



Target-induced catalytic hairpin assembly formation of functional Y-junction DNA structures for label-free and sensitive electrochemical detection of human serum proteins

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ABSTRACT

The construction of simple and highly sensitive aptasensors will significantly advance the monitoring of proteins. In this work, we have developed a convenient and label-free protein-induced catalytic hairpin assembly signal amplification approach for aptamer-based sensitive electrochemical detection of thrombin in human serums. The target thrombin binds the aptamer-containing hairpin probes immobilized on the sensor electrodes and triggers catalytic assembly of other two hairpins to form many G-quadruplex Y-junction DNA structures *in situ*. These G-quadruplex structures further associate with hemin to form G-quadruplex/hemin complexes, which are then subject to electrochemical measurement and generate substantially amplified current output for label-free and sensitive detection of thrombin. The developed aptasensor shows a dynamic range of 0.01–1.0 nM and a detection limit of 6.0 pM for thrombin detection. The sensor can also discriminate the target thrombin against other non-target proteins and exhibits promising results for detecting thrombin in human serums. With the successful construction of the thrombin aptasensor, our signal amplified method can be potentially applied to detect other target molecules by choosing appropriate aptamer/ligand pairs.

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1. Introduction

Aptamers are artificial functional oligonucleotides (single-stranded DNA or RNA) isolated from large random-sequence nucleic acid libraries by an *in vitro* evolution process termed systematic evolution of ligands by exponential enrichment (SELEX) [1,2]. The *in vitro* selected aptamers can bind a wide range of target molecules, such as proteins, peptides, small molecules, whole cells and organic/inorganic ions with high specificity and affinity [3–7]. Besides their binding affinity, aptamers also feature with several significant advantages, such as easy chemical modification, long-term storage, reversible folding without loss of activity and simple synthesis, over the conventional antibodies or biomimetic affinity receptors [8,9]. Because of these unique properties, aptamers have been increasingly employed as desirable bio-recognition elements for the fabrication of different aptasensors [10–12]. Until now, a

large number of aptasensors have been extensively reported since the first introduction of aptamers in 1990 [13,14]. Many of these sensors involved in using thrombin as the target molecules because of the important roles of thrombin in physiological and pathological coagulations.

Thrombin, a specific allosteric serine protease generated from inactive zymogen prothrombin via a series of enzyme cleavage reactions in humans, is the central protease in the blood coagulation cascade [15]. Thrombin also exhibits perfect procoagulant properties by converting soluble fibrinogen into insoluble strands of fibrin that can promote the formation of blood clots to prevent bleeding [16,17]. Moreover, thrombin can also catalyze many coagulation-related reactions, such as inflammation, cardiovascular diseases, wound healing and so on [18,19]. As a consequence, abnormal concentration of thrombin is tightly associated with various diseases such as Alzheimer's disease and cancers [20,21]. Taking into account the importance of thrombin in biochemical studies and clinical diagnosis, the development of sensitive, simple and selective methods for monitoring thrombin is thus urgently demanded. Over the past decades, different assay techniques including sur-

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face plasmon resonance [22], colorimetry [23], fluorescence [24], electrochemiluminescence [25] and electrochemistry [26] have been widely developed for detecting thrombin. Among these techniques, the electrochemical approach has attracted more attentions because of its distinct advantages of high sensitivity, simplicity, miniaturization, rapid response and low cost.

In order to realize sensitive detection of thrombin, a variety of effective signal amplification approaches, such as rolling circle amplification (RCA) [27], nanomaterial-based signal amplification [28], enzyme-aided recycling amplification [29] and hybridization chain reaction (HCR) [26] have been reported. In addition, two or more of these amplification methods have been incorporated into one single assay system to further improve the sensitivity [30]. Although these sensing strategies can offer high sensitivity, the shortcomings of easy contamination, time/sample-consuming operation processes and high cost of these assay approaches have triggered the development of new alternatives for sensitive, selective, and convenient detection of thrombin. In recent years, the DNA-triggered self-assembly has gained increasing attention owing to the unique properties of DNA molecules (e.g., highly specific Watson-Crick base pairing capability, excellent biocompatibility, and the capability for significant signal amplifications) [31]. Despite that the non-enzyme-based assembly of nucleic acids has been widely used for sensitive detection of nucleic acids (DNA and RNA) [32,33], the target-induced hairpin assembly as an alternative signal amplification method for the detection of proteins has been rarely reported [34]. In response, based on target-induced catalytic hairpin assembly formation of DNA Y-junction structures with multiple G-quadruplex arms on the sensor electrode, we report herein a simple, non-enzymatic and label-free amplified electrochemical aptasensor for highly sensitive and selective detection of thrombin. The DNA Y-junction structures are commonly formed by the hybridization of three complementary DNA sequences and exhibit distinct advantages in terms of target versatility, high selectivity and stability as probes for different biosensing applications because of their template enhanced hybridization effect [35,36]. The resulting G-quadruplex sequences on the Y-junction structures can associate with hemin to form G-quadruplex/hemin complexes, and direct electron transfer between the surface-confined hemin and the electrode generates significantly enhanced current responses for sensitive and convenient monitoring of thrombin.

2. Experimental section

2.1. Materials and reagents

Thrombin, bovine serum albumin (BSA), trypsin (Try), lysozyme (Lyso), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), dimethyl sulfoxide (DMSO) and 6-mercaptohexanol (MCH) were supplied by Sigma (St. Louis, MO, USA). Hemin purchased from Aladdin Reagents (Shanghai, China) was dissolved in DMSO to make the stock solution (1 mM) and stored at -20°C for further use. Tris-HCl, 4-(2-hydroxyethyl) piperazine-1 ethanesulfonic acid sodium salt (HEPES) and all oligonucleotides (Table 1) were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Other reagents were of analytical grade and ultrapure water (resistance $>18\text{ M}\Omega\text{-cm}$) was used throughout all experiments.

2.2. Preparation of the sensing electrode

Prior to sensor modification, the working gold electrode (AuE, 3 mm in diameter) was first successively polished with 0.3 and 0.05 μm alumina slurry after being immersed in a freshly prepared piranha solution (mixture of concentrated H_2SO_4 and 30% H_2O_2 at the volume ratio of 3:1) for 30 min. Following sonication in ultra-

pure water, ethanol and ultrapure water for 5 min respectively, the AuE was transferred into freshly prepared H_2SO_4 solution (0.5 M) for electrochemical pretreatment [37]. Washed thoroughly with distilled water and dried with nitrogen, the cleaned AuE was incubated with 10 μL SH-HP1 (0.3 μM) in Tris-HCl buffer (10 mM Tris-HCl, 1 mM EDTA, 10 mM TCEP, 1 mM MgCl_2 and 0.1 M NaCl, pH 7.4) overnight at room temperature (Note: to form perfect stem-loop structures, all hairpin DNAs were annealed at 95°C for 5 min and gradually cooled to room temperature at a rate of $1^{\circ}\text{C min}^{-1}$). After careful rinsing with Tris-HCl buffer, the SH-HP1-assembled electrode was blocked with 1 mM MCH for 2 h to remove the non-specific DNA adsorption. This is followed by rinsing with ultrapure water to obtain the MCH/SH-HP1/AuE interface for thrombin detection.

2.3. Amplified thrombin sensing protocol

For thrombin detection, different MCH/SH-HP1/AuE sensor electrodes were incubated with various concentrations of thrombin (10 μL) in buffer (20 mM Tris-HCl, 100 mM NaCl, 10 mM KCl, and 10 mM MgCl_2 , pH 7.5) containing the annealed HP2 (1.0 μM) and HP3 (1.0 μM) for 2 h at room temperature, followed by washing with buffer. After that, the sensor electrode was incubated with 20 mM HEPES buffer (50 mM KCl, 200 mM NaCl, 1% DMSO, pH 8.0) containing 0.2 mM hemin for 30 min to allow the signal probes to form G-quadruplex/hemin complexes.

2.4. Electrochemical measurements

All electrochemical measurements were performed on a CHI 621D electrochemistry workstation (CH Instruments, Shanghai, China) with a conventional three-electrode system: a platinum wire auxiliary electrode, Ag/AgCl reference electrode and the modified AuE working electrode. Cyclic voltammetry (CV) was recorded in 0.1 M KCl solution containing 1 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ with the potential range from -0.1 to 0.6 V at a scan rate of 50 mV s^{-1} . Differential pulse voltammetry (DPV) was carried out in N_2 -saturated 20 mM HEPES buffer (50 mM KCl, and 200 mM NaCl, pH 8.0), by scanning the potential range from -0.15 to -0.5 V with pulse amplitude of 50 mV and sample width of 16.7 ms.

3. Results and discussion

3.1. Design principle for the amplified thrombin sensing strategy

The detailed mechanism of our label-free and amplified thrombin aptasensor is illustrated in Scheme 1. Due to the significant signal amplification by the target-induced catalytic hairpin assembly formation of the G-quadruplex/hemin Y-junction structures and electrochemical reduction of hemin, the proposed strategy can achieve sensitive and label-free detection of thrombin. The catalytic assembly formation of the Y-junction structures is basically based on the toehold-mediated strand migration reactions between nucleic acids [38,39]. The hairpin structures are designed with long stem regions to ensure a relatively thermodynamic steady state of the hairpin structures [40] and to avoid spontaneous hybridizations between any two or three of the hairpins in the absence of the target molecules. To achieve this goal, the assembly initiation sequences (toeholds) for HP2 (the cyan region in SH-HP1) and HP3 (the yellow region in HP2) are respectively locked in the stems of SH-HP1 and HP2. The thiol-modified hairpin probe (SH-HP1) containing the sequence of the thrombin aptamer is anchored on the AuE surface via the formation of Au-S covalent bond, followed by blocking with MCH to prepare the sensor interface. Thus, in the absence of the target thrombin, these hairpin structures are metastable and the assembly of the three hairpins is

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