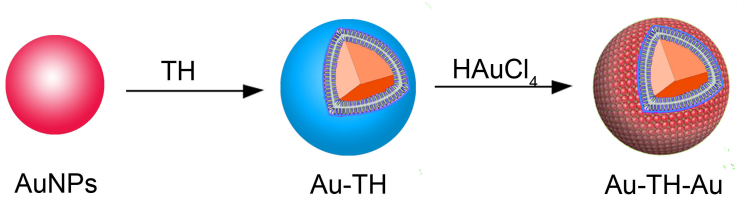

Fig S1 The schematic diagram of the procedure used to prepare Preparation of Au-TH-Au nanocomposite.

Preparation of secondary aptamer

60 μL thiol-terminated TBA (100 nM) was added into prepared Au-TH-Au solution and stirred for 16 h. Then, centrifugation at 12 000 rpm for 30 min to remove unbounded TBA and the sediment was resuspended in 2 mL Tris-HCl buffer (pH 7.4). Subsequently, 0.5 mg hemin was put into the above solution with an extra reaction time of 2 h. 200 μL bovine serum albumin (BSA, w/w, 1%) was implemented to block remaining active sites on Au surface with an reaction time of 40 min. The finally solution (secondary aptamer) was then subjected to centrifugalization at 12 000 rpm for 30 min and the secondary aptamer was then resuspended in 2 mL Tris-HCl buffer for further use.

In order to testy that the prepared Au-TH-Au nanocomposites could improve the hemin and TH immobilization amount and achieve amplified detection of thrombin, a contrastive secondary aptamer was also prepared: analogously, 300 μL of 3 mM thionine (TH) was added into 2 mL double distilled water. Owing to the $-NH_2$ groups for TH, cross-linking agent glutaraldehyde was used to link the TH and amido-terminated thrombin binding aptamer (TBA', 60 μL, 100 nM) with the incubation time of 20 min. 0.5 mg hemin was put into the above solution with an extra reaction time of 2 h. The finally solution (contrastive secondary aptamer) was then also subjected to centrifugalization at 12 000 rpm for 30 min and resuspended in 2 mL Tris-HCl buffer for further use.

Fabrication of pseudobienzyme electrochemical aptasensor

The assay protocol of the electrochemical aptasensor was fabricated as follows: Firstly, cleaned glassy carbon electrode (GCE) was electrodeposited a gold nanoparticles (dep-Au) layer under the potential of -0.2 V for 30 s. Then, 20 μL TBA (2.0 μM) solution was dropped onto the surface of the dep-Au/GCE surface and incubated for 16 h at room temperature. After that, the modified electrode was immersed in BSA solution (0.1%) for 40 min incubation to block nonspecific binding sites. The modified electrode was then dropped with 20 μL of a fixed concentration of thrombin for 40 min. Lastly, in order to catch the secondary aptamer on electrode surface through "sandwich" reactions, 20 μL of prepared secondary aptamer was put on the thrombin/BSA/TBA/dep-Au/GCE surface with a incubation time of 40 min.

Experimental measurements

The fabricated aptasensor was dropped with 20 μL of a fixed concentration of thrombin for 40 min. The aptasensor was placed into an electrolytic cell containing 1 mL 0.1 M PBS (pH 7.0) and 4.8 mM NADH. The DPV measurement was taken: the potential was range from -0.5 to 0.1 V, modulation amplitude was 0.05 V, pulse width was 0.06 s, and sample width was 0.02 s. The CV was taking from -0.2~0.6 V (vs. SCE) at 50 mV/s in 0.10 M PBS containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (pH 7.0). EIS measurements were carried out also in the presence of 0.10 M PBS containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (pH 7.0). The bias potential was 0.17 V, alternative voltage was 5 mV and frequency range was 50 MHz-10 KHz.

RESULTS AND DISCUSSION

Hemin/G-quadruplex Electrocatalyst Kinetics

The apparent Michaelis–Menten constant K_m stands for the biological activity of immobilized enzyme. In our work, K_m was calculated according to the Michaelis-Menten equation[7]:

$$1/I_{ss} = 1/I_{max} + K_m/I_{max}\, C.$$

Where, I_{ss} is the steady-state current after the addition of substrate, C is the NADH concentration, and I_{max} is the maximum current measured under saturated substrate conditions. K_m can be obtained by the analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current versus NADH concentration. From data we can obtained the equation:

$$1/I_{ss} = -6E\text{-}05/C - 0.0855.$$

Therefore, the K_m value in our work was determined to be 0.70 mM and the I_{max} value is -11.696. Owing to the hemin can intercalate into thiol-terminated thrombin binding aptamer (TBA) to from hemin/G-quadruplex, the concentration of hemin/G-quadruplex was estimated by assuming that the TBA used for secondary aptamer fabrication is wholly bind to Au-TH-Au nanocomposites. The concentration of hemin/G-quadruplex is equal to that of TBA. According to the equation:

$$I_{max} = nFAk_{cat}\Gamma$$

Where n is the number of electrons in the electrochemical reaction, Γ is the surface concentration of hemin/G-quadruplex in the film (mol cm^{-2}), A is the electrode area (cm^2), F is Faraday's constant, turnover number (k_{cat}) is the catalytic rate constant (s^{-1}). We can also obtain the k_{cat} value of 30.4 s^{-1}.

The amplification of Au-TH-Au nanoparticles

Gold nanoparticles have the advantages of large specific surface area and superior conductivity, which could effectively improve the immobilization amount of thionine and hemin/G-quadruplex components, achieving amplified detection of thrombin. In order to testify that conclusion, a

comparison that employing modified electrode "sandwich" with TH-hemin/G-quadruplex (contrastive secondary aptamer) and proposed secondary aptamer was made, respectively. As can be seen from Fig S2, the electrode that "sandwich" with TH-hemin/G-quadruplex (A) showed a much lower reduction peak currant with inefficient bioelectrocatalysis in contrast to that of proposed secondary aptamer (B), suggesting a desirable amplification of Au-TH-Au nanoparticles.

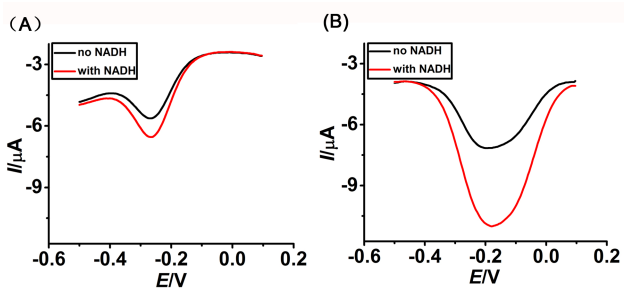

Fig S2. The modified electrode "sandwich" with TH-hemin/G-quadruplex (A) and proposed secondary aptamer (B) investigated in PBS (pH 7.0) and PBS containing 4.8 mM NADH, respectively.

Optimization of detection condition

In order to obtain a higher sensitivity of the electrochemical aptasensor, the experimental conditions, such as primary TBA concentration, incubation and NADH concentration, were optimized. As shown in Fig S3A and Fig S3B, the peak current decreased with the increasing of the primary TBA concentration, and reached plateau regions at the at the concentration of 2μM. Thus, the optimal primary TBA concentratione were chosen at 2μM.

The incubation time is an important parameter affecting the analytical performance of aptasensor. At room temperature, the CV responses for thrombin (10 nM) decreased with the increasing incubation time and then tended to constant values after 40 min (Fig S3C), which showed the saturated binding both between the thrombin and the primary TBA and between the complexed thrombin and secondary aptamer. Therefore, an incubation time of 40 min was selected for the sandwich-type assay.

The concentration of NADH in electrolytic cell played a key role for the bioelectrocatalytic effect. Therefore, before testing the target thrombin, we first studied the effect of NADH concentration. The

aptasensor after incubation of 10 nM thrombin was investigated PBS with different NADH concentration. The change of NADH concentration was achieved by adding different volumes of high-concentration NADH (320 mM) in 2 mL PBS. As shown in Fig S3D, the peak current increased with the increase of NADH and then leveled off after adding 30 μL of 320 mM NADH (equivalent to the concentration of NADH in PBS reached to 4.8 mM), suggesting a saturated state of NADH. Thus, 4.8 mM NADH in PBS was employed throughout the detection process.

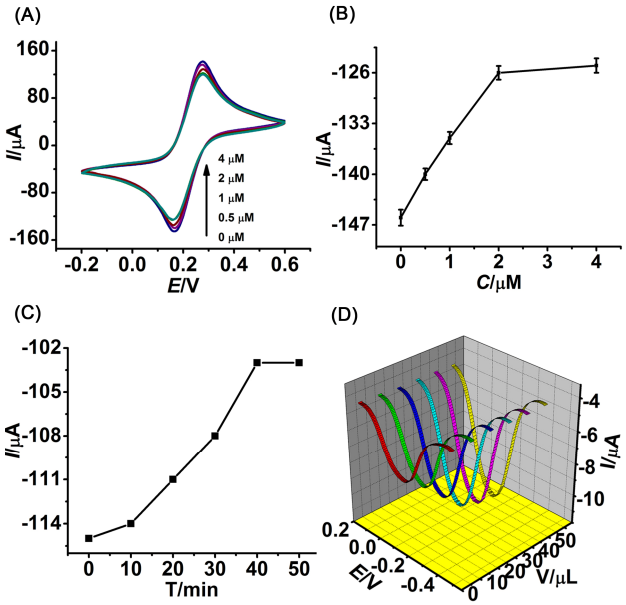

Fig S3. Effect of primary TBA concentration (A and B), incubation time (C) and NADH concentration (D).

Comparisons of proposed aptasensor with other detection methodologies as direct thrombin detection

With hemin/G-quadruplex simultaneously served as NADH oxidase and HRP-mimicking DNAzyme, a pseudobienzyme amplifying strategy was obtained in our work, which makes the proposed strategy among the most sensitive approach for aptamer-based thrombin monitoring. In order to testify that our proposed strategy showed a desirable sensitivity, comparisons of proposed aptasensor with other detection methodologies as direct thrombin detection were listed as follows:

Table 2

Comparisons of proposed aptasensor with other detection methodologies as direct thrombin detection.

Analytical method	Detection limit	Linear range	Ref.
CV	40 pM	0.12~ 46 nM	8
fluorescence	1 pM	0.01~0.12 nM	9
DPV	0.2 nM	0.8~15 nM	10
ECL	0.35 pM	0.0005~0.8 nM	11
EIS	----	0.5~500 nM	12
SV	22.6 nM	1~500 nM	13
QCM	----	50~200 nM.	14
SPR	----	0.1~150 nM	15
DPV	0.15 pM	0.0005~20 nM	Our work

Abbreviation: cyclic voltammetry (CV); differential pulse voltammetry (DPV); electrochemiluminescent (ECL); UV-vis absorbance measurements (UV-vis); electrochemical impedance spectroscopy (EIS); stripping voltammetry (SV); quartz crystal microbalance (QCM); surface plasmon resonance (SPR).

From the Table 2 we can see that our proposed aptasensor exhibits a much higher sensitivity, which provides a vigorous evidence of our strategy for highly sensitive detection of thrombin.

References

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