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# Mediator-free triple-enzyme cascade electrocatalytic aptasensor with exonuclease-assisted target recycling and hybridization chain reaction amplification

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ABSTRACT

The amplified sensitive detection of protein is essential to biomedical research as well as clinical diagnosis. Here, we developed an ultrasensitive mediator-free triple-enzyme cascade electrocatalytic aptasensor for thrombin detection on the basis of exonuclease-assisted target recycling and hybridization chain reaction (HCR) amplification strategy. The double strands constructed by the hybridization of thrombin binding aptamer ($S_1$) with its complementary strand ($S_2$) were firstly assembled on the electrode. Upon addition of target to the system, the $S_1$ recognized thrombin and left off electrode to make space for assembly of hybrid-primer probe ($H_0$). Then, the $H_0$ triggered the HCR to form the multi-functional hemin/G-quadruplex DNAzyme nanowires. In the mediator-free triple-enzyme cascade electrocatalytic amplification system, the hemin/G-quadruplex DNAzyme nanowires here simultaneously played three roles: the redox probe, NADH oxidase and HRP-mimicking DNAzyme, respectively, which effectively avoided the fussy redox probe and enzyme labeling process, serving a useful alternative or supplement to conventional assays that typically suffer from complexity and poor sensitivity. Additionally, in order to improve the assembly amount of hemin/G-quadruplex DNAzyme nanowire, the exonuclease-assisted target recycling amplification was used for the continuous removal of $S_1$. As a result, the proposed method can detect thrombin specifically with a detection limit as low as 20 fM.



## 1. Introduction

The amplified sensitive detection of protein is being greatly motivated by its potential applications in biological studies, clinical diagnostics, and treatment (Xue et al., 2012a; Xue et al., 2012b; Akter et al., 2012). The Recently appeared hybridization chain reaction (HCR) amplifying technique, which is a promising autonomous isothermal replication process with a DNA fragment, serves as an initiator and triggers a cascade of hybridization events to simply in situ yield long DNA nanowires (Dirks and Pierce, 2004; Jiang et al., 2012; Li et al., 2012). In this context, an enormous amount of signal tags such as electrochemical redox probes (Zhou et al., 2012; Chen et al., 2012a; Zhang et al., 2012), quantum dots (Song et al., 2012; Liu et al., 2013; Zhong et al., 2011), fluorophor (Yang et al., 2012) and electrochemiluminescent indicators (Chen et al., 2012b) thus can be assembled with an amplified signal output. Among the various HCR-based biosensors, electrochemical biosensors, being simple, portable and low-cost, are particularly attractive for protein detection (Zhou and Dong, 2011). In order to further improve sensitivity, HCR-based electrochemical biosensor is commonly coupled with the enzyme amplification. This is because the enzyme not only entails the enzymatic reaction taking place in the close vicinity of the biosensor but also allows its amplification according to the catalytic activity of the enzyme, providing the possibility to achieve high sensitivity (Velde et al., 2000; Yuan et al., 2013). Until now, some enzymes such as horseradish peroxidase (HRP) (Wang et al., 2012), alkaline phosphatase (ALP) (Zhao et al., 2012) have been successfully used for developing sensitive HCR-induced electrochemical biosensors. Nevertheless, these biosensors were involved in the extra enzyme modification or conjugation steps, which were rather costly, time-consuming, and sophisticated. Therefore, adopting new designs to solve the problems is beneficial for the signal amplification.

The emerging of catalytic nucleic acids (DNAzymes) may resolve this issue. The flexibility of DNAzymes to encode in the base sequences of DNA, together with the reduced nonspecific binding properties of nucleic acids, provides a simple, stable feature against hydrolysis and heat treatment alternative to protein enzyme for assay applications (Chen et al., 2009; Shimron et al., 2012). The typical DNAzyme is the

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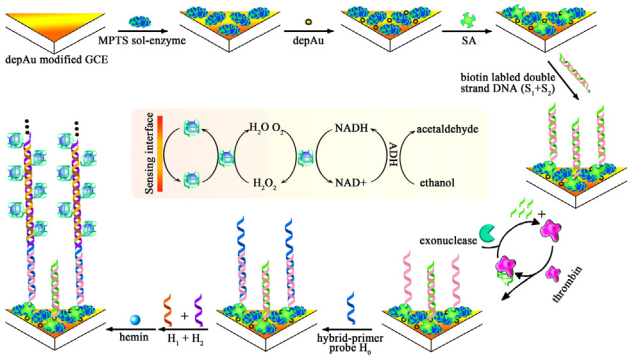
hemin/G-quadruplex, which is formed by the interlacing of hemin into a single-stranded guanine-rich nucleic acid. If properly designed, the hemin/G-quadruplex DNAzyme can in situ be propagated during the formation of HCR-induced long DNA nanowires. This process avoided the fussy enzyme labeling process and successfully obtained the protein enzyme-like activity. Based on the superior properties, Zhou et al.,(2013) and Tang et al., (2012) utilizing the formed hemin/G-quadruplex DNAzyme nanowires as HRP-mimicking DNAzyme proposed electrochemical immunosensor for protein detection, where redox probes were assembled on electrode *via* electrostatic adsorption and covalent bonding, respectively. Although these biosensors showed relatively high sensitivity, they still need the fussy redox probe labeling process. More importantly, they are all the monoenzyme-catalyzed amplification system. It was found that the signal amplification efficiency of multienzyme-catalyzed amplification system is obviously superior to that of monoenzyme-catalyzed amplification system. To the best of our knowledge, however, there are few reports focusing on the bienzyme-catalyzed electrochemical biosensor with HCR amplification, not mention the triple-enzyme cascade electrocatalytic amplification (Yuan et al., 2013).

Willner (Golub et al., 2011) have proved that the hemin/G-quadruplex can mimic the activity of horseradish peroxidase as well as NADH oxidase. Inspired by this property, we fabricated an ultrasensitive mediator-free triple-enzyme cascade electrocatalytic electrochemical aptasensor based on the HCR-induced multi-functional hemin/G-quadruplex DNAzyme nanowires simultaneously acting as an NADH oxidase and HRP-mimicking DNAzyme. Thrombin, a kind of serine protease that plays an important role in the thrombosis and hemostasis (Yin et al., 2009; Xue et al., 2012; Yuan et al., 2011), is used as the model target. The double strands composed by thrombin binding aptamer ($S_1$) and its complementary strand ($S_2$) were firstly assembled to the electrode. After the target recognition, $S_1$ bonded with thrombin and left off the electrode to make space for assembly of hybrid-primer probe ($H_0$), which thus successfully triggered the HCR to form the multi-functional hemin/G-quadruplex DNAzyme nanowires. In the electrolyte of 0.1 M PBS (pH 7.0) containing $NAD^+$ and alcohol, the ADH embedded in 3-D silicate network catalyzed the transformation of $NAD^+$ into NADH. At this point, the hemin/G-quadruplex firstly acts as an NADH oxidase, which catalyzed the oxidation of NADH by $O_2$ to reform $NAD^+$ with the concomitant local formation of high concentration of $H_2O_2$. And here came the hemin/G-quadruplex acting as an HRP-mimicking DNAzyme that quickly bioelectrocatalyzed the reduction of produced $H_2O_2$ with the aim of dramatically improving the self oxidation–reduction reaction of hemin/G-quadruplex. Additionally, in order to further improve the sensitivity, we also adopted the exonuclease-assisted target recycling amplification. For this amplification, the thrombin binding aptamer $S_1$ could be protected from exonuclease-catalyzed digestion upon hybriding with its complementary strand, while digested when binding to the target thrombin. Therefore, the thrombin could be easily released from the thrombin–thrombin binding aptamer complex, which resulted in continuous removal of $S_1$ from the electrode surface, making more space for assembly of $H_0$ to form multi-functional hemin/G-quadruplex DNAzyme nanowires (Scheme 1). With the exonuclease-assisted target recycling and HCR amplification, the proposed mediator-free triple-enzyme cascade electrocatalytic electrochemical aptasensor exhibited good sensitivity and selectivity.




**Scheme 1.** Schematic illustration of the multi-functional hemin/G-quadruplex DNAzyme nanowires-based mediator-free triple-enzyme cascade electrocatalytic electrochemical aptasensor with exonuclease-assisted target recycling and hybridization chain reaction amplification.

## 2. Experimental section

### 2.1. Materials

β-nicotinamide adenine dinucleotide hydrate ($NAD^+$), (3-mercaptopropyl)trimethoxysilane (MPTS), chloroauric acid ($HAuCl_4$), alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae*, thrombin, hemin, bovine serum albumin (BSA) and hemoglobin (HB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Streptavidin (SA) was obtained from Biocell Biotechnology (Zhengzhou, China). $RecJ_f$ exonuclease was obtained from New England Biolabs (Beijing) Ltd. (Beijing, China). All the oligonucleotides used in the present study were synthesized and purified by HPLC by Shanghai Shenggong Biotechnology Co. (Shanghai, China) and used without further purification (see Table S1). Aptamer stock solutions obtained by dissolving oligonucleotides in 20 mM Tris–HCl buffer (pH 7.4) contaning 140 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$. 0.10 M sodium phosphate buffer (PBS, pH 7.0) containing 0.32 mM $NAD^+$ and 0.85 M alcohol were employed to investigate the performance of electrodes. All other chemicals used in this investigation were of analar grade unless mentioned otherwise. Double distilled water was used throughout this study.

For the assembly of three-dimensional (3-D) silicate network with numerous ADH on electrode, we prepared the MPTS sol–enzyme according to the literature with slight modification (Jena and Raj, 2006). The MPTS sol was firstly obtained by putting 24 μL MPTS and 10 μL HCl solution (0.1 M) into 2 mL of water. Then, the mixture was stirred vigorously for 30 min. Subsequently, 8 mg ADH was added into 2 mL of prepared MPTS sol and the mixture was stirred for 2–3 min for the encapsulation of enzymes into the network. The resulting sol–gel biocomposites were stored at 4 °C for further use.

### 2.2. Instrumentation

The scanning electron micrographs were performed with a scanning electron microscope (SEM, S-4800, Hitachi Instrument, Japan). X-ray photoelectron spectroscopy (XPS) measurements were performed with a VG Scientific ESCALAB 250 spectrometer, using Al Ka X-ray (1486.6 eV) as the light source (Thermofisher, England). The pH measurements were performed with a pH meter (MP 230, Mettler-Toledo, Switzerland). Electrochemical impedance spectroscopy (EIS) and differential pulse voltammograms (DPV) were performed on a CHI 660D electrochemical workstation (Shanghai Chenhua Instrument, China) using a conventional three-electrode system with a modified glassy carbon electrode as working electrode (GCE, $\Phi$=4 mm), a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference electrode.

### 2.3. Fabrication of the electrochemical aptasensor

Prior to immobilization, the bare GCE was polished carefully with 0.3 and 0.05 μm alumina slurries and washed ultrasonically