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A label-free aptasensor for highly sensitive detection of ATP and thrombin based on metal-enhanced PicoGreen fluorescence

Kaiyu Wang [a,b,c,1], Jian Liao [a,b,1], Xiangyue Yang [a,b], Meng Zhao [a,b], Min Chen [a,b], Weirong Yao [c], Weihong Tan [d,e,f,*], Xiaopeng Lan [a,b,c,**]

[a] Institute of Laboratory Medicine, Fuzhou General Hospital of Nanjing Military Command, Fuzhou 350025, PR China
[b] Department of Laboratory Medicine, Dongfang Hospital, Xiamen University, Fuzhou 350025, PR China
[c] Department of Laboratory Medicine, Fuzong Clinical College, Fujian Medical University, Fuzhou 350025, PR China
[d] Center for Research at the Bio/Nano Interface, Department of Chemistry and Shands Cancer Center, University of Florida, Gainesville, Florida 32611, United States
[e] Plant Pathology Lab, North Florida Research and Education Center, University of Florida, Quincy, Florida 32351, United States
[f] Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Bio-Sensing and Chemometrics, College of Chemistry and Chemical Engineering, College of Biology, Collaborative Innovation Center for Chemistry and Molecular Medicine, Hunan University, Changsha 410082, PR China

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ABSTRACT

A label-free fluorescence aptasensor for highly selective and sensitive detection of ATP and thrombin was developed by using PicoGreen (PG) as signal molecule and surface-bound metal-enhanced fluorescence (MEF) substrates (silver island films, SIFs) as signal enhancers. On binding with ATP or thrombin, aptamers undergo structure switching, leading to a reduction of fluorescence intensity of PG. Chang of fluorescence intensity can be magnified by SIFs. The limit of detection for ATP and thrombin is 1.3 nM and 0.073 nM, respectively. The fluorescence quenching efficiency is linear in the logarithmic scale with ATP concentration range from 10 nM to 100 μM (R^2=0.995) and thrombin concentration range from 0.1 nM to 100 nM (R^2=0.997). The coefficients of variation of the intra-assay reproducibility and inter-assay reproducibility for ATP (10 μM) assay are 3.8% and 5.2%, respectively. In addition, the aptasensor is stable and can be reliably used for ATP measurement in biological samples. Overall, the aptasensor can be a useful and cost effective tool for the specific detection of ATP, thrombin and potentially other biomolecules in biological samples.

1. Introduction

Aptamers are a new type of recognition molecule composed of single-strand nucleic acid molecules (ssDNA or RNA) that fold into special 3-D structures and specifically bind to targets with high affinity (Ellington and Szostak, 1992; Tuerk and Gold, 1990). Aptamers can bind a wide range of targets, including metal ions (Liu et al., 2009), small molecules (Huizenga and Szostak, 1995), proteins (Bock et al., 1992), pathogenic microorganisms (K.Y. Wang et al., 2011) and cells (Shangguan et al., 2006). Aptamers have several advantages over antibodies due to their low molecular weight, high stability, and ease of chemical synthesis and modification (Bunka and Stockley, 2006). Therefore, aptamers have potential applications in clinical diagnostics, molecular imaging, biomarker identification and targeted therapy (Liu et al., 2013).

Multiple types of aptasensors have been developed by combining aptamers as recognition molecules with fluorescent (K.Y. Wang et al., 2011), colorimetric (Liu and Lu, 2006), electrochemical (Huang et al., 2013) and nanomaterial techniques (Medley et al., 2011). Among them, aptasensors combined with fluorescent molecules have advantages in terms of sensitivity, simplicity, speed, linear range, and homogeneity (R.E. Wang et al., 2011). The reporters used in the fluorescent aptasensor can be either labeled or label-free (Sassolas et al., 2011). Labeled fluorescent reporters are fluorescence clusters or fluorescence donors that have been chemically modified into aptamers (Huang et al., 2007). In contrast, label-free reporters are free dyes or fluorescent cationic conjugated polymers that have been selectively combined to the specific sequence or structure of the nucleic acid (Zhu et al., 2011). Aptasensors using label-free fluorescent reporters are cheaper, have simpler operation, and the potential alteration of binding

* Corresponding author. Tel.: +86 731 88821884.
** Corresponding author. Tel.: +86 591 22859490.
E-mail addresses: tan@chem.ufl.edu (W. Tan), lanxp@sina.com (X. Lan).
[1] These authors contributed equally to this work.

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profiles that sometimes occur upon fluorescent labeling can be avoided.

Cyanine dyes, extremely sensitive detection reagents for double-stranded DNA (dsDNA), have been used successfully in the development of label-free fluorescent aptasensors (Kong et al., 2013; Lv et al., 2013). We have found that the sensitivity and dynamic range of targets detected by label-free aptasensors are usually determined by the sensitivity, photostability and dynamic range of the fluorescent dye with which the aptasensor is combined (Wang and Liu, 2008). Metal-enhanced fluorescence (MEF), which involves the interaction between MEF substrates (surface-bound or free metal nanoparticles) and adjacent fluorescence clusters, has been used to increase the sensitivity and photostability of fluorescent reporters (Deng et al., 2013). As a result of this interaction between MEF substrates and adjacent fluorescence clusters, spontaneous emission rate, fluorescent quantum yields and the photostability of these fluorescence clusters increase (Dragan et al., 2010b). Currently, MEF has shown promise in biological tests, such as immunoassays (Xie et al., 2006) and single-molecule imaging (Deng et al., 2011).

In the present study, we developed a highly sensitive aptasensor by using cyanine dye PicoGreen (PG) as signal molecule and surface-bound MEF substrates (silver island films, SIFs) as signal enhancers. To the best of our knowledge, this is first time that MEF strategy was used in a label-free fluorescent aptasensor, which may potentially be used for sensitive detection of a wide range of aptamer binding targets.

2. Experimental

2.1. Materials and reagents

All the nucleic acid sequences were synthesized and purified with High Performance Liquid Chromatography (HPLC) by Invitrogen (Guangzhou, China) (ESI Table 1†). PG (200 ×) was purchased from Invitrogen (Guangzhou, China). Silver nitrate (99.9%), ammonium hydroxide (30%), D-glucose, human thrombin, mouse immunoglobulin G (IgG), lysozyme, bull serum albumin (BSA) and premium quality APS-coated glass slides (75 × 25 mm^2) were purchased from Sigma-Aldrich (St Louis, MO, USA). FBS was purchased from Hyclone (Logan, UT, USA). ATP, ADP, GTP, CTP and TTP were from Amresco (Solon, OH, USA).

2.2. Aptamer/cDNA duplex preparation

Aptamer (5 mL, 100 mM) and its complementary DNA (cDNA, 5 mL, 100 mM) were mixed in buffer (20 mL, 20 mM Tris–HCl, pH = 8.3). The nucleic acid mixture was heated in an incubator and maintained at 90 °C for 10 min, followed by slow cooling to room temperature. The hybridized aptamer/cDNA duplex was stored at −20 °C.

2.3. SIF preparation

SIFs were prepared as above (Aslan et al., 2005; Dragan et al., 2010a). In brief, the glass slides coated with amine were merged in Tollern's solution mixed with silver nitrate and ammonium hydroxide. A 4.8% (V/W) D-glucose solution was used to prevent silver nitrate from forming silver islands on the surface of the glass slides. Electric resistance was monitored in order to control the thickness and silver particle size of the SIFs. The glass slides were took out of the solution when electric resistance was 3–10 Ω cm^{-1}. In the present study, the time was 2 min.

2.4. The common method of target measurement using label-free aptasensor

To a micropore plate, 40 μL aptamer/cDNA solution (50–5000 nM bp) and 50 μL 2 × binding buffer containing different concentrations of target were added, followed by addition of 10 μL PG dye at different PG/Base ratios. The mixture was incubated at room temperature for 5–30 min. After incubation, 50 μL of the mixture was added onto the surface of glass slides with or without SIFs and covered with a coverslip. PG fluorescence intensity and spectrum of the mixture on the slides were measured with a fiber optic spectrometer (Ocean Optics, Dunedin, FL, USA). The PG on the slides was excited with a continuous laser at a wavelength of 473 nm, and emission was collected in the range of 460–690 nm.

To optimize conditions for the measurement system, different aptamer/cDNA concentration, PG/Base ratio and NaCl concentration in the binding buffer were tried. The 2 × binding buffer for measurement of ATP contained 20 mM Tris–HCl, 100 mM NaCl and 20 mM $MgCl_2$, pH 7.5. For thrombin, it contained 200 mM Tris–HCl, 100 mM NaCl, 40 mM $MgCl_2$ and 20 mM KCl, pH 8.0.

2.5. Practical analysis of label-free aptasensor

The present study chose commonly used serum and bacterial extraction solution as samples. ATP concentration in these samples was measured to evaluate the effectiveness of the method. FBS was diluted 10-fold with 2 × ATP binding buffer, and ATP was added to diluted FBS at different final concentrations to prepare mimic serum samples. To measure bacterial ATP, *Escherichia coli* (*DH5α*) culture was treated with 2.5% trichloroacetic acid (TCA), filtered with 0.22 μM membrane and diluted 10-fold with 2 × ATP binding buffer. The ATP concentration in serum and bacterial extraction was measured using the above method. In addition, ATP in bacterial extraction was measured by firefly luciferase assay using an ATP Determination Kit (Invitrogen, Guangzhou, China).

3. Results and discussion

3.1. Fabrication process of the aptasensor

The measurement system is represented in Scheme 1, which includes aptamer (red), cDNA (black), PG (heptagon), target (pentagon) and SIFs, where SIFs are the membrane materials formed by the 200-Å (radius) silver ellipsoids that covered 40% of the amino-functionalized glass surface (ESI Fig. S1†). First, the aptamer is hybridized with equal volume of its cDNA to form the aptamer/cDNA duplex, followed by addition of solution containing target molecules and PG dye. After incubation, the mixture is added to the SIFs and sandwiched between a glass slide and a coverslip. The fluorescent signal intensity is then measured. The target competitively binds to the aptamer, resulting in dissociation of aptamer/cDNA and formation of aptamer/target complex and free cDNA. The binding event causes the fluorescence intensity of the reaction system to be quenched. The amount of target molecules can be quantified by monitoring the quenching of fluorescence intensity, which is proportional to the amount of dissociated aptamer/cDNA. SIF is used to make the fluorescence of PG/DNA complex stronger and more stable, which leads to increased sensitivity. As its principle show, the aptasensor could be easily designed and enable the detection of various molecules for which an aptamer with capability of structure switching upon target binding. Furthermore, modification of aptamers and silver ellipsoids, which are necessary for the labeled fluorescent aptasensors established by core–shell nanoparticles (Li et al., 2014; Lu et al., 2014), are not needed to for our aptasensor.