Biosensors and Bioelectronics 49 (2013) 226–230

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

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

A new method for the detection of adenosine based on time-resolved fluorescence sensor

Kai Zhang [a,*], Ke Wang [a,*], Minhao Xie [a], Lan Xu [a], Xue Zhu [a], Shiyang Pan [b], Qun Zhang [b], Biao Huang [a]

[a] Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi, Jiangsu 214063, China
[b] The First Affiliated Hospital of Jiangsu Province, Nanjing, Jiangsu 210029, China

ARTICLE INFO

Article history:
Received 27 March 2013
Received in revised form
7 May 2013
Accepted 20 May 2013
Available online 25 May 2013

Keywords:
Aptasensor
Adenosine
Complex biological fluids
Time-resolved fluorescence

ABSTRACT

In this work, we report a thrombin binding aptamer complex based time-resolved fluorescence sensor for small molecule detection. The sensor employs two strands (DNA1 and DNA2) of oligonucleotides. This two strands of oligonucleotides contain two aptamer (α-aptamer and β-aptamer) respectively. DNA1 and DNA2 were labeled with biotin and DIG at the 3′-end, respectively. Binding of the α-aptamer and β-aptamer to the thrombin promotes the hybridization between the complementary stem sequences attached to the two oligonucleotide sequences. The hybridization then brings biotin to be hidden in the shield part on DNA1, shielding biotin from being approached by the streptavidin modified on the microplate due to the steric hindrance effect of the shield part of DNA1. Result in the thrombin–aptamer complex cannot be modified on the surface of microplate which further leads to no signal reported. The strategy integrates the distinguishing features of aptamer and fluorescent techniques. As a proof-of-principle, adenosine in serum was detected with a detection limit of 0.5 nM. A nice detection limit and linear relationship were obtained.

1. Introduction

Sensitive and selective detection of small molecules in their native environments plays an important role in medical diagnosis and academic research (Yang et al., 2005; Zhang et al., 2013b). Many methods for the analysis of small molecules have become indispensable tools in their characteristic studies (Lu et al., 2008). Fundamentally, assays that allow discovery of sensitive and selective detection of small molecules is of significant importance for the diagnosis, and prevention of many diseases. So, the key factors, including a highly selective small molecule recognition element and a novel signal enhancement mechanism, should be integrated for the advance of the successful assay method (Yang et al., 2005). Among many molecular recognition elements, aptamers have gained increasing attention due to its unique characteristic in this area (Ferapontova et al., 2008; Shangguan et al., 2006). These artificially selected oligonucleotides which are generated by an in vitro selection technique known as SELEX from an oligonucleotide pool with immense combinatorial random sequences are able to bind various targets (from small molecules to whole cells) with desirable selectivity, specificity, and affinity, allowing them to rival antibodies in diagnostic applications (Huang et al., 2008; Nimjee et al., 2005; Shangguan et al., 2006). Nucleic acid synthetic chemistry also facilitates conjugation of these aptamer sequences to fluorescent dyes, antibodies, or other electrochemistry labels (Liu et al., 2008; Wang et al., 2012a; Wu et al., 2012; Xuan et al., 2012; Zhang et al., 2012, 2007; Zhang and Zhang, 2012). While the selected aptamers are targeting enzymes, the aptamer-based sensors (aptasensors) also provide a promising tool for the analysis of small molecules. So the aptamers hold great promise for the biosensing of disease-related small molecules and for the development of small molecules arrays as well.

Much attention has been paid to the fluorescence method, compared to other signal enhancement mechanisms, on account of its excellent sensitivity within modulating the label emission in different types of physicochemical interactions (Mukamel et al., 2012; Qian et al., 2012; Shim et al., 2012; Wang et al., 2012b; Zhen et al., 2012). However, because of diffusion and natural fluorescence of various compounds like sensor itself or proteins in biological samples when used with complicated biological samples, conventional fluorescent dyes suffer from serious limitations of sensitivity due to the high background signal. In other words, in histological samples observations may be obscured by background fluorescence. In this context, it is highly required to develop longer lifetime fluorescence strategies for the assay of small molecules.

One of the best approaches to decrease background interference is to use time-resolved fluorescent reagents (Kim et al., 2008). Lanthanide ion compounds show highly desirable spectral

* Corresponding authors. Tel.: +86 510 85514482; fax: +86 510 85520770.
E-mail addresses: zhangkai@jsinm.org (K. Zhang), wangke@jsinm.org (K. Wang).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.05.030

characteristics, including long fluorescence emission life times (Eu^{3+} ions has a longer lifetime on the order of milliseconds) and large Stokes' shift (200–300 nm) (Conlon et al., 2008; Karhunen et al., 2011; Ouyang et al., 2011; Wu et al., 2010). These advantages allow lanthanide ion compounds to be used in time-resolved fluorescence detection, reducing the background interference from ubiquitous endogenous fluorescent components. For example, lanthanide ion compounds were successfully applied in time-resolved fluorescence assay (TRFIA) and DNA-based sensors by covalently labeling the lanthanide ion chelates on the proteins or nucleic acid molecules (Ouyang et al., 2011; Yang et al., 2005; Zhang et al., 2013a).

Here, attempting to integrate the advantages of the lanthanide ion compounds and aptamer, we report here our attempt to realize signal-on and time-resolved fluorescence assay of adenosine by employing conjugation of a Eu^{3+} complex and anti-DIG as a tag. Adenosine was chosen as a model target in this study because it was a major local regulator of tissue function (Zhang et al., 2008).

2. Experimental section

2.1. Materials and chemicals

Thrombin, adenosine, *N*-(3-dimethylaminopropyl)-*N*-ethylearbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and N^1-(*p*-isothiocyanatobenzyl) diethylenetriamine-N^1, N^2, N^3, N^4-tetraacetic acid (DTTA) were obtained from Sigma-Aldrich. The oligonucleotides used here were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd., China. 96-well microplates (300 μL per well) were obtained from Corning (Costar, Corning, New York, USA). Tris-base and other chemicals were all of analytical grade. All solutions were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead) to a specific resistance of >18 MΩ cm.

The probe sequence is as follows:

DNA1: 5′-ACCTGGGGGAGTATT*GCGGAGGAAGGT*TTTTT**GGTTGGT GTGGTTGG**TTTTTTTTTTTTTTT-DIG-3′,
DNA2: 5′-**AGTCCGTGGTAGGGCAGGTTGGGGTGACT**TTT TT*ACCTTCCTCCGC*TTT-biotin-3′
DNA3: 5′-*GCGGAGGAAGGT*TTTTT**GGTTGGTGTGGTTGG** TTTTTTTTTTTTTTT-DIG-3′

DNA1 contains the following elements: (1) the sequences with double underline and single underline is adenosine aptamer, (2) the italic sequence is a linker complementary to the italic sequence of DNA2, (3) the sequence shown in bold is α-aptamer of thrombin, and (4) the underlined crewel sequence is the shield of biotin on DNA2. DNA2 contains two functional regions: (1) the sequence shown in bold is β-aptamer of thrombin, the italic sequence is a linker complementary to the italic sequence of DNA1. DNA3, a part of DNA1, does not contain the sequence: ACCTGGGGGAGTATT (shield part of DNA1).

The sequence shown in the bold is an adenosine aptamer. The stock solutions of the probe were prepared with a final concentration of 1 μM in Tris-buffer (pH 8.0 10 mM Tris, 1 mM EDTA).

2.2. Preparation of Eu–anti-DIG

Anti-DIG was first dispersed in bicarbonate buffer (50 mM Na_2CO_3–$NaHCO_3$, pH 9.6) for 200 μL (1.2 mg mL^{-1}). After centrifuging and washing with bicarbonate buffer, 20 μL of Eu-chelate of N^1-(*p*-isothiocyanatobenzyl) diethylenetriamine-N^1, N^2, N^3, N^4-tetraacetic acid (DTTA) was added to the solution, and then the mixture was stirred at 25 °C overnight. The unbound substances were removed after centrifuging and dialysising with rinsing buffer (pH 7.8 Tris–HCl buffer with 0.9% NaCl and 0.05% NaN_3). The final product was stored at 4 °C for future use.

2.3. Preparation of streptavidin coated plate

The activation solution (200 mM EDC and 50 mM NHS) was dropped on the surface to activate the carboxyl group which is on the surface of the plate for 10 min at room temperature. The activated plate surfaces were incubated with biotin (5 mg mL^{-1} biotin–PEO–amine in 0.1 M MES buffer, PH 5.0) for 10 min. Then the plates were incubated with 1 M BSA for 2 h to block the residual activated carboxyl groups. The biotinylated plates were incubated in 5 μg mL^{-1} streptavidin (SA) in 0.1 M PBS for 10 min to immobilize the streptavidin. After six times washing, plates were stored at −20 °C before use.

2.4. Preparation of thrombin–aptamer complex

According to the previous report, due to the different K_d of α-aptamer (~100 nM) and β-aptamer (~0.5 nM) to human thrombin, the need for the concentration of α-aptamer is higher than that of β-aptamer (at the ratio of 5:1). So, 1 μM thrombin was treated with 1 μM DNA1 and 200 nM DNA2 in Tris–HCl buffer (20 mM Tris, pH 7.4, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM NaCl and 0.02% Tween-20) at 37 °C for 30 min to make the thrombin completely assemble with DNA1 and DNA2. Subsequently, the product was stored at −20 °C for future experiments.

2.5. The detection of adenosine

The 25 μL of target solution was added to the 25 μL of 100 nM thrombin–aptamer complex solution in 100 μL Tris–HCl buffer and the mixture was equilibrated at 37 °C for 30 min in streptavidin coated microplate. After being washed by washing buffer for six times, the modified plate was treated with 200 μL enhancement solution (ES) buffer (pH 3.2, 15 mM 2-naphthoyltrifluoroacetone (β-NTA), 50 mM tri-*n*-octylphosphine oxide (TOPO), and 1% Triton X-100 (v/v)) for 5 min before fluorescence assay.

Eu^{3+} is labeled by the chelate (DTTA) which has a strong binding ability on the anti-DIG. After specific binding reaction between DIG and anti-DIG has been performed and the non-bound fraction of the Eu–anti-DIG has been efficiently washed away, Eu^{3+} may be dissociated and released from the lanthanide chelate label, which then enter an enhancement solution and micelle environment in which it coordinates new ligand and is measured via fluorescence.

The fluorescent emission spectra of the Eu^{3+} was carried out with an AutoDELFIA-1235 automatic analyzer (Perkin-Elmer, WALLAC). All the measurements were performed by independent experiments with repetition for at least three times, and the error bars have been shown in the figures.

2.6. Confirmation of the effect of the shield part.

For the shield part effect experiments, thrombin was treated with DNA3. All other conditions are the same as in the experimental section of the detection of adenosine.

3. Results and discussion

In this work, we described a novel and general time-resolved fluorescence based method for adenosine detection, combining affinity recognition of aptamers and steric hindrance effect of a part