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Fluorescent sensing of thrombin using a magnetic nano-platform with aptamer-target-aptamer sandwich and fluorescent silica nanoprobe

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ABSTRACT

A method for fluorescent sensing of thrombin was developed by using fluorescent silica nanoparticles as the fluorescence signal probes and magnetic beads as target enriching platform. RuBpy-doped silica nanoparticles are highly photostable and provide significant fluorescent signal amplification as compared with single dye molecules. TBA1 is a 15-mer DNA aptamer which binds exosite I of thrombin (Fibrinogen Binding Site), while TBA2 is a 29-mer DNA aptamer binding to exosite II of thrombin (Heparin Binding Domain). In this work, 15-mer thrombin-binding aptamer (15-A) was immobilized onto the surface of RuBpy-doped silica nanoparticles, and 29-mer thrombin-binding aptamer (29-A) was immobilized onto the surface of magnetic beads, and sandwich aptamer complexes were formed upon the addition of target thrombin molecules with the two kinds of aptamer-modified particles, and therefore quantitative analysis of thrombin was performed by detecting the fluorescence intensity of the complexes after magnetic separation of the targets. The results show that the detection limit of thrombin is 0.70 nM, and this method has some advantages such as speed, high sensitivity, high specificity and low cost.

1. Introduction

Aptamers are a piece of RNA or DNA which can specifically conjugate to some target molecule such as small molecules, protein, nucleic acid, cells, microorganism and so on [1–7]. In addition, they have every high specificity and affinity [8,9]. Aptamer is selected from the plenty of nucleic acid database in vitro by the procedure of systematic evolution of ligands by exponential enrichment (SELEX) [10]. Compared with antibody, aptamer has the advantages of low-cost, easy to be separated, easy to be modified, high affinity, reversible denaturation and so on.

Thrombin is a kind of multifunctional serine protease which can change fibrinogen to fibrous protein [11,12]. Besides the function of coagulation and anticoagulation, thrombin also plays a key role in the disease of atherosis, apoplexy and so on. So it is significant to detect the number of thrombin which may become indication of the disease [13,14]. The aptamers for thrombin have been widely researched [15]. Up to now, there are two kinds of aptamers which both can form G-quadruplex with α-thrombins in different sites [16]. One aptamer (TBA1) is a 15-mer DNA aptamer which binds exosite I of thrombin (Fibrinogen Binding Site), while the other aptamer (TBA2) is a 29-mer DNA aptamer binding to exosite II of thrombin (Heparin Binding Domain), and their dissociation constants are 100 nM and 0.5 nM [16–18], respectively.

RuBpy-doped silica nanoparticles (RSiNPs) are highly photostable and provide significant fluorescent signal amplification as compared with single dye molecules of RuBpy. In addition, the silica matrix can provide a low polarity and limited environment which can decrease the nonradiative decay and increase the quantum yield of the dye molecules [19,20]. Moreover, they have the advantages of good dispersibility, easy synthesis, facile modification and low cost [21–28]. Herein we attempt to construct a detection approach for thrombin by using fluorescent silica nanoparticles as the fluorescence signal probes and magnetic sphere as the carrier platform.

2. Material and methods

2.1. Chemicals and materials

Tris (2,2′-bipyridyl) Ruthenium(II) Chloride Hexahydrate (RuBpy), Triton X-100, n-hexanol, cyclohexane, tetraethylorthosilicate (TEOS), ammonium hydroxide (NH_4OH, 25–28 wt%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide ehydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), bisbenzimide (hoechst33258) were purchased from Sigma Chemical Co.(St.

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Louis. MO). Dynabeads® MyOne™ Carboxylic Acid (1 μm) was purchased from Invitrogen. Carboxyethlsilanetriol sodium salt (CEOS) was purchased from ABCR Chemical Co. (Germany). The DNA oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology & Services Co. (China), and have the following sequences: 29-A: 5'-NH_2-TEG linker-AGT CCG TGG TAG GGC AGG TTG GGG TGACT-3' 15-A: 5′-biotin-TEG linker-GGT TGG TGT GGT TGG-3′ Complementary strand of 15-A: 5′-CCAAC-CACACCAACC-3′.

2.2. Instrumentation

The size and uniformity of carboxyl-modified silica NPs were measured with a transmission electron microscope (JEOL, JEM100CXII, Japan). The modified carboxyl on the surface of NPs was confirmed using an IR spectrometer (FT-IR, Nicolet-5700, USA). Fluorescence spectra and fluorescence intensity were measured using a fluorescence spectrophotometer (Hitachi, F-4600, Japan) equipped with a 150 W xenon lamp.

2.3. Synthesis of carboxyl-modified silica nanoparticles

RuBpy doped carboxyl-modified silica nanoparticles were synthesized using the water-in-oil (W/O) reverse microemulsion method [23,29,30]. The microemulsion consisted of a mixture of 1.77 mL of Triton X-100, 7.5 mL of cyclohexane, 1.6 ml of n-hexanol, 400 μL of water, 80 μL of 0.1 M RuBpy aqueous solution and 100 μL of TEOS that was stirred for 30 min at room temperature, and then 60 μL of NH_4OH was added. The aqueous ammonia served both as a reactant (H_2O) and a catalyst (NH_3) for the hydrolysis of TEOS. The reaction was allowed to continue for 24 h at room temperature, then postcoating of carboxyl-modified silica was performed by adding another 50 μL of TEOS and 50 μL of CEOS. This polymerization was allowed to proceed for 24 h. After the reaction completed, the nanoparticles were isolated by acetone, followed by centrifuging and washing with ethanol three times and deionized water one time. The NPs were finally resuspended in 4 mL deionized water and stored at 4 °C until use.

2.4. Immobilization of aptamer (15-A) onto silica NPs

500 μL of silica NPs (~8 mg) were reacted with 50 μL of 1 mg/mL avidin in the presence of 3 mg EDC and 5 mg NHS in 1 mL of 0.01 M PBS (pH 7.2) for 4 h at room temperature with continuous shaking. Afterward, silica NPs were separated and washed three times by centrifugation. Then, avidin-conjugated silica NPs were incubated with 30 μL of 10 μM 15-A for another 2 h in PBS. Finally the resultants were washed three times and stored at 4 °C in Tris–HCl (pH 7.4, 20 mM Tris)for further use.

2.5. Quantitative detection of aptamer (15-A) on the silica NPs surface

20 μL of 10 μM complementary strand of 15-A and different concentrations of standard 15-A (ranging from 0 to 0.16 μM) were added to the hybridization solution (0.01 M PBS, pH 7.2) to keep the final volume at 500 μL in each solution. After incubating for 60 min at 60 °C, 50 μL of aqueous solutions of 2 μM hoechst33258 were added into the solution. The fluorescence was measured at λex/λem=360 nm/450 nm with excitation and emission slits 10 nm and 5 nm, respectively. The fluorescence intensity was plotted as a function of the concentrations of 15-A. The number of immobilized 15-A on the silica NPs can be quantitatively calculated from the regression equation of standard 15-A.

2.6. Immobilization of aptamer (29-A) onto magnetic beads

The carboxylated magnetic beads were functionalized with 29-A according to the procedure from the manufacturer. Firstly, 2 mg of magnetic beads were washed twice in MES (100 mM, pH 5), and then incubated with 3.5 nmol 29-A in the presence of 1 mg EDC in 100 μL 100 mM MES overnight at room temperature. Afterward, the aptarmer modified magnetic beads were incubated for 30 min in 500 μL Tris–HCl (250 mL of 1 M pH 8.0 Tris and 1 mL 10% Tween-20, diluted to 1 L with purified water) and washed three times. Finally the resultants were stored in 500 μL Tris–HCl (50 mM Tris–HCl, pH 7.4) at 4 °C for further use.

2.7. Quantitative detection of aptamer (29-A) on the surface of magnetic beads

Quantitative detection of aptamer was based on the fact that DNA molecules exhibits maximum absorption in the wavelength of 260 nm. Different concentrations of standard 29-A (ranging from 0 to 1.4 μM) were added to the solution (100 mM MES, pH 5.0) to keep the final volume at 500 μL in each solution. The absorption intensity was plotted as a function of the concentrations of 29-A. The number of immobilized 29-A on the magnetic beads can be quantitatively calculated from the regression equation of standard 29-A by detecting the absorption of DNA from the collected supernatant indirectly.

2.8. Hybridization procedure and detection of thrombin

20 μL of 15-A conjugated silica NPs (~0.32 mg), 6 μL of 29-A conjugated magnetic beads (~24 μg) and different concentrations of thrombin were incubated in Tris–HCl (pH 7.4, 20 mM Tris, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/mL)at room temperature for 2 h with continuous shaking. Then the suspension was put onto a magnetic scaffold and washed three times in Tris–HCl (pH 7.4, 20 mM Tris, 140 mM NaCl, 5 mM KCl, 0.1% Tween). Finally, they were resuspended in 100 μL of Tris–HCl (pH 7.4, 20 mM Tris). The fluorescence was measured at λex/λem=450 nm/605 nm with excitation and emission slits set at 10 nm and 5 nm, respectively. Four major factors (reaction temperature, reaction time, K^+ concentration, concentrations of 15-A conjugated silica NPs dosage and 29-A conjugated magnetic beads) were optimized, and each factor had three levels (Data not shown). Signal-to-background ratio (S/B) was selected as the evaluating index, which was calculated to be the ratio of fluorescence intensity of a sample to that of the blank. The experiment was arranged according to the orthogonal layout $L_{16}(4^5)$.

3. Results and discussion

3.1. Working principle

The principle for fluorescence sensing of thrombin is shown in Scheme 1. Firstly, 29-mer thrombin-binding aptamer (29-A) is immobilized onto the surface of magnetic beads (MB), then 15-mer thrombin-binding aptamer (15-A) is immobilized onto the surface of RuBpy-doped silica nanoparticles (SP). Finally, the sandwich aptamer complexes are formed upon the addition of target thrombin molecules with the two kinds of aptamer-modified particles, and therefore quantitative analysis of thrombin can be performed by detecting the fluorescence intensity of the complexes after magnetic separation of the targets.