Biosensors and Bioelectronics 34 (2012) 232–237

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

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

An aptamer-capture based chromogenic assay for thrombin

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ARTICLE INFO

Article history:
Received 5 December 2011
Received in revised form 3 February 2012
Accepted 6 February 2012
Available online 15 February 2012

Keywords:
Aptamer
Thrombin
Magnetic beads
Enzyme reaction
Chromogenic substrate

ABSTRACT

A simple chromogenic assay for human alpha thrombin is developed through aptamer affinity capture and a subsequent enzyme reaction. Thrombin is captured on the aptamer-modified magnetic beads, and catalyzes the conversion of chromogenic substrates to optically measured products. The measurement of the generated products by an absorbance spectrometer allows for the final quantification of thrombin. This assay shows high sensitivity by taking advantage of sample enrichment and enzyme amplification, and exhibits good specificity by involving the selective aptamer binding and the specific enzyme reaction. A concentration detection limit of 40 fM can be reached when the tripeptide substrate of tosyl-Gly-Pro-Arg-p-nitroanilide is used in a 24 h enzyme reaction, and the use of 2 h enzyme reaction in the assay enables the detection of 400 fM thrombin for a rapid analysis. This assay can be applied to detect thrombin in dilute human serum.

1. Introduction

As affinity ligands, aptamers can specifically bind to targets ranging from small molecules to proteins and cells, showing advantages over antibodies such as good stability, ease of production, and facile modification for labeling and immobilization. (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Jayasena, 1999; Klussmann, 2006) Therefore, the aptamer-based assays and biosensors have attracted increasing interests since the discovery of aptamers. (Tombelli et al., 2005; Hamula et al., 2006; Mairal et al., 2008; Song et al., 2008; Cho et al., 2009; Liu et al., 2009; Sassolas et al., 2011)

Human α-thrombin is a trypsin-like serine protease, can selectively cleave the peptide substrate at Arg residues. (Maragoudakis and Tsopanoglou, 2009) It plays important roles in blood coagulation, inflammation, angiogenesis, and tumor biology, and can be used as a therapeutic and a biomarker for diagnosis of some diseases, such as pulmonary metastasis, diseases associated with coagulation abnormalities, and synovial inflammation. (Maragoudakis and Tsopanoglou, 2009; Kitamoto et al., 2008) A sensitive and specific assay for thrombin is desired. Numerous assays and biosensors have been developed for thrombin using aptamers, by detecting electrochemical signals, optical signals, and etc. (Tombelli et al., 2005; Hamula et al., 2006; Mairal et al., 2008; Pai et al., 2008; Song et al., 2008; Cho et al., 2009; Liu et al., 2009; McMullan et al., 2011; Zhao et al., 2011a) The widely used aptamers for thrombin include a 15-mer DNA aptamer (denoted as Apt15) binding to the fibrinogen recognition site of thrombin (Bock et al., 1992) and a 29-mer DNA aptamer (denoted as Apt29) interacting with the heparin-recognition site of thrombin. (Tasset et al., 1997) Aptamer-based sandwich assays have been demonstrated for thrombin detection to improve selectivity and sensitivity, (Centi et al., 2007; He et al., 2007; Li et al., 2007; Song et al., 2008; Cho et al., 2009; Liu et al., 2009; Zhao et al., 2009, 2011a) but these assays need a pair of aptamers, or one aptamer and one antibody, and labeling of the reporter ligands.

The binding-sites on human α-thrombin of these two aptamers (Apt15 and Apt29) are away from the active site of thrombin, so the binding of the aptamers does not significantly affect the proteolytic activity of thrombin toward small-molecule peptide substrates. (Wu et al., 1992; Liu et al., 2002; Petrera et al., 2009; Dobrovolsky et al., 2009; Zhou et al., 2010; Mazurov et al., 2011) Taking advantage of the activity of thrombin toward these small-molecule substrates, aptamer-capture based assay can be developed for the detection of thrombin. Mir et al. demonstrated that it was possible to detect captured thrombin by measuring the thrombin reaction products, though the reported sensitivity was low. (Mir et al., 2006) Centi et al. reported the detection of thrombin using aptamers and magnetic beads by electrochemically measuring the enzymatic product of thrombin, and the reported detection limit was about 175 nM. (Centi et al., 2008) As the immuno-affinity capture assays for enzymes used fluorogenic substrate to achieve high sensitivity (Bagramyan et al., 2008), aptamer-capture based fluorogenic assays enabled a sensitive detection of thrombin in a wide range by using the aptamer-captured thrombin to convert fluorogenic substrates to fluorescent product (Muller et al., 2011; Zhao et al., 2011b), and the detection of 2 fM thrombin could be achieved (Zhao et al.,

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doi:10.1016/j.bios.2012.02.009

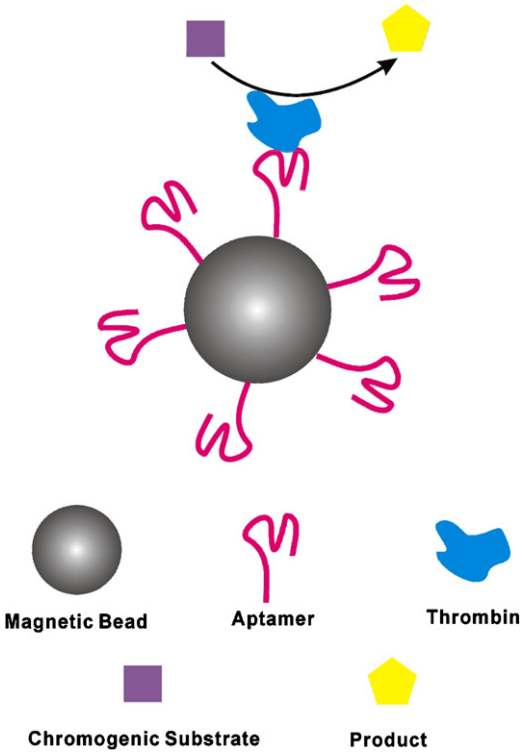

Fig. 1. Schematic diagram shows the principle of the aptamer-capture based chromogenic assay for human α-thrombin. Thrombin is selectively captured on the aptamer-modified magnetic beads, and the enriched thrombin catalyzes the conversion of the chromogenic substrate to the product. The optical absorbance measurement of the generated product enables the quantification of thrombin.

2011b), but the fluorogenic assay required a relatively expensive instrument for the fluorescence measurement.

Herein, we describe an aptamer-capture based assay for human α-thrombin by using the aptamer-modified magnetic beads and the chromogenic substrates of thrombin. Fig. 1 shows the principle of this assay. The aptamer recognizing thrombin is modified on the magnetic beads as a capture ligand. Thrombin is specifically captured by the aptamer on the magnetic beads, and separated from sample mixture. The captured thrombin catalyzes the hydrolysis of the chromogenic peptide substrate with p-nitroanilide, generating the product of p-nitroaniline, and then the absorbance of the p-nitroaniline is measured by a spectrometer to achieve the determination of thrombin. Taking advantage of the sample preconcentration by the aptamer-modified magnetic beads and the enzymatic amplification, a detection limit of 40 fM can be reached when a 24 h enzyme reaction is applied. The involvement of the selective aptamer capture and the specific enzymatic reaction enables a high specificity of the assay. This aptamer-based chromogenic assay is simple and easy to operate. In addition, the visual color change of the enzyme reaction solution allows for an instrument-free colorimetric detection for the semi-quantitative measurement of thrombin.

2. Experimental

2.1. Chemicals and apparatus

Human transferrin, bovine serum albumin (BSA), hemoglobin from bovine blood, and trypsin (from bovine pancreas) were purchased from Sigma. Protease K was ordered from Merck. Porcine pancreatic elastase (PPE) and chymotrypsin were obtained from Ruibio. Human α-thrombin, human β-thrombin, and human γ-thrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). The chromogenic substrates of thrombin: N-P-tosyl-Gly-Pro-Arg-p-nitroanilide acetate (Product No. T1637; denoted as T1637 in this work) and β-Ala-Gly-Arg p-nitroanilide diacetate (Product No. T3068; denoted as T3068 in this work) were obtained from Sigma. Pooled human serum was obtained from Zhongke Chenyu Biotechnology in Beijing. The biotinlyated 15-mer DNA aptamer (Apt15) and 29-mer DNA aptamer (Apt29) against human α-thrombin had the following sequences: 5′-biotin-GGT TGG TGT GGT TGG-3′ and 5′-biotin-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′, respectively. One 36-mer DNA oligo with biotin label as a control DNA had the following sequence: 5′-biotin-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′. The other DNA oligo control was poly A36 with a biotin label at 5′ end. All DNA oligos were synthesized and purified by Sangon Biotech (Shanghai, China). Streptavidin coated magnetic beads were purchased from New England Biolabs (2 μm in diameter; Product No. S1421s). Solvents and other reagents were in analytical grade. The following buffers A and B were used. Buffer A contained 50 mM Tris–HCl, 2 M NaCl and 0.1% Tween 20 (pH 7.4). Buffer B contained 20 mM Tris–HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (pH 7.4). A UV–vis spectrophotometer (HITACHI U3010) was used to record the absorbance signals.

2.2. Preparation of the aptamer-modified magnetic beads

Streptavidin-coated magnetic beads (250 μL, 4 mg/mL) were added into a 0.5 mL centrifuge tube and separated from the solution by using a magnetic separator. The collected beads were washed once with 100 μL of buffer A and redispersed in 100 μL of buffer A. Biotinlyated aptamers at 7 μM (100 μL) were heated at 90 °C for 3 min, cooled at room temperature, and then was added to the above beads suspensions. The mixture of the aptamers and magnetic beads incubated for 2 h under gently shaking. The magnetic beads were separated from the unreacted reagents by the magnetic separator, and the reaction solution was collected for the UV measurement of the concentration of the unreacted aptamer. The obtained aptamer-modified magnetic beads were washed with buffer B containing 0.1% Tween 20 three times. The aptamer-modified magnetic beads were finally redispersed in 100 μL of buffer B containing 0.1% Tween 20, and stored at 4 °C prior to use. By measuring the UV absorbance at 260 nm the concentration of the aptamer solution could be determined. The amount of the aptamers attached on the magnetic beads was estimated by measuring the decrease of aptamer concentration in the aptamer solution after the aptamer immobilization. The total amount of the Apt15 attached on the magnetic beads (1 mg) in 100 μL of final solution was about 440 pmol. The total amount of the Apt29 on the magnetic beads (1 mg) in 100 μL of final solution was about 300 pmol. The biotinlyated DNA controls were modified on the magnetic beads by a similar procedure.

2.3. Procedure of the aptamer-capture based assay for human α-thrombin

For the 5 μL target sample analysis, 45 μL of buffer B containing 1 mg/mL BSA, 5 μL of human α-thrombin at different concentrations, and 1 μL of the Apt15-modified magnetic beads (or the Apt 29-modified magnetic beads) were pipetted into a 0.5 mL centrifuge tube, and the mixture was incubated on a shaker at room temperature for 30 min. The beads were magnetically separated and rinsed three times with 50 μL of wash solution (buffer B containing 0.1% Tween 20). Next, the magnetic beads were redispersed in 20 μL of buffer B containing the chromogenic substrate (1.8 mM β-Ala-Gly-Arg p-nitroanilide or 0.28 mM tosyl-Gly-Pro-Arg-p-nitroanilide), and the mixture was incubated at 37 °C for