Biosensors and Bioelectronics 47 (2013) 265–270

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

# Aptamer/thrombin/aptamer-AuNPs sandwich enhanced surface plasmon resonance sensor for the detection of subnanomolar thrombin

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ARTICLE INFO

*Article history:*
Received 4 December 2012
Received in revised form
17 January 2013
Accepted 4 February 2013
Available online 20 March 2013

*Keywords:*
Surface plasmon resonance
Aptamer
Thrombin
Gold nanoparticles
Sandwich assay

ABSTRACT

A sensitive and selective aptamer/thrombin/aptamer-AuNPs sandwich enhanced surface plasmon resonance (SPR) sensor has been developed for real-time detection of subnanomolar thrombin. In this protocol, one thiol-modified thrombin aptamer (TBA29) was immobilized on gold nanoparticles (AuNPs) via Au–S bonding. The other biotinylated thrombin aptamer (TBA15) was grafted onto streptavidin pretreated SPR gold film through biotin–streptavidin recognition. The presence of thrombin would then induce the formation of a double aptamer sandwich structure on the SPR gold film and results in obvious enhancement of SPR signal, which was proportional to the concentration of thrombin. This proposed assay took advantage of sandwich binding of two affinity aptamers for increased specificity, AuNPs for signal enhancement, as well as SPR signal readout for real-time detection. The SPR signal had a good linear relationship with thrombin concentration in the range of 0.1–75 nM, and the detection limit for thrombin was determined to be as low as 0.1 nM. It was found that aptamer functionalized AuNPs enhanced the signal of SPR response and thus increased the limit of detection 4-fold and 5-fold compared to direct detection format without AuNPs. This sensor also showed good selectivity for thrombin without being affected by some other proteins, such as BSA and lysozyme. Furthermore, this proposed SPR sensing platform was successfully applied to thrombin analysis in diluted human serum samples.



## 1. Introduction

Thrombin is the main effector protease of the coagulation cascade, which converts circulating fibrinogen to fibrin monomer, and then polymerizes to form fibrin, the fibrous matrix of blood clots (Coughlin, 2000). Thrombin also can act as a hormone to regulate platelet aggregation, endothelial cell activation, and other important responses in vascular biology (Coughlin, 2000). Under normal conditions, the concentration of thrombin in blood varies from nM to low μM level during the coagulation progress (Arai et al., 2006). Therefore, it is very important to develop a sensor toward thrombin detection with high sensitivity and selectivity for research and also clinical diagnosis applications.

Aptamers are artificial single-strand DNA or RNA sequences (more recently, peptides) that fold into secondary and tertiary structures making them bind to certain targets (small molecules, nucleic acids, proteins, and even entire cells) with extremely high affinity and specificity, which are selected with a combinatorial method called systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Iliuk et al., 2011; Robertson and Joyce, 1990; Tuerk and Gold, 1990). Compared to traditional recognition element antibody, aptamers have a number of unique features, such as minimal immunogenicity, synthesis convenience, ease of chemical modification, and structural stability and flexibility, which make them an ideal alternative candidate for developing aptamer sensors (aptasensors) (Iliuk et al., 2011; Liu et al., 2009). To date, two thrombin aptamers have been widely used, one is a 15-mer DNA aptamer (denoted as TBA15) binding to the fibrinogen-recognition exosite of thrombin with a $K_d$ around 100 nM (Bock et al., 1992), and the other one is a 29-mer DNA aptamer (denoted as TBA29) bound to the heparin-binding exosite of thrombin with higher affinity ($K_d$ = 0.5 nM) (Tasset et al., 1997).

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0956-5663/$ - see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bios.2013.02.004

Recently, various aptasensors have been reported for the detection of thrombin by using different signal readout assays including electrochemistry (Centi et al., 2007; He et al., 2007; Li et al., 2008; Wang et al., 2011b; Xiao et al., 2005; Zhang et al., 2009), piezoelectric (Bini et al., 2007), quartz crystal microbalance (QCM) (Chen et al., 2010), colorimetry (Pavlov et al., 2004; Wang et al., 2008; Zhao and Wang, 2012), fluorescence (Huang et al., 2010), and surface enhanced Raman scattering (SERS) assays (Wang et al., 2007b). Among them, SPR aptasensor has been reported for the detection of thrombin and has attracted more and more interest (Kim et al., 2009; Lee et al., 2008; Li et al., 2007; Mani et al., 2011; Tombelli et al., 2005).

SPR is a powerful analytical technique for label-free and real-time detection of chemical and biological species and biomolecules interaction analysis (Homola, 2008). SPR sensors measure the change in refractive index of the solvent near the surface that occurs during complex formation or dissociation (Rich and Myszka, 2000). However, SPR assays generally suffer from low signal intensity and nonspecific binding, resulting in low assay sensitivity and poor selectivity. Accordingly, compared to the direct detection format by using only one recognition element to capture and label the target molecules, the sandwich assay format offers high sensitivity and high selectivity.

Recent technological developments mostly focus on the application of AuNPs-based strategies to further enhance the SPR response (He et al., 2000; Lyon et al., 1998). Many AuNPs enhanced SPR sensors have been developed for the detection of protein (Lyon et al., 1998; Wang et al., 2009b), nucleic acid (He et al., 2000; Wang et al., 2007a; Yao et al., 2006) and small molecules (Golub et al., 2009; Mitchell and Lowe, 2009; Mitchell et al., 2005; Wang et al., 2009a, 2011a; Wang and Zhou, 2008). AuNPs-based SPR aptasensor combined with sandwich assays has been demonstrated for protein detection to improve selectivity and sensitivity (Fernández et al., 2012; Kwon et al., 2012; Wang et al., 2009b), which generally used a pair of antibodies, or one aptamer and one antibody together.

In the present study, a new method based on AuNPs enhanced SPR aptasensor for the real-time detection of thrombin was proposed. A sandwich format was easily fabricated with aptamer/thrombin/aptamer-AuNPs system on SPR sensor based on the fact that thrombin can simultaneously bind to different aptamers at two exosites. During the sensing process, biotinylated TBA15 was immobilized onto the streptavidin coated sensor chip. In the presence of thrombin, the TBA29-AuNPs could further bind to thrombin and form a sandwich sensing system on the sensor chip. Taking advantages of the amplifying effect of Au nanoparticles and the specificity of aptamer, a sensitive and selective SPR sensor for real-time detection of thrombin was fabricated. This method was successfully applied to detect thrombin in diluted human serum samples. With more aptamers being selected for their target proteins, we envision this strategy would be holding great potential to be used as a simple, sensitive, selective, and universal platform for the detection of other proteins.

## 2. Experimental

### 2.1. Materials

Human thrombin (2777 NIH unit/mg, MW 37.4 kDa), tris(hydroxymethyl)aminomethane (Tris), lysozyme from chicken egg, tris(2-carboxyethyl)phosphine (TCEP), and AuNPs (10 nm diameter, OD 1, stabilized suspension in citrate buffer) were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Roche Diagnostics (Mannheim, Germany). Labeled DNA oligonucleotides were synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China), and purified by HPLC. The sequences of three employed oligomers are given below:

*TBA15*: 5′-Biotin-$(CH_2)_6$-TTT TTG GTT GGT GTG GTT GG-3′
*TBA15C*: 5′-Biotin-$(CH_2)_6$-TTT TTG GTG GTG GTT GTG GT-3′
*TBA29*: 5′-SH-$(CH_2)_6$-TTT TTT TTT TTT TTT AGT CCG TGG TAG GGC AGG TGG GGG TGA CT-3′

All reagents were of analytical grade and used without further purification. Deionized water was used for the preparation of aqueous solution.

### 2.2. Preparation of TBA29-functionalized AuNPs (TBA29-AuNPs)

AuNPs modified by TBA29 were prepared according to the literature with some modifications (Liu and Lu, 2006). Briefly, 9 μL of 1 mM TBA29 was pretreated with 1.5 μL of 10 mM TCEP and 1 μL of 500 mM acetate buffer (pH 5.2) for 1 h. 3 mL of AuNPs was transferred to the NaOH-treated glass vials, and then the TECP-treated TBA29 was added, followed by incubation at room temperature in dark for 16 h. 30 μL of 500 mM Tris acetate (pH 8.2) and 300 μL of 1 M NaCl were slowly added into the AuNPs solution. The mixture was aged for an additional 24 h at room temperature in dark. Centrifuge the TBA29-AuNPs at 16,000*g* at room temperature for 25 min twice to remove the excess TBA29. At last, TBA29-AuNPs were dispersed in 25 mM Tris acetate (pH 8.2) containing 300 mM NaCl and then stored at 4 °C before use.

### 2.3. SPR apparatus

SPR experiments were performed on a two-channel Biacore X™ apparatus and SA chips with streptavidin covalently immobilized on a carboxymethylated dextran matrix (Biacore, Uppsala, Sweden). Running buffer was HBS-EP (pH 7.4), which contains 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) Surfactant P20 (Biacore, Uppsala, Sweden). With this Biacore technology, the SPR angle change is reported as resonance units (RU), where 1000 RU corresponds to an angle change of 0.1 degree. For most of the proteins, binding of 1 ng/mm$^2$ of protein at the dextran surface is required to cause a signal change of 1000 RU (Wilson, 2002). All buffers for experiments were filtered (0.22 μm) and degassed before use. All experiments were conducted at a sample flow rate of 5 μL/min and at an operating temperature of 25 °C. The SPR response obtained from the detection channel (Fc2) was normalized by subtracting the signal simultaneously acquired from the control channel (Fc1), which could eliminate nonspecific binding and buffer-induced bulk refractive index changes.

### 2.4. TBA15 immobilization

TBA15 was immobilized on the Fc2 of SA chip according to the literature with some modifications (Zhang et al., 2012). Briefly, the SA chip was firstly cleaned with three consecutive 1 min injections of a solution of 1 M NaCl in 50 mM NaOH before the immobilization. TBA15 (1 μM in HBS-EP) was injected into the Fc2 of the SA chip for 10 min. The sensor chip was subsequently washed with 5 μL of 50 mM NaOH to remove all solutes from the flow cell except TBA15 binding to the SA chip by biotin–streptavidin system. Fc1 was used as the background control. The resulting chip was used as a sensing surface for detecting thrombin.

### 2.5. In situ SPR measurement

The fabrication of sandwich format consists of two steps. Firstly, various concentrations of thrombin in HBS-EP were injected into both Fc1 and Fc2 on the immobilized SA chip followed by washing SPR cells