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A novel "signal-on/off" sensing platform for selective detection of thrombin based on target-induced ratiometric electrochemical biosensing and bio-bar-coded nanoprobe amplification strategy

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ABSTRACT

A novel dual-signal ratiometric electrochemical aptasensor for highly sensitive and selective detection of thrombin has been designed on the basis of signal-on and signal-off strategy. Ferrocene labeled hairpin probe (Fc-HP), thrombin aptamer and methyl blue labeled bio-bar-coded AuNPs (MB-P3-AuNPs) were rationally introduced for the construction of the assay platform, which combined the advantages of the recognition of aptamer, the amplification of bio-bar-coded nanoprobe, and the ratiometric signaling readout. In the presence of thrombin, the interaction between thrombin and the aptamer leads to the departure of MB-P3-AuNPs from the sensing interface, and the conformation of the single stranded Fc-HP to a hairpin structure to take the Fc confined near the electrode surface. Such conformational changes resulted in the oxidation current of Fc increased and that of MB decreased. Therefore, the recognition event of the target can be dual-signal ratiometric electrochemical readout in both the "signal-off" of MB and the "signal-on" of Fc. The proposed strategy showed a wide linear detection range from 0.003 to 30 nM with a detection limit of 1.1 pM. Moreover, it exhibits good performance of excellent selectivity, good stability, and acceptable fabrication reproducibility. By changing the recognition probe, this protocol could be easily expanded into the detection of other targets, showing promising potential applications in disease diagnostics and bioanalysis.

1. Introduction

Highly sensitive, selective and accurate determination of biomolecule with easy operation, low consumption and short assay time has attracted considerable interest in various fields such as clinical diagnosis, gene therapy, and biomedical studies (Li et al., 2010; Wang et al., 2009; Zhang et al., 2013a, 2013b). Various biosensors, including fluorescent (Hu et al., 2014; Huang et al., 2012; Xue et al., 2016; Zuo et al., 2010), chemiluminescent (Arakawa et al., 2015; Yu et al., 2016; Zong et al., 2014), colorimetric (Gupta et al., 2016; Wu et al., 2016), electrochemical (He et al., 2016; Mahshid et al., 2015; Mao et al., 2015; Woo et al., 2014; Taghdisi et al., 2016), and Raman spectroscopic (Lierop et al., 2011; Sikirzhytski et al., 2012) have been designed for the detection of biomolecules.

Aptamers are single stranded DNA or RNA oligonucleotides isolated from random sequence libraries in vivo selection (SELEX) through an iterative process of adsorption, recovery, and amplification (Ellington and Szostak, 1990; Tuerk and Gold, 1990). They could form well-ordered structures to bind a wide range of specific targets (e.g., small molecules, proteins, amino acids, and even cells) with high specificity and affinity (Stojanovic et al., 2001; Yamamoto and Kumar, 2000; Michaud et al., 2003; Labib, et al., 2016). Until now, aptasensors have been established for selective recognition and detection of molecules with mechanical (Bini et al., 2007), optical (Fredriksson et al., 2002), or electronic readouts (Willner and Zayats, 2007). Among these achievements, electrochemical aptasensor (Jo et al., 2015; Wang et al., 2015b; Wu et al., 2015; Xue et al., 2015; Zhang et al., 2007; Zhang et al., 2013a), has attracted intense interest in the design of platform for biological analytes due to its characters of easy fabrication, simple signal production, and amenability to miniaturisation and multiplexing.

Thrombin is a coagulation protein in the blood stream that has played pivotal roles in many physiological processes and relates to a multitude of diseases (Bichler et al., 1996; Holland et al., 2000). Low concentrations of thrombin (50 pM to 100 nM) mediate neuroprotection against ischemia and environmental insults such as oxidative stress, hypoglycemia, hypoxia, and growth supplement deprivation. High concentrations of thrombin, however, are shown to cause degeneration and cell death in both astrocyte and hippocampal neuron (Vaughan et al., 1995; Striggow et al., 2000). Therefore, the quantita-

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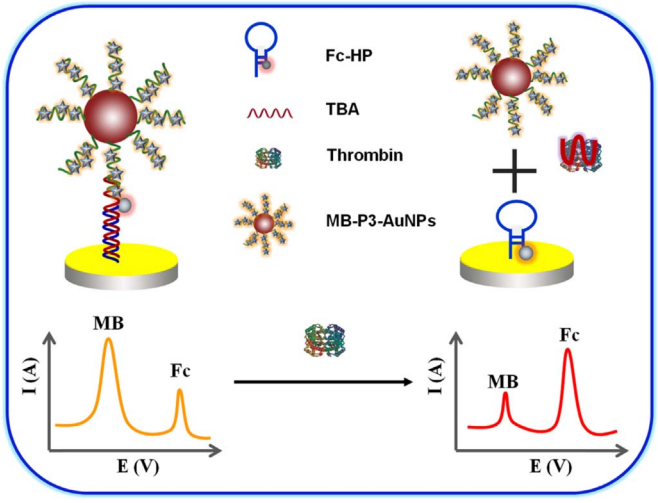
tive detection of thrombin is extremely important in both clinical practice and diagnostic applications.

Electrochemical indicators, such as ferrocene (Fc), methylene blue and other redox tags, have been used to label DNA probes for the development of electrochemical biosensors (Cunningham et al., 2014; Farjami et al., 2011; Lou et al., 2013; Xiao et al., 2005). By designing artful target-induced conformational change on the electrode surface, "signal-on" or "signal-off" biosensors have been proposed for the detection of DNA and proteins due to the distance of a redox tag to an electrode surface was altered. For example, Plaxco et al. developed a novel "signal-on" aptasensor based on target induced strand displacement of the thrombin aptamer hybridized MB-tagged oligonucleotide thus increases the concentration of MB at the electrode surface (Xiao et al., 2005). In contrast, Xiao et al. reported a biosensor based on target-induced strand release of Fc-tagged poly(A) from the poly(A)/poly(T) hybridized duplex to fulfill the "signal-off" determination of mercury (Lou et al., 2013). Despite recent advances in this field, both the "signal-on" and "signal-off" biosensors transduce the target reorganization event into the structural change of the probe, and then output detectable signal directly through the labeled molecule in a "one target to one response signal" manner.

However, it is difficult to confirm whether the observed "signal-on" or "signal-off" changes are due to target binding or deterioration of the sensing surface because of the hard-to-avoid variations in electrode areas, DNA loading densities, and nontarget-induced reagent dissociation, which can lead to relatively low reproducibility, robustness, and reliability of the results. The ratiometric detection technology that utilizes two electrochemical labels simultaneously for robust detection of one specific target has been introduced to improve the robustness and reproducibility (Du et al., 2014; Ren et al., 2015). However, the traditional ratiometric biosensor is not sensitive enough, because a single target only opens a single signaling capture probe and makes a pair of Fc/MB labeled probe conformational change which may limit the total signal and the corresponding sensitivity. To overcome this problem, the strategies based on enzyme-aided amplification have been proposed (Gao et al., 2015a, 2015b; Shen et al., 2015; Xiong et al., 2015). To the best of our knowledge, the technology combining the advantages of the high specificity of ratiometric detection and nano-amplification has not been reported.

In this work, a simple and sensitive electrochemical biosensor for the detection of thrombin has been developed based on the dual-signaling ratiometric electrochemical method and methyl blue labeled bio-bar-coded AuNPs (MB-P3-AuNPs) probe amplification strategy. The detailed concept of strategy is illustrated in Scheme 1. The sensing platform was constructed via stepwise assembly of ferrocene labeled hairpin probe (Fc-HP), thrombin aptamer, and MB-P3-AuNPs to form a recognition structure on gold electrode. In the presence of target thrombin, MB-P3-AuNPs was released from the electrode surface and Fc approached to the electrode due to the formation of a hairpin structure. These changes led to the decrease of the oxidation peak current of MB (I_{MB}), accompanied by the increase of that of the Fc (I_{Fc}), which led to "signal-off" and "signal-on" elements for dual-signal electrochemical ratiometric readout (Scheme 1).



Scheme 1. Schematic illustration of the dual-signal ratiometric electrochemical thrombin aptasensor.

2. Experimental

2.1. Materials and reagents

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 6-Mercapto-1-hexanol (MCH) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma-Aldrich (USA). Bovine serum albumin (BSA), Myoglobin (Mb) and Hemoglobin (Hb) were purchased from Alfa Aesar. Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), MB and trisodium citrate were obtained from Shanghai Reagent Co. (Shanghai, China). Oligonucleotides were synthesized and purified by Sangon Biotech. Co., Ltd. (Shanghai, China), and their sequences were listed as follows:

TBA: 5′-*AGT CCG TGG* ***TAG GGC AGG TTG GGG TGA CT***-3′.

Fc-HP: 5′ -SH-$(CH_2)_6$-***AGT CAC CCC AAC CTG CCC TA***T GAC T-Fc-3′.

P3: 5′- *CCA CGG ACT* GGG AGG GAG GGA GGG A-$(CH_2)_6$-SH-3′.

The binding regions between TBA and Fc-HP as well as TBA and P3 were shown in italics with bold and italics with underlined, respectively. All these reagents were used as received without further purification. The clinical serum samples were from the People's Hospital of Liaocheng. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all assays. DNA was stored in Tris-HCl (10 mM, pH 8.0) containing 1 mM ethylenediaminetetraacetic acid. Tris-HCl buffer (10 mM, pH 7.4) containing 100 mM NaCl was employed for hybridization and washing.

2.2. Apparatus

The transmission electron microscopic (TEM) image was observed on a JEM-2100 transmission electron microscope (JEOL Ltd., Japan) with an accelerating voltage of 200 kV. The UV–vis absorption spectrum was observed with a Lambda750 UV–vis spectrophotometer (PerkinElmer). The dynamic light-scattering measurement and the ζ potential were carried out using a Zetasizer Nano ZSP instrument (Malvern Instruments Ltd.). All work was performed at room temperature (about 25 °C) unless otherwise mentioned. The electrochemical measurements were performed on a CHI 760 C electrochemical workstation (CH Instruments Inc., U.S.A.) at room temperature with a conventional three-electrode system composed of a platinum wire as counter, Ag/AgCl as reference and the Au electrode as working electrode, respectively.

2.3. Preparation of MB-P3-AuNPs nanoprobe

First, the colloidal AuNPs were prepared according to the previous protocol with modification (Frens, 1973). Briefly, 100 mL of 1 mM $HAuCl_4$ solution was boiled with vigorous stirring, and 10 mL of 38.8 mM trisodium citrate solution was quickly added to the boiling solution. The solution turned deep red, indicating the formation of AuNPs. Followed by continued stirring and cooling down, the resulting Au colloidal solution was stored in brown glass bottles at 4 °C before use. Prior to attachment to the AuNPs, the thiolated P3 was dissolved in 10 mM Tris-HCl buffer (containing 100 mM NaCl and 10 mM TCEP, PH 7.4) and incubated in the dark for 1 h to reduce disulfide bonds. Next, the deprotected P3 was self-assembled onto the AuNPs by incubation at room temperature for at least 16 h with gently stirring