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An ultrasensitive electrochemical aptasensor with autonomous assembly of hemin–G-quadruplex DNAzyme nanowires for pseudo triple-enzyme cascade electrocatalytic amplification†

Yali Yuan, Yaqin Chai,* Ruo Yuan,* Ying Zhuo and Xianxue Gan

We for the first time fabricate a pseudo triple-enzyme cascade electrocatalytic electrochemical aptasensor by using the alcohol dehydrogenase (ADH) as well as the autonomously assembled hemin–G-quadruplex that simultaneously acted as an NADH oxidase and HRP-mimicking DNAzyme.

Enzyme-amplified bioaffinity electrodes, especially electrochemical aptasensors with superior properties such as stability, reusability and availability for almost any given protein,[1] have attracted substantial research efforts, and have emerged as viable alternatives to detect and quantify trace amounts of biomarkers in biological studies, clinical diagnostics, and treatment.[2] Among the enzyme-amplified electrochemical aptasensors, the most frequently used one is the monoenzyme-catalyzed amplification system.[3] The enzyme not only entails the enzymatic reaction taking place in the close vicinity of the aptasensor but also allows its amplification according to the catalytic activity of the enzyme, leading to the possibility of developing highly sensitive biosensors.[4] However, as many literature studies demonstrated, the signal amplification efficiency of a bienzyme-catalyzed amplification system is obviously superior to that of a monoenzyme-catalyzed amplification system. This is because *in situ* biocatalytic formation of substrates by one enzyme could generate high local concentrations on the surface of another catalytic enzyme leading to kinetic enhancements.[5] Therefore, numerous electrochemical aptasensors with bienzyme-catalyzed amplification have been reported.[6] Well, how about the triple-enzyme-catalyzed amplification system? To date, we have not seen use of this amplification strategy in electrochemical sensors. The main reason for this can be attributed to the fact that the coimmobilization of three enzymes on the electrode surface would involve in the spatial distribution of each enzyme. And each enzyme on the electrode surface is limited, which influences the sensitivity of electrochemical sensors to a certain extent.

Hemin–G-quadruplex is a class of catalytic nucleic acid (DNAzyme) formed by guanine-rich nucleic acid sequences and intercalated hemin.[7] It possesses several unique features, such as it is relatively easy to label, less expensive to produce, and more stable against hydrolysis and heat treatment.[8] To date, the hemin–G-quadruplex structures have commonly been shown to exhibit horseradish peroxidase-type catalytic functions and utilized extensively in DNAzyme sensors for signal amplification.[9] Very recently, Willner[10] *et al.* proved that the hemin–G-quadruplex could act as a horseradish peroxidase-mimick as well as an NADH oxidase. Inspired by this property, we for the first time constructed a pseudo triple-enzyme cascade electrocatalytic electrochemical aptasensor by using the alcohol dehydrogenase (ADH) as well as the hemin–G-quadruplex that simultaneously acted as an NADH oxidase and HRP-mimicking DNAzyme. This strategy is interesting to resolve the fussy labeling process, deactivation and spatial distribution of each enzyme, and thus successfully realize the triple-enzyme-catalyzed amplification. More importantly, this fascinating strategy can diminish the concentration reduction of the produced substrate caused by diffusion from one enzyme to another enzyme, providing a possibility of *in situ* production of high local concentrations on the surface of another catalytic enzyme leading to kinetic enhancements.

In this contribution, in order to improve the immobilization amount of hemin–G-quadruplex catalytic structure, the hybridization chain reaction (HCR), a real protein-free isothermal amplification technique that relies on the autonomous self-assembly of two single DNA strands,[11] was employed for the formation of long hemin–G-quadruplex DNAzyme nanowires. The fabrication process and assay principle of the pseudo triple-enzyme electrochemical aptasensor are depicted in Scheme 1 (see ESI† for experimental details). Thrombin, a highly specific serine protease that plays a central role in cardiovascular diseases and anti-clotting therapeutics, served as a target model. A thrombin binding aptamer conjugated with the initiator strand that triggered HCR (TBA-S_0) is used as a secondary aptamer. Two auxiliary DNA single-strands S_1 and S_2 are designed for

Key Laboratory on Luminescence and Real-Time Analysis, Ministry of Education, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, PR China. E-mail: yuanruo@swu.edu.cn; Fax: +86-23-68252277; Tel: +86-23-68252277

† Electronic supplementary information (ESI) available: Experimental section, the electrochemical characterization of the stepwise modified electrodes, comparisons of the proposed aptasensor with other reported aptasensors for thrombin detection and the determination of thrombin added in the human blood serum. See DOI: 10.1039/c3cc42874e

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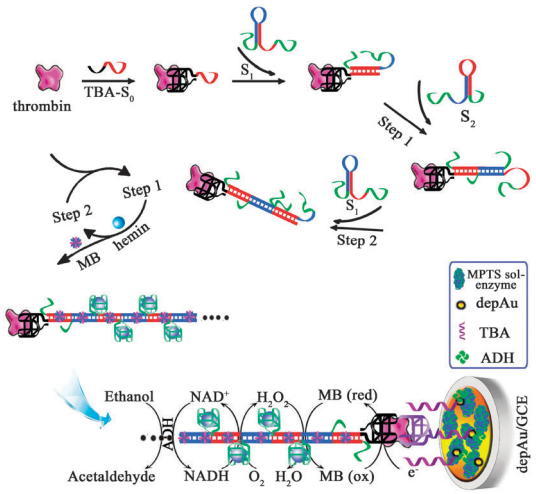

Scheme 1 Illustration of the electrochemical aptasensor with autonomous assembly of hemin–G-quadruplex DNAzyme nanowires for pseudo triple-enzyme cascade electrocatalytic amplification.

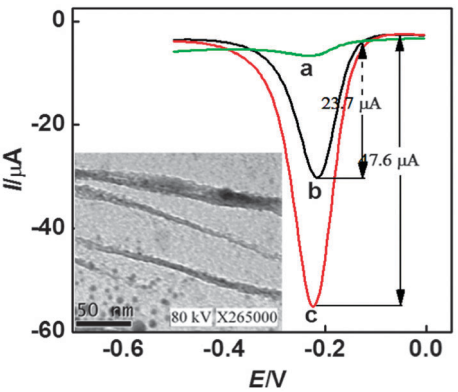

Fig. 1 DPV responses of the aptasensor with the incubation of thrombin and TBA-S_0 (a) and further treated with the autonomous assembly of the hemin–G-quadruplex DNAzyme nanowires (b) obtained in the electrolyte of PBS. (c) The aptasensor with the incubation of thrombin and autonomous assembly of the hemin–G-quadruplex DNAzyme nanowires obtained in the electrolyte of PBS containing 0.32 mM NAD^+ and 1.0 M alcohol. The inset shows the TEM image of hemin–G-quadruplex DNAzyme nanowires.

the DNAzyme nanowire reaction. In the sandwich-type reaction, the DNA initiator carried on the TBA-S_0 paired with the auxiliary single-strand S_1, following which a cascade of hybridization events occurs between the two auxiliary single-strands and finally leads to an autonomous assembly of micrometer-long nanowires with substantial hemin–G-quadruplex catalytic structure. Herein, utilizing the electrostatic interaction, positively charged methylene blue (MB) was interlaced in the formed DNAzyme nanowires and then served as an electron mediator. In the electrolyte of 0.1 M PBS (pH 7.0) containing NAD^+ and alcohol, the ADH on the electrode surface catalyzes the transformation of ethanol into acetaldehyde, which is accompanied by the formation of NADH from NAD^+. Then, the hemin–G-quadruplex firstly acts as an NADH oxidase, where the produced NADH is oxidized by O_2 to reform NAD^+ with the concomitant local formation of high concentration of H_2O_2. Then the hemin–G-quadruplex acting as an HRP-mimicking DNAzyme quickly bioelectrocatalyzes the reduction of the produced H_2O_2 with the aim of dramatically improving the oxidation–reduction reaction of electron mediator MB. Thus, an amplified electrochemical signal could be obtained through the protein-free isothermal amplification technique HCR and the pseudo triple-enzyme cascade electrocatalytic system.

As described above, the amplification of the electrochemical signal was initially derived from the formation of the long hemin–G-quadruplex DNAzyme nanowires. Therefore, one question to be answered first was whether the HCR could be carried out in the sensor when the primers were labeled with TBA. To clarify this issue, the TBA-S_0 after incubation with S_1 + S_2 was characterized using transmission electron microscopy (TEM). As seen in the inset in Fig. 1, a long nicked DNA nanowire was observed, suggesting that the initiator strand on the TBA could trigger a HCR in the presence of auxiliary single-strands S_1 and S_2. The formed long nanowires could provide numerous electron mediators MB and hemin–G-quadruplex catalytic structures, and finally generate significantly amplified electrochemical signals for thrombin detection. As shown in Fig. 1a, the aptasensor with the incubation of thrombin (1 nM) and TBA-S_0 displays neglectable DPV response in the electrolyte of PBS. However, after the aptasensor was treated with the autonomous assembly of the hemin–G-quadruplex DNAzyme nanowires, an obvious increase in DPV response could be obtained in the electrolyte of PBS (Fig. 1b), suggesting that the negatively charged nanowires could gather numerous electron mediators MB to the working electrode through electrostatic interaction, significantly amplifying the electrochemical signal. Additionally, the aptasensor treated with the autonomous assembly of the hemin–G-quadruplex DNAzyme nanowires was also investigated in the electrolyte of PBS containing 0.32 mM NAD^+ and 1.0 M alcohol (Fig. 1c); the DPV response further increased largely owing to the pseudo triple-enzyme cascade electrocatalytic amplification. Based on the results of TEM and the DPV assay, we might conclude that the HCR and pseudo triple-enzyme cascade electrocatalytic system could be utilized for amplified detection of thrombin.

Furthermore, we also made a comparison of the signal amplification efficiency between the pseudo triple-enzyme cascade electrocatalytic system and the pseudobienzyme system. The aptasensor with the incubation of 1 nM thrombin and autonomous assembly of the hemin–G-quadruplex DNAzyme nanowires was investigated in a different electrolyte by using the DPV technique. Fig. 2a shows the DPV response of the aptasensor investigated in the electrolyte of PBS only. Owing to the electron mediator MB interlaced in the formed DNAzyme nanowires, a readily detectable electrochemical signal could be seen. After the addition of 0.32 mM NADH (Fig. 2b), 0.32 mM NAD^+ and 1.0 M alcohol (Fig. 2c) in the electrolyte, respectively, the DPV responses increased obviously. However, the DPV response in 0.32 mM NAD^+ and 1.0 M alcohol (pseudo triple-enzyme cascade electrocatalytic system) increased much higher than that in 0.32 mM NADH (pseudobienzyme system), indicating the feasibility and superiority of the proposed pseudo triple-enzyme cascade electrocatalytic amplification strategy as expected.

The sensitivity of the described strategy towards the target thrombin was examined. Fig. 3A shows the current changes in the proposed aptasensor in response to the varying concentrations of thrombin. The peak current increased significantly with the increase in target concentration and showed a good linear relationship

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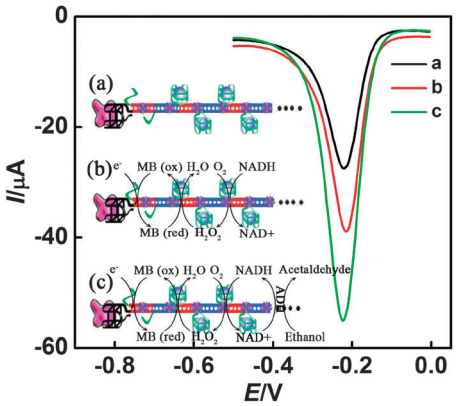

Fig. 2 DPV responses of the aptasensor with the incubation of thrombin and the autonomous assembly of the hemin–G-quadruplex DNAzyme nanowires obtained in the electrolyte of PBS (a), PBS containing 0.32 mM NADH (b), and PBS containing 0.32 mM NAD^+ and 1.0 M alcohol (c).

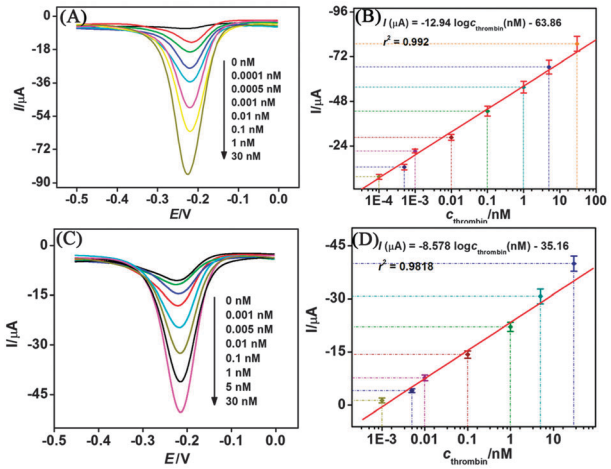

Fig. 3 DPV responses of the aptasensor with the incubation of various thrombin concentrations and the autonomous assembly of the hemin–G-quadruplex DNAzyme nanowires obtained in the electrolyte of PBS with (A) and without (C) 0.32 mM NAD^+ and 1.0 M alcohol. (B) and (D) The corresponding calibration curves (the background signals have been subtracted).

between the peak currents and the logarithms of thrombin concentrations in the range from 0.0001 to 30 nM with a detection limit of 0.08 pM (Fig. 3B), which confirms that the designed pseudo triple-enzyme cascade electrocatalytic amplification strategy can be successfully applied in thrombin detection. To further confirm the mechanism of our strategy, the DPV assay investigated in the electrolyte of PBS only was carried out under the same conditions. Fig. 3C shows the corresponding DPV response with the increasing thrombin concentrations. As a result, the linear ranges span the concentration of thrombin from 0.001 to 30.0 nM with a detection limit of 0.6 pM (Fig. 3D). Additionally, the analytical performance of the proposed aptasensor has been compared with those of other reported aptasensors. The detailed results are shown in Table S1 (see ESI†). The higher sensitivity and the wider linear range of the proposed aptasensor investigated in PBS containing 0.32 mM NAD^+ and 1.0 M alcohol effectively demonstrated the significant amplification of HCR and the pseudo triple-enzyme cascade electrocatalytic system as expected.

For the specificity study, the proposed aptasensor was evaluated by challenging it against several other possible interferences, *e.g.* BSA, Hb, IgG and L-cysteine. As indicated in Fig. S3 (see ESI†), no obvious currents were obtained toward these interferences alone. However, the presence of target thrombin yielded a significant increase in the current. Even when BSA, Hb, IgG and L-cysteine coexisted with thrombin, the current response was almost the same as that with thrombin only, indicating that the aptasensor is highly selective to thrombin and only the target thrombin triggers the HCR process efficiently.

The feasibility of this pseudo triple-enzyme cascade electrocatalytic aptasensor was validated by testing thrombin in a human real serum sample. Since no thrombin exists in a healthy human serum sample, here, we spiked thrombin of different concentrations into 10-fold-diluted serum samples (obtained from Ninth People's Hospital of Chongqing, China) to examine the feasibility of the proposed aptasensor in a real serum sample. As seen in Table S2 (see ESI†), the recoveries varied from 95.3 to 108.4%, exhibiting an optional scheme for determination of thrombin in clinical diagnostics.

In conclusion, we have fabricated a novel pseudo triple-enzyme cascade electrocatalytic electrochemical aptasensor by using the alcohol dehydrogenase (ADH) as well as a hemin–G-quadruplex that simultaneously acted as an NADH oxidase and HRP-mimicking DNAzyme. The highlight of this work is to adequately utilize the bifunctional hemin–G-quadruplex for resolving the fussy labeling process, deactivation and spatial distribution of each enzyme, and thus to successfully realize the triple-enzyme-catalyzed amplification.

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