Biosensors and Bioelectronics 50 (2013) 351–355

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

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

Pyrophosphate-regulated Zn^{2+}-dependent DNAzyme activity: An amplified fluorescence sensing strategy for alkaline phosphatase

Rong-Mei Kong [a], Ting Fu [b], Ni-Na Sun [a], Feng-Li Qu [a,*], Shu-Fang Zhang [a], Xiao-Bing Zhang [b]

[a] The Key Laboratory of Life-Organic Analysis, College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu, Shandong 273165, PR China
[b] State Key Laboratory for Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

ARTICLE INFO

Article history:
Received 19 May 2013
Received in revised form
28 June 2013
Accepted 28 June 2013
Available online 5 July 2013

Keywords:
Pyrophosphate
DNAzyme
Alkaline phosphatase
Fluorescence

ABSTRACT

In this work, based on the fact that pyrophosphate (PPi) could regulate the activity of Zn^{2+}-dependent DNAzyme, we for the first time report a fluorescence turn-on sensing system for alkaline phosphatase (ALP) with improved sensitivity via nonprotein-enzymatic signal amplification. A catalytic and molecular beacon (CAMB) design was employed to further improve its sensitivity. Taking advantage of the strong interactions between PPi and the Zn^{2+}, the cofactor Zn^{2+} was caged, and the DNAzyme activity was effectively inhibited. The introduction of ALP, however, could catalyze the hydrolysis of PPi and release free Zn^{2+}, resulting in the activation of DNAzyme to catalyze the cleavage of the molecular beacon substrate with a remarkable increase of fluorescent signal. These optimized designs together allow a high sensitivity for ALP, with a detection limit of 20 pM observed, much lower than previously reported methods. It has also been used for detection of ALP in human serum with satisfactory results, demonstrating its potential applications in clinical diagnosis.

1. Introduction

DNAzymes are nucleic acids which are isolated from combinatorial oligonucleotide libraries by in vitro selection (Robertson and Joyce, 1990; Silverman, 2005). Similar to protein enzymes, DNAzymes depend on specific metal ions or neutral molecules as cofactors, and show high catalytic hydrolytic cleavage activities toward certain substrates (Freisinger and Sigel, 2007; Sigel and Pyle, 2007). Moreover, compared with protein enzymes, DNAzymes possess some unique features, such as more stable than enzymes, versatility in modification, and can be denatured and renatured many times without losing their catalytic activities toward substrates. Therefore, DNAzymes have been widely applied to construct sensing systems for a variety of cofactors from metal ions to neutral molecules (Liu and Lu, 2003, 2007; Xiang and Lu, 2011; Kong et al., 2011). The autocatalytic amplification property of DNAzyme has also attracted great interest in recent years. By conjugating with recognition units via various allosteric strategies, they have also been employed as biocalaysts to design nonprotein-enzymatic assay platforms for amplified detection of targets other than cofactors, including nucleic acids, proteins and other biomolecules (Wang et al., 2011; Qi et al., 2012; Xiang and Lu, 2012; Zhao et al., 2013).

Alkaline phosphatase (ALP, EC 3.1.3.1) is one of the most commonly assayed enzymes in clinical practice, since it is often used as important biomarker in the diagnosis of many diseases. The abnormal levels of ALP in serum have been verified to be associated with the occurrence and development of certain diseases such as bone disease, liver dysfunction (Colornbatto et al., 1996a, Colornbatto et al., 1996b), breast and prostatic cancer (Lorente et al., 1999), and diabetes (Rao and Morghom, 1986). It is therefore important to develop rapid and high-throughput assay methods for ALP activity. A number of assay methods have been developed in recent years for the detection of ALP activity, including colorimetry (Choi et al., 2007; Wei et al., 2008), fluorescence (Liu and Schanze, 2008; Kawaguchi et al., 2011; Chen et al., 2013, 2010; Freeman et al., 2010; Zhang et al., 2013), surface-enhanced Raman spectroscopy (Ingram et al., 2009), electrochemistry (Kazakeviciene et al., 2008; Murata et al., 2009; Miao et al., 2011; Ino et al., 2012), and electrochemiluminescence methods (Jiang and Wang, 2012). Among them, fluorescence assays have drawn considerable attention due to the high sensitivity, convenience, cost-effective, and easy to scale-up to a high-throughput screening format. While most of these fluorescence sensing systems offer satisfactory sensitivity towards ALP with a detection limit located at nM level, an even higher sensitivity is desired if these systems are to be used in complex biological samples to ensure large signal to noise ratios to counteract background interference of biological system.

ALP has broad substrate specificity, and several substrates have been employed to design assay methods for ALP. As one of the many nature substrates for ALP, pyrophosphate (PPi) is unique

* Corresponding author. Tel./fax: +86 537 4456301.
E-mail address: fengliquhn@hotmail.com (F.-L. Qu).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.06.064

because it has a lower pH optimum compared to other substrates (Fernley, 1971). In addition, PPi can strongly chelate with certain metal ions (Su et al., 2013), such as Hg^{2+}, Zn^{2+}, and Cu^{2+}, while the presence of ALP could catalyze the hydrolysis of PPi and release free metal ions. To develop a fluorescence sensing system with improved sensitivity, we turn our attention to DNAzymes, since they possess autocatalytic amplification property and could provide amplified signal for recognition events (Liu et al., 2009). Most importantly, the metal ion cofactors are indispensable for most DNAzymes to maintain their catalytic function, and it could be speculated that the activity of Zn^{2+}-dependent DNAzyme could be inhibited by PPi due to the formation of the PPi–Zn–PPi complex with high stability (stability constant log $K^{PPi-Zn}=11.0$), while ALP could re-activate the DNAzyme via catalyzing the hydrolysis of PPi and release free cofactor, and such regulation can be adopted to develop sensing system for ALP. Herein, based on the fact that PPi could regulate the activity of Zn^{2+}-dependent DNAzyme, we for the first time report a fluorescence turn-on sensing system for ALP with improved sensitivity via nonprotein-enzymatic signal amplification. A catalytic and molecular beacon (CAMB) (Zhang et al., 2010) design was employed to further improve its sensitivity via realizing the true enzymatic multiple turnover of DNAzyme. The proposed sensing system shows an improved sensitivity and a high selectivity for pyrophosphate. It has also been used for detection of ALP in human serum with satisfactory results.

2. Experimental section

2.1. Materials and reagents

ALP (EC 3.1.3.1) was purchased from Sigma Co. Ltd. (St. Louis, MO, USA). Bovine serum albumin (BSA), human serum albumin (HSA), immunoglobulins G (IgG), streptavidin (SA), and lysozyme were purchased from Dingguo Biotech. Co. (Beijing, China). All other chemicals were obtained from Shanghai Chemical Reagents (Shanghai) and used without further purification. The serum samples are kindly provided by the Qufu Normal University hospital and stored at 4 °C. The buffers involved in this work were composed of 100 mM NaCl and 25 mM HEPES (pH 7.4), and the pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter. Milli-Q water (resistance > 18 MΩ cm) was used in all experiments. DNA oligonucleotides used in this work were synthesized and purified by Takara Biotechnology Co., LTD. (Dalian, China), and their sequences are shown as follows:

MB: 5′-/FAM/-CCACCAC AATGTTATAC AGGTACTAT *rAG* GAAG-TTGAG TTACGAGGCG GTGGTGG-/BHQ1/-3′

DNAzyme: 5′-CTCAACTTC TCCGAGCCGGTCGAA ATAGTACCT-3′

All fluorescence measurements were carried out on an F-7000 spectrometer (Hitachi, Japan). The instrument settings were chosen as follows: $\lambda_{ex}=494$ nm (slit 5 nm), $\lambda_{em}=518$ nm (slit 5 nm), PMT detector voltage = 950 V.

2.2. Procedure for optimization of detection conditions

To investigate the effect of MB concentration for DNAzyme cleavage, 50 μM of Zn^{2+} was incubated with different CAMB conditions, by varying the concentration of MB but fixing the concentration of DNAzyme at 100 nM at 37 °C for 25 min to detect the fluorescence changes.

The investigation of Zn^{2+} concentration for DNAzyme cleavage to obtain the best fluorescence responses was evaluated. Briefly, different concentrations of Zn^{2+} were first mixed with 150 nM MB probe and 100 nM DNAzyme in 100 μL HEPES buffer (100 mM NaCl, 25 mM HEPES, pH 7.4) for 25 min at 37 °C, then the fluorescence intensity was recorded.

The investigation of PPi regulation of DNAzyme activation was performed in 100 μL HEPES buffer (100 mM NaCl, 25 mM HEPES, pH 7.4). First, different concentrations of PPi were mixed with 100 μM of Zn^{2+} for 25 min at 37 °C. Then, adding 150 nM of MB substrate and 100 nM of DNAzyme into the sample solution simultaneously and incubated for another 25 min at 37 °C. Finally, the fluorescence changes were recorded.

2.3. Procedure for fluorescence detection of ALP activity

The experiments performed in 100 μL HEPES buffer (100 mM NaCl, 25 mM HEPES, pH 7.4). First, the assay was conducted by incubating 100 μM of Zn^{2+}, 500 μM of PPi, and a varying concentration of ALP for 25 min at 37 °C for the ALP catalytic reaction. Then, adding 150 nM of MB probe and 100 nM of DNAzyme into the sample solution simultaneously, and, finally, put the sample into fluorescence spectrometer quickly and monitor the real-time fluorescence changes at 518 nm, or incubating the mixture at 37 °C for 25 min to detect the fluorescence changes. For ALP detection in real complex samples, the experiments were conducted similar to that in buffer solution just containing 1% diluted human serum.

3. Results and discussion

3.1. The design of amplified sensing system for ALP

As one of the many nature substrates for ALP, PPi is unique because it has a lower pH optimum compared to other substrates. In addition, PPi can strongly chelate with certain metal ions, such as Hg^{2+}, Zn^{2+}, and Cu^{2+}, while the presence of ALP could catalyze the hydrolysis of PPi and release free metal ions. Therefore, PPi was chosen as the substrate for ALP in our design. The Zn^{2+}-dependent DNAzyme was chosen as the catalytic unit for the sensing system, since it possesses highly catalytic activity to afford a high sensitivity for the system and adopts Zn^{2+} as cofactors to maintain its functionality. It could be speculated that the activity of Zn^{2+}-dependent DNAzyme could be inhibited by PPi due to the formation of the PPi–Zn–PPi complex with high stability (stability constant log $K^{PPi-Zn}=11.0$), while ALP could re-activiate the DNAzyme via catalyzing the hydrolysis of PPi and release free cofactor. Such regulation constitutes the basis for the amplified detection of activity of ALP with our sensing system.

The sensing strategy of the DNAzyme-based amplified sensing system for ALP is illustrated in Scheme 1. The 17E strand of Zn^{2+}-dependent DNAzyme could hybridize with a hairpin-structured MB substrate to form the CAMB system. In the absence of ALP, the Zn^{2+} cofactor was caged by PPi due to the formation of a stable complex between them. In such case, the 17E DNAzyme activity is suppressed, and cannot catalyze the cleavage of the MB substrate, which affords a low fluorescence background for the sensing system. The introduction of ALP, however, will catalyze the hydrolysis of PPi into phosphate, release free Zn^{2+} cofactor, and activate the 17E DNAzyme to catalyze the cleavage of the MB substrate. The quenched MB fluorophore/quencher pair is then separated, and thereby produces a dramatic increase of fluorescent signal and a free DNAzyme strand. The released free DNAzyme strand can then hybridize with another MB and trigger the second cycle of cleavage. Eventually, each released DNAzyme strand can undergo many cycles to trigger the cleavage of many MBs, providing an amplified detection signal for the target ALP.

3.2. Optimization of detection conditions

To achieve the best fluorescent performance of the designed CAMB system, the concentration of MB substrate was first