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Target-programmed and autonomous proximity binding aptasensor for amplified electronic detection of thrombin

Jianmei Yang[a], Yulan Wu[b], Chunfang Gan[b,*], Ruo Yuan[a], Yun Xiang[a,*]

[a] Key Laboratory of Luminescent and Real-Time Analytical Chemistry, Ministry of Education, School of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, PR China

[b] College of Chemistry and Materials Science, Guangxi Teachers Education University, Nanning 530001, PR China

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ABSTRACT

The development of sensitive and simple approaches capable of monitoring trace amounts of protein biomarkers is appealing for disease diagnosis and treatment. Towards this end, we have developed an electrochemical sensing platform for sensitive and simple detection of protein biomarkers by using thrombin as the model target molecules via a target-programmed proximity binding amplification approach. The binding of thrombin to the aptamer sequences in the partial dsDNA duplex probes induces the release of the ssDNA trigger strands, which catalyze subsequent assembly formation of many methylene blue (MB)-tagged proximate DNA motifs with the presence of the DNA fuel strands through cascaded toehold-mediated strand displacement reactions. Due to the proximity-binding effect, these MB-tagged proximate DNA motifs anneal with the capture probes on the sensor surface with significantly enhanced stability against the corresponding single component counterpart, thereby pulling the MB tags close to the sensor surface and generating substantially amplified signal responses for sensitive determination of thrombin down to 23.6 pM. In addition, such aptasensor can specifically discriminate thrombin from other interference proteins, and can also be utilized to monitor thrombin in diluted serum samples, demonstrating its great potential for sensitive determination of proteins for early disease diagnosis.

1. Introduction

Sensitive and specific detection of protein biomarkers plays a vital significance in early diagnosis of various diseases. However, sensitively monitoring trace amounts of protein biomarkers is still of a current challenge because of the lack of polymerase chain reaction (PCR)-like techniques to amplify the target protein molecules. To meet such demands for highly sensitive detection of proteins, many different signal enhancement strategies such as PCR-integrated immunoassays (Sano et al., 1992; Niemeyer et al., 2005), rolling circle amplification (Wu et al., 2015; Gao et al., 2015), nanomaterial- and enzyme-assisted signal boost means (Song et al., 2014; Zhang et al., 2013a; Tan et al., 2014; Xue et al., 2012) have been extensively used as powerful tools for improving the sensitivity for protein detection. However, these methods are inherently complicated, expensive, and time-consuming due to the requirements of enzymes or nanomaterials. Therefore, the construction of sensitive and simple approaches without using any nanomaterials or enzymes will undoubtedly benefit the detection of proteins.

Toehold-mediated strand displacement reaction (TSDR) is an attractive approach that was first demonstrated by Yurke et al. for the fabrication of a DNA-fuelled molecular machine (Yurke et al., 2000). In a typical TSDR, an invading single-stranded DNA (ssDNA) binds to an exposed overhang region (known as a toehold) on a double-stranded DNA (dsDNA) to initiate branch migration, which eventually leads to the displacement of the originally-bound short ssDNA from the dsDNA (Wang et al., 2017; Song and Liang, 2012). The whole process of TSDR can occur without using any enzymes at room temperature (Li et al., 2015), which endows TSDR the distinctive advantages for the construction of enzyme-free signal amplification means substantially more robust than previous enzyme-assistant strategies (Eckhoff et al., 2010). Moreover, TSDR has been widely applied for the development of digital logic circuits (Qian and Winfree, 2011), chemical reaction networks (Soloveichik et al., 2010), entropy-driven catalytic reactions (Zhang et al., 2007a), and synthetic transcriptional clocks (Franco et al., 2011). In these systems, the invading strand can be cyclically reused through a series of TSDRs, which makes TSDR a powerful signal amplification approach. Indeed, the extensive application of TSDR as sensitive sensing systems for signal amplified determination of DNA (Duan et al., 2015), RNA (Shi et al., 2015), proteins (Kim et al., 2016), and small molecules (Li et al., 2016) demonstrates that TSDR has significant

* Corresponding authors.
E-mail addresses: ganchunfang2008@126.com (C. Gan), yunatswu@swu.edu.cn (Y. Xiang).

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potential for improving detection sensitivity.

The proximity binding assay, which relies on simultaneous recognition of one molecule by two affinity ligands, is a promising approach for protein analysis (Li et al., 2013a; Yang et al., 2016). The two complementary DNA probes linked to the affinity ligands (termed as proximity probes) are brought into close proximity upon binding to the target molecule, which increases the local concentrations of the two complementary DNA affinity probes and enhances the hybridization efficiency between them, resulting in the formation of more stable complexes (Zhang et al., 2013b, 2012). By successful application of the proximity binding effect for the construction of electrochemical sensors, Zhang et al. developed a proximity-dependent surface hybridization assay approach, which involved the annealing of the proximate affinity probes with the capture probes immobilized on the sensor surface, for the detection of protein (Zhang et al., 2007b). Compared with previously reported methods for electrochemical detection of proteins, such sensing approach exhibits the obvious advantage of simplicity. Yet, for highly sensitive detection of low levels of disease-related proteins biomarkers, further improvement in sensitivity is demanded.

Aptamers are single-stranded oligonucleotides (DNA or RNA) in vitro selected for specifically binding to proteins or other targets with high affinity (Khonsari and Sun, 2017; Li et al., 2013b). Owing to their unique advantages in terms of high affinity, selectivity, stability, and low cost, aptamers exhibit significant potentials over traditional antibodies as recognition probes for the detection of proteins (Xu et al., 2013; Li et al., 2018). Moreover, aptamers can also be easily manipulated and modified due to their nucleic acid nature, which makes them particularly useful for bioanalysis purposes (Hu et al., 2012; Yang et al., 2017). Herein, by integrating the advantages of the aptamer recognition probes and the proximity binding effect, as well as the convenient signal amplification nature of TSDR, we report a target-programmed proximity binding electronic biosensing platform for amplified and sensitive determination of the serum thrombin protein biomarker. Thrombin is a serine protease which plays critical roles in various life processes, such as, thrombosis, hemostasis and other coagulation-related reactions (Li et al., 2012b). The variation in the concentration of thrombin also relates to many diseases, including thromboembolic diseases, inflammation reaction and cardiovascular diseases (Jiang et al., 2013b). Therefore, it is of great importance to monitor thrombin for clinical research and diagnosis purpose. Specific bindings between thrombin and the two distinct corresponding aptamers result in the release of the trigger strands from the complementary aptamer sequences, which initiate subsequent cascaded TSDRs to recycle the trigger strands and to attach massive proximate methylene blue (MB)-modified DNA motifs on the sensor surface via autonomous proximity-based surface hybridizations. In consequence, the surface-confined MB-tags on the DNA motifs can produce significant electrochemical current responses for sensitive determination of thrombin.

2. Experimental section

2.1. Materials and reagents

Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 6-mercaptohexanol (MCH), thrombin, bovine serum albumin (BSA), lysozyme, and human serum were provided by Sigma-Aldrich (St. Louis, MO). Immunoglobulin G (IgG) was bought from Abcam Co., Ltd (Shanghai, China). The oligonucleotide sequences and Tris-HCl were received from Sangon Biotechnology Inc. (Shanghai, China). The sequences of the oligonucleotides were listed as follows. Thrombin Aptamer (TBA): 5′-GTTATG GGTTGGTGTGGTTGG-3′; Trigger 1 (T1): 5′-CCAACCCATAA CCGAACTGCC-3′; S1: 5′-GGAGAGA GGCAGTTCGGTTATG GGTTGG TTTTTTTTTTTTTTTTTTTT GTGTGTCT-MB-3′; S2: 5′-CATAACCGAAC TGCC TCTCTCC TTTTTTTTTTTTTTTTTTTT GTGTGTCT-MB-3′; Protect Probe (P1): 5′-CATAACCGAACTGCC TCTCT-3′; Capture Probe (CP): 5′-SH-$(CH_2)_6$-TTTT AGACACAC-3′. MCH solution (0.1 M) was first prepared in ethanol and was further diluted to 1 mM by Tris-HCl buffer.

2.2. Preparation of the sensing electrode

Pretreatment of electrodes was first carried out by following our previous protocol (Yang et al., 2015). Briefly, the gold electrode (AuE, 3 mm diameter) was soaked in a fresh piranha solution for 30 min, polished with alumina slurry (0.3 and 0.05 μm, respectively), and then sonicated with ethanol and ultrapure water. After that, the AuE was electrochemically cleaned in H_2SO_4 (0.5 M) within the potential range from −0.3–1.55 V until the appearance of the stable characteristic voltammetric peaks. Finally, after rinsing with water and drying with nitrogen, the AuE was used for probe immobilization.

The capture probe (CP) was first reduced 1 h by 10 mM TCEP and then diluted to 1 μM by using Tris-HCl buffer (pH 7.4, containing 20 mM Tris-HCl, 200 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$). Tris-HCl buffer was used throughout all experiments. Then, 10 μL of 1 μM CP was dropped on the AuE for a 2 h incubation at room temperature. After washing with buffer and further incubating with 10 μL MCH (1 mM) for 2 h, the AuE was washed again and finally dried under nitrogen.

2.3. Amplified electrochemical detection of thrombin

To prepare thrombin recognition probes, the mixture of thrombin aptamer (TBA) and trigger1 (T1) or the S1 and the protect probe (P1) were separately annealed at 90 °C for 5 min, and then slowly cooled down to 25 °C to obtain the TBA/T1 or S1/P1 duplexes. Subsequently, the CP-modified sensing interface was incubated with 10 μL the mixture of TBA/T1 (0.2 μM), S1/P1 (0.2 μM), S2 (0.2 μM), and various concentrations of thrombin at 37 °C for 120 min. Finally, the sensor was subjected to electrochemical measurements after washing with buffer.

2.4. Electrochemical measurements

All electrochemical experiments were completed by using our previously reported procedure (Dou et al., 2016) on an electrochemical workstation (CHI 621D) with the modified AuE working electrode, a platinum counter electrode, and Ag/AgCl (3 M KCl) reference electrode. Cyclic voltammetry (CV) with the potential range from −0.1–0.6 V was carried out in KCl solution (0.1 M) with the presence of $[Fe(CN)_6]^{3-/4-}$ (1 mM) at a 50 mV s^{-1} scan rate. Square wave voltammetry (SWV) was recorded in Tris-HCl buffer from −0.5 to −0.1 V with the step potential of 4 mV at an amplitude of 25 mV and a frequency of 25 Hz.

3. Results and discussion

3.1. Design principle of the target-programmed proximity binding aptasensor

Scheme 1 displays the principle of the proposed sensitive electrochemical thrombin assay strategy. The sensing interface is constructed by the immobilization of the thiolated capture probes (CPs) onto the gold electrode (AuE) via Au-S interaction. Initially, the trigger strand (T1) is partially hybridized with the thrombin aptamer (TBA) to inhibit the binding between T1 and the toehold 1 region in S1 for subsequent TSDR. Similarly, the protect probe (P1) is hybridized with S1 to block the toehold 2 region in S1 to avoid TSDR between S1/P1 and S2 without the addition of thrombin. Both S1 and S2 possess a short 3′-MB-labeled tail sequence (eight-nucleotide) complementary to the CP strand immobilized on the electrode surface with a predesigned low melting temperature (T_m = 28 °C, verified by Mfold online software). In this case, under the experimental temperature (37 °C), TBA/T1, S1/P1 and S2 can co-exist with the absence of the target thrombin, and no free T1 can be generated to trigger the downstream two TSDRs as shown in Scheme 1, avoiding the association of S1 and S2. Therefore, the MB-labeled tail sequences of S1 and S2 are unable to spontaneously