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# A cascade signal amplification strategy for surface enhanced Raman spectroscopy detection of thrombin based on DNAzyme assistant DNA recycling and rolling circle amplification

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ABSTRACT

A sensitive protocol for surface enhanced Raman spectroscopy (SERS) detection of thrombin is designed with R6G-Ag NPs as a signal tag by combining DNAzyme assistant DNA recycling and rolling circle amplification (RCA). Molecular beacon (MB) as recognition probe immobilizes on the glass slides and performs the amplification procedure. After thrombin-induced structure-switching DNA hairpins of probe 1, the DNAzyme is liberated from the caged structure, which hybridizes with the MB for cleavage of the MB in the presence of cofactor $Zn^{2+}$ and initiates the DNA recycling process, leading to the cleavage of a large number of MB and the generation of numerous primers for triggering RCA reaction. The long amplified RCA product which contained hundreds of tandem-repeat sequences, which can bind with oligonucleotide functionalized Ag NPs reporters. The attached signal tags can be easily read out by SERS. Because of the cascade signal amplification, these newly designed protocols provides a sensitive SERS detection of thrombin down to the femolar level (2.3 fM) with a linear range of 5 orders of magnitude (from $10^{-14}$ to $10^{-9}$ M) and have high selectivity toward its target protein. The proposed method is expected to be a good clinical tool for the diagnosis of a thrombotic disease.



## 1. Introduction

Thrombin is a coagulation protein in the blood stream that has played significant roles in many life processes and relates to a multitude of diseases (Bichler et al., 1996; Holland et al., 2000). It is usually regarded as a tumor marker in the diagnosis of pulmonary metastasis and the high or low concentration of thrombin in blood is associated with coagulation abnormalities (Wang et al., 2009; Zhang et al., 2009a, 2009b). Therefore, the quantitative detection of thrombin is extremely important in both clinical practice and diagnostic (Liu et al., 2009). The current clinical methods for protein detection rely heavily on antibodies. Although these conventional strategies provide accurate and sensitive detection of proteins, there are still some inconveniences that exist, such as the utilization of radioactive substances, enzyme labeling, time-consuming processes, and technical expertise as well as sophisticated equipment (Shuman and Majerus, 1976; Bichler et al., 1991; Zhu et al., 2000). Thus, development of protein sensing methods that are rapid, simple, sensitive, selective, on-site and cost-effective is still highly desirable.

To overcome these problem, the strategies based on aptamers as recognition element to construct thrombin biosensor have been developed, which owing to lots of advantages, such as the ease of labeling, excellent stability and high affinity and selectivity towards thrombin compared to traditional molecular recognition system (Xiao et al., 2005; He et al., 2007; Li et al., 2008). Recently, various sensitive detection modes based on aptamers including electrochemical (Baker et al., 2006; Zhao et al., 2011; Peng et al., 2014; Sun et al., 2014), fluorescence (Wang et al., 2004; Lin et al., 2006; Huang et al., 2010; Chi et al., 2011), colorimetry (Huang et al., 2005; Li et al., 2012a, 2012b, 2012c, 2012d; Li et al., 2014), surface plasmon resonance (Wang et al., 2008, 2010), electrochemiluminescence (Huang and Zhu, 2009; Wang et al., 2011a, 2011b; Li et al., 2012a, 2012b, 2012c, 2012d) and surface enhanced Raman spectroscopy (Hu et al., 2008; Li et al., 2012a, 2012b, 2012c, 2012d; He et al., 2013) techniques have been used for detecting thrombin. Among these aptasensors, Raman spectra aptasensors are the most attractive due to their advantages of being excited at any wavelength, alleviated photobleaching, and showing narrow peak widths and fast response over the fluorescent counterparts (Cho et al., 2008; Ye et al., 2013; Zheng et al., 2014). Despite its

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significant advantages, the traditional Raman aptasensor is not sensitive enough because of that traditionary 1:1 binding ratio of aptamer and analyte, limiting the total signal gain and corresponding sensitivity (Kim et al., 2010; Li et al., 2012a, 2012b, 2012c, 2012d).

In order to improve the sensitivity of these aptasensors, DNA recycling amplification methods via nuclease, such as polymerase-mediated strand displacement amplification (Qiu et al., 2011), exonuclease III (Gao et al., 2013; Liu et al., 2014a, 2014b) and nicking endonuclease (Xue et al., 2010, 2012; Zheng et al., 2012) aided signal amplification, wherein a single target can be amplified to cyclically produce multiple hybridization events, have increasingly become attractive alternatives for the detection of trace levels of protein. Recently, DNA recycling was also achieved by DNAzyme to promote signal amplification, which can realize simple detection, high sensitivity and low detection limit (Wang et al., 2011a, 2011b). DNAzymes are catalytic DNA sequences isolated via in vitro selection (Breaker, 2000). Cofactor-dependent DNAzymes can often be generated by varying cofactors and cofactor concentrations during the selection process. DNAzymes show high catalytic hydrolytic cleavage activities toward specific substrates, and can be denatured and renatured many times without losing their catalytic activities toward substrates (Lu et al., 2012). This unique advantage makes DNAzymes ideal biocatalysts for amplified sensing applications (Liu et al., 2014a, 2014b).

This work further combined the DNAzymes assisted DNA recycling with rolling circle amplification (RCA) for SERS detection of thrombin. RCA, as an advanced DNA amplification technique alternative to polymerase chain reaction, can achieve significant signal amplification via the production of thousands of repeated sequences under mild reaction conditions and with speediness, high efficiency, and specificity. Thus, it has widely been employed for the analysis of proteins and nucleic acids by coupling with electrochemistry (Zhang et al., 2009a, 2009b), SERS (Hu and Zhang, 2010), and colorimetry (Li et al., 2010). In this paper, molecular beacon (MB) immobilized on a substrate by a highly amino–epoxy interaction to recognize the DNAzymes by thrombin-induced structure-switching DNA hairpins, and then produce MB fragment structure with $Zn^{2+}$ (Brown et al., 2003) for triggering the RCA reaction (Scheme 1). Subsequently, oligonucleotide functionalized Ag NPs reporters bind with long repeated DNA sequences of RCA products, which were used for SERS analysis. The combination of dual signal amplification ways led to the attachment of a mass of Ag NPs reporters on the prolonged MB fragments, leading to an extremely sensitive strategy for detection of thrombin.

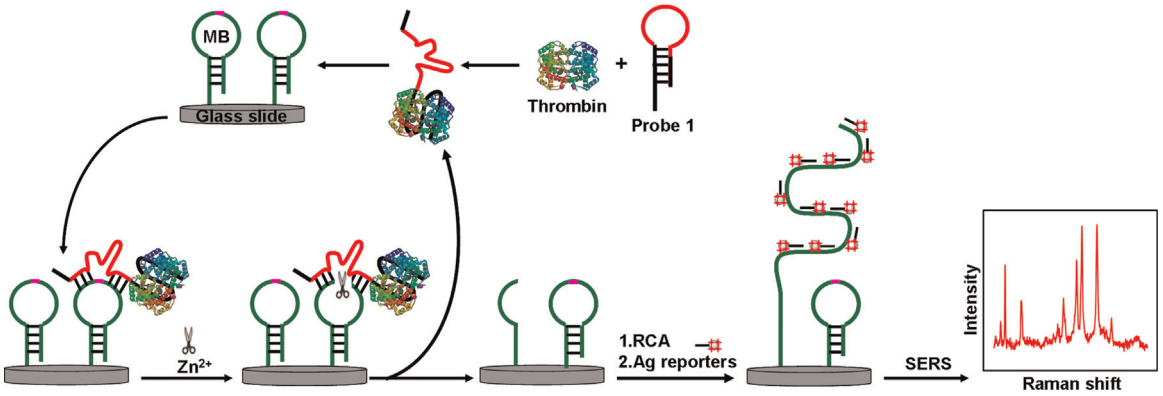
## 2. Experimental

### 2.1. Materials and reagents

Zinc chloride, rhodamine 6G (R6G), glycidoxypropyltrimethoxysilane (GPTMS) (98%), thrombin, bovine serum albumin (BSA), mouse IgG, and fibrinogen were purchased from Sigma-Aldrich Inc. T4 ligase, phi29 DNA polymerase and the mixtures of deoxyribonucleotides (dNTPs) were obtained from Fermentas Biotechnology Co. Ltd. (Canada). Silver nitrate ($AgNO_3$) and trisodium citrate were obtained from Shang-hai Reagent Co. (Shanghai, China) and other chemicals were of analytical grade and purchased from Beijing Chemical Reagent Co. (Beijing, China). Milli-Q water (resistance $>18\ M\Omega\ cm$) was used in all experiments. Phosphate-buffered saline (PBS, 0.1 M) of various pHs were prepared by mixing the stock solutions of $NaH_2PO_4$ and $Na_2HPO_4$. The washing buffer was PBS (0.1 M, pH 7.4) containing 0.05% (w/v) Tween-20. Thrombin aptamer and DNA were obtained from Takara (Dalian, China). The detail base sequences is shown in Table 1.

### 2.2. Preparation and modification of Ag NPs

To provide a suitable metal surface for enhancing the SERS signal, a suspension of citrate-reduced Ag NPs was produced using a modified procedure with the conditions specified by Lee et al. (Lee and Meisel, 1982). Briefly, 200 mL aqueous solution of $10^{-3}$ M $AgNO_3$ was boiled under vigorous stirring, then 5 mL of 35 mM sodium citrate solution was added, and the resulting mixture was kept boiling for 1 h. The colloidal solution was stored at 4 °C and protected from room light. Before DNA loading, the thiol functionality on the oligonucleotide probes was deprotected by treatment with 1.7 equivalents of TCEP for 1 h by using acetate buffer (0.05 mM, pH 5.2) at room temperature. The Ag NPs (3 mL, 2.5 nM) were functionalized with the deprotected thiol oligonucleotides (30 μL, 12 μM) by incubation at room temperature for at least 16 h with gently stirring and an additional 24 h after the concentration of NaCl had been increased to 100 mM. In order to obtain the DNA-conjugated Ag NPs, dependence of SERS intensity for 1.0 pM target thrombin on the concentration of DNA have been investigated in Supporting information. The resulting detection probe attached Ag NPs were purified three times by centrifugation at 8000 rpm for 15 min, redispersed in 10 mM PBS and stored at 4 °C. 10 μL of R6G solution (1 mM) was added to 1.0 mL of probe attached Ag NPs (25 nM) for reaction under stirring for 12 h. The R6G co-assembled with the probe on Ag NPs surface by thiol group. The obtained reporter Ag NPs were rinsed by centrifugation and redispersed in 1 mL PBS (0.01 M, pH 7.4).




**Scheme 1.** Schematic illustration of SERS assay for thrombin detection based on DNAzyme assistant DNA recycling and rolling circle amplification.