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A convenient sandwich assay of thrombin in biological media using nanoparticle-enhanced fluorescence polarization

Qiaoli Yue [a,*], Tongfei Shen [a], Lei Wang [a], Shuling Xu [a], Haibo Li [a], Qingwang Xue [a], Yuanfu Zhang [a], Xiaohong Gu [b], Shuqiu Zhang [b], Jifeng Liu [a,**]

[a] Department of Chemistry, Liaocheng University, Liaocheng 252059, PR China
[b] Shandong Provincial Key Lab of Test Technology on Food Quality and Safety, Shandong Academy of Agricultural Sciences, Jinan 250100, PR China

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ABSTRACT

A new aptamer biosensor was presented for the detection of thrombin in this work, which was based on fluorescence polarization (FP) using silica nanoparticles as enhancement probe. The silica nanoparticles covered by streptavidin were tagged with a thrombin aptamer (5′-biotin-GGTTGGTGTGGTTGG-3′), which was bound to the surface of silica nanoparticle through the specific interaction between streptavidin and biotin. In the presence of thrombin, it induced the aptamer to form quadruplex structure. When the other thrombin aptamer labeled with fluorescein (5′-FAM-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′) was added to the above system, a sandwich structure can form at the surface of silica nanoparticles. The fluorescence polarization was therefore enhanced and quantification between fluorescence polarization signal and concentration of thrombin was built. The sensor provided a linear range from 0.6 to 100 nM for thrombin with a detection limit of 0.20 nM ($3.29S_B/m$, according to the recent recommendation of IUPAC) in a homogeneous media. The same linear range was obtained in spiked human serum samples with a slightly higher detection limit (0.26 nM), demonstrating high anti-interference of the sensor in a complex biological sample matrix. And the sensor can be used to monitor spiked concentration of thrombin level in real human plasma with satisfactory results obtained.

1. Introduction

Aptamers, the nucleic acid sequences (DNA or RNA) selected in vitro from large combinatorial pools to bind to specific targets (Beissenhirtz and Willner, 2006; Ellington and Szostak, 1990; Toro et al., 2008), can fold into a variety of different structures. This characteristic can be used in the development of techniques for aptamer isolation (Ellington and Szostak, 1990). During the procedure, DNA or RNA was screened from a randomly generated population of sequences for their ability to bind a desired molecular target with high affinity and specificity (Tuerk and Gold, 1990). Aptamers can be simply synthesized via cost-effective and readily automated routes (Chakraborty et al., 2009; Tang et al., 2007). Due to the advantages of aptamers such as ease of isolation and modification, tailored binding affinity, and resistance against denaturation, they are suitable candidates for the use of protein detection (Tennico et al., 2010).

Thrombin, a protein involved in the blood coagulation cascade, was the first biological macromolecule exploited for aptamer selection (Crawley et al., 2007). Thrombin is a “trypsin-like” serine protease protein, which converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions (Shuman, 1986). Activation of thrombin is crucial in physiological and pathological coagulation. Blood from a ruptured cerebral aneurysm clots around a cerebral artery and releases thrombin, which can induce an acute and prolonged narrowing of the blood vessel, potentially resulting in cerebral ischemia an infraction. Because of the thrombin's importance in anticlotting therapeutics, thrombin-binding aptamer has been studied extensively (Bock et al., 1992).

Thrombin binding aptamers (TBA) consist of two G-quartet conformations that selectively bind to specific and different epitopes of human α-thrombin (Smirnov and Shafer, 2000): (1) a 15-mer DNA aptamer (TBA1) which binds exosite I of thrombin (fibrinogen binding sites) with nanomolar affinity (Paborsky et al., 1993); (2) a 29-mer DNA aptamer binding to exosite II of thrombin (heparin-binding aptamer) with sub-nanomolar affinity (Tasset et al., 1997). These properties facilitated the development of aptamer sandwich assays for the detection of thrombin with higher specificity than direct immunoassays including electrochemical (Numnuam et al., 2008; Radi et al., 2006; Zheng et al., 2007), fluorescence (Li et al., 2007; Li and Ho, 2008), absorbance (Pavlov et al., 2004), colorimetric (Wei et al., 2007; Xu et al., 2009), or surface-enhanced Raman scattering (SERS) methods (Cho et al., 2008; Hu et al., 2009). The aptasensors mainly used three types of detection ways including the fluorescence or electrochemical methods based on the aptamer

* Corresponding author.
** Corresponding author. Tel./fax: +86 635 8239001.
E-mail addresses: yueqiaoli@gmail.com (Q. Yue), liujifeng111@gmail.com (J. Liu).

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duplex to target/aptamer complex switching in the presence of thrombin, the colorimetric or SERS sensor based on the conformational change of a single-stranded aptamer to the target-induced quadruplex structure, and the sandwich-based biosensor using aptamer-functionalized gold nanoparticles based on the multiple binding sites of thrombin for DNA aptamers. For the aptasensors there have been a few methods reported to determine thrombin in biological samples (Cho et al., 2008; Hu et al., 2009; Zheng et al., 2007). Furthermore, signal amplification was seldom used especially for fluorescence relative techniques (Wang and Liu, 2009).

Biomaterial–nanoparticle hybrid systems based on a nanoparticle-amplified technique are extensively used in different bioanalytical applications (Niemeyer, 2001). Metallic nanoparticles such as Au nanoparticles (Liao et al., 2012; Pavlov et al., 2004), semiconductor nanoparticles including silica nanoparticles (SNPs) (Deng et al., 2012), quantum dots (Dong et al., 2010) and magnetic nanoparticles (Jie and Yuan, 2012; Shen et al., 2013; Yue et al., 2013) have been employed to substantially improve the performance and sensitivity of various biosensing systems for the absorbance, molecularly imprint, capillary column, electroluminescence, fluorescence and fluorescence polarizatioin assays, respectively.

To achieve a high selectivity and sensitivity, we take advantage of the specificity of aptasensor and the signal amplification of the separation of aptamer-functionalized SNPs to develop a sandwich assay for optical detection of thrombin in biological media. SNPs are selected as the enhancement probe due to the high density of silica, which facilitates easy separation of SNPs during the synthesis, modification and detection steps through centrifugation. Moreover, SNPs have been proven to be a biocompatible and versatile substrate for probe immobilization (Wu et al., 2009). In this work, the fluorescence polarization (FP) technique was employed for the detection of thrombin based on the specific binding of DNA and thrombin and the signal amplification effect of SNPs. The SNPs-enhanced aptasensor can detect 0.2 nM thrombin with good selectivity and shows high anti-interference in human serum as well as in plasma samples. The present biosensing system was finished in homogeneous media without the separation of bound or unbound analyte due to the unique properties of FP and was therefore fast, simple, and accurate (Checovich et al., 1995).

2. Experimental section

2.1. Apparatus

All FP measurements were carried on a LS 55 spectrofluorometer (Perkin Elmer, USA) equipped with a xenon lamp excitation source and a Hamamatsu (Japan) 928 PMT, using a 90° angle detection for solution samples and a 1.0 cm length sample cell used as a sample carrier. The absorption spectra of aptamers were measured using a Lambda 25 spectrophotometer (Perkin Elmer, USA). The circular dichroism (CD) measurements were obtained on a J-810 CD spectrometer (Jasco, Japan). The CD spectra displayed were the average of five repeated scans recorded from 200 to 310 nm in a 1 cm-path length quartz cell at a scanning rate of 50 nm/min. Atomic fluorescence measurements were performed on a PF6 atomic fluorescence spectrophotometer (AFS) (Persee, China). All pH measurements were made with a pHs-3C digital pH meter (Shanghai Leici Device Works, China) with a combined glass-calomel electrode.

2.2. Chemicals and reagents

About 30, 50 and 100 nm sized SNPs coated with streptavidin were purchased commercially (Micromod, GmbH, Germany). Human α-thrombin, albumine egg (EA), lysozyme (Lys), L-tyrosine (Tyr), L-phenylalanine (Phen), human serum albumin (HSA), L-tryptophan (Trp), bovine albumin V (BSA), bovine hemoglbin G (BHG), and glucose oxidase (GOD) were ordered from Sigma. The TBAs used in this study have the following sequences: 5′-biotin-GGTTGGTGTGGTTGG-3′ (biotin-TBA1) used to be modified on the surface of SNPs, and 5′-FAM-AGTCCGTGGTAGGGCAGGTTGGGGT-GACT-3′ (FAM-TBA2) used to supply fluorescence signal as well as to bind thrombin were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. The DNA aptamers were put into 95 °C water bath for 10 min and then cooled to room temperature before using for the preparation of stock solutions. Reagents used for buffer preparation such as Tris (hydroxymethyl)-aminomethane (Tris) and HEPES were purchased from Fluka (Buchs, Switzerland). The composition of the buffer used for the experiment is reported as 10 mM HEPES, pH 6.0, 10 mM KCl and 1 mM $MgCl_2$. KCl, $MgCl_2$, NaH_2PO_4, Na_2HPO_4, NaOAc, HOAc, $Na_2B_2O_7$ and other common metal salts used were obtained from Beijing Chemical Works (Beijing, China). All chemicals were commercial products and used as received without further purification. Milli-Q water ($\geq$ 18.2 MΩ) was used to prepare all stock solutions and buffer solutions used in the assay.

2.3. Procedures for thrombin sensing

About 5.0 μg ml^{-1} SNPs coated with streptavidin was treated directly in 10 mM HEPES solution by dilution from stock solution to 2.0 ml. 10 nM biotin-TBA1 was added to 1.0 ml SNPs dispersion in thrombin reaction buffer (200 μl). And then the dispersion was incubated for about 20 min at room temperature to form composite of SNPs and DNA linkers (denoted as SNPs@TBA1). Subsequently, FAM-TBA2 was added to the system of SNPs@TBA1. Finally, human α-thrombin (0.1 μM) with various volumes were added to the mixture of SNPs@TBA1 and FAM-TBA2 system to yield final thrombin concentrations of 0.6–100 nM. The resulting mixtures were incubated for 20 min at 37 °C and then used for the fluorescence polarization measurement. Parallel experiments were conducted using EA, Lys, Tyr, Phen, HSA, Trp, BSA, BHG, and GOD as interferences in buffer solution under the same experimental conditions.

2.4. Preparation of plasma samples

Standard solutions of thrombin were added to plasma to test the performance of the sensor in complex matrixes. The samples should be pretreated before determination since the addition of thrombin to plasma, which contains all the proteins involved in the coagulation cascade including fibrinogen, leads to the formation of fibrin and to rapid sample clotting. To avoid the coagulation of the sample, fibrinogen should be precipitated from plasma before the addition of thrombin in the preparation of spiked samples.

The selective precipitation of the plasma sample was carried out based on the use of $(NH_4)_2SO_4$ as precipitant referred the prior report (Bini et al., 2007), 1.25 ml of 2 M $(NH_4)_2SO_4$ and 1.0 ml of 0.1 M NaCl were added to 0.25 ml plasma solution, after suspension of 10 min, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was eluted in a NAP column for rapid desalting and buffer exchange. For the raw plasma and the eluted solution, the content of protein was evaluated by spectrophotometric measurements at 280 nm, and the loss of protein content was detected after precipitation of fibrinogen.

3. Results and discussion

3.1. Principle of the SNPs-enhanced FP sensor for thrombin

The thrombin sensor was constructed on the basis of aptamer-based fluorescence polarization, as shown in Scheme 1. The