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A transformer of molecular beacon for sensitive and real-time detection of phosphatases with effective inhibition of the false positive signals

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ABSTRACT

Molecular beacons (MBs) have shown great potential in measurement of enzyme activities. However, currently available methods for monitoring of phosphatases only use MBs as a signal reporter. Extra substrates for the phosphatases are needed to hybridize to the MB either as a primer or as a template. Moreover, few MB-based methods have been used to detect enzyme activities in real biological samples due to insufficient sensitivity or false positive interference signals caused by nonspecific nucleases. In this work, a novel type of fluorescent probe was designed and synthesized for monitoring of phosphatases by integrating the DNA substrate and the signaling structures into a single molecule. Such a new design not only significantly simplified the probing system and greatly enhanced the sensitivity, but also offered a practical way to guard against the false-positive signal problems in the application to real samples. The unique design of the assay format should be widely applicable to many other enzymatic assays using oligonucleotide fluorescent probes.

1. Introduction

Molecular beacons (MBs) are fluorescent oligonucleotide probes widely used for DNA and RNA detection (Santangelo et al., 2004; Tyagi and Kramer, 1996; Tyagi et al., 2008). They also showed substantial potential in the study of protein-DNA interactions, especially in monitoring of the nucleic acid enzyme activities (Li et al., 2000; Tang et al., 2003, 2005; Wang et al., 2009). Phosphatases play crucial roles in many biological processes involving genetic transduction, cell metabolism and neuronal functions. Sensitive detection of the activity of phosphatases is of great value for monitoring of the differentiation of embryonic stem cell, diagnosis of disease and development of effective biomedical countermeasures (Bronstein et al., 1996; Steiner et al., 2010; Wolf, 1994).

Previously, we and other groups have developed methods for monitoring of the activity and kinetics of T4 polynucleotide kinase phosphatase and calf intestinal alkaline phosphatase (ALP) by using conventional MBs (Ma et al., 2007; Song et al., 2010). These methods are very useful for screening enzymes and their inhibitors. However, they all employed separate DNA substrates as a primer or a template and only use MBs as a signal reporter. Besides, their application to quantify the phosphatase activities in real biological samples is still impaired by the lack of sensitivity and the false-positive signals caused by nonspecific enzymatic degradation.

Here we designed and synthesized a novel fluorescent probe by integration of the substrate of phosphatases and the signaling structure into a single molecule and modification of the loop strand close to the fluorescent label at 5′-end with a C3 carbon spacer (Fig. 1A), which significantly simplifies the probing system and enhances the detection sensitivity. The basic principle of the new assay format is illustrated in Fig. 1. As shown in Fig. 1A, the probe is a single-strand molecule that forms self-complementary structure at both ends, which appears like a transformer of conventional MBs. The 5′-end is labeled with a carboxyfluorescein (FAM) dye, which is quenched by a Black Hole Quencher (BHQ-1) labeled at the complementary nucleotide after the hybridization. The 3′-end is modified with a phosphate group and serves as the substrate of the target phosphatases. BHQ-1 is designed to be labeled at the T-base so as not to influence the polymerase extension reaction, whereas a C3 carbon spacer is inserted in the middle of the loop close to 5′-end to block the extension reaction from the 3′-end and ensure high fluorescence intensity after the reaction (Cradic et al., 2004).

The specific sequences of the proposed oligonucleotide fluorescent probe are listed in Table 1. Typical time courses of the reactions with ALP as a model phosphatase are shown in Fig. 1B. As can be seen, the probe exhibits very low and stable background signals when the system only contains exonuclease-deficient Klenow fragment DNA polymerase (KF^-) and dNTPs. Upon the addition of target phosphatases, the 3′-PO_4 termini of the probe is hydrolyzed and the

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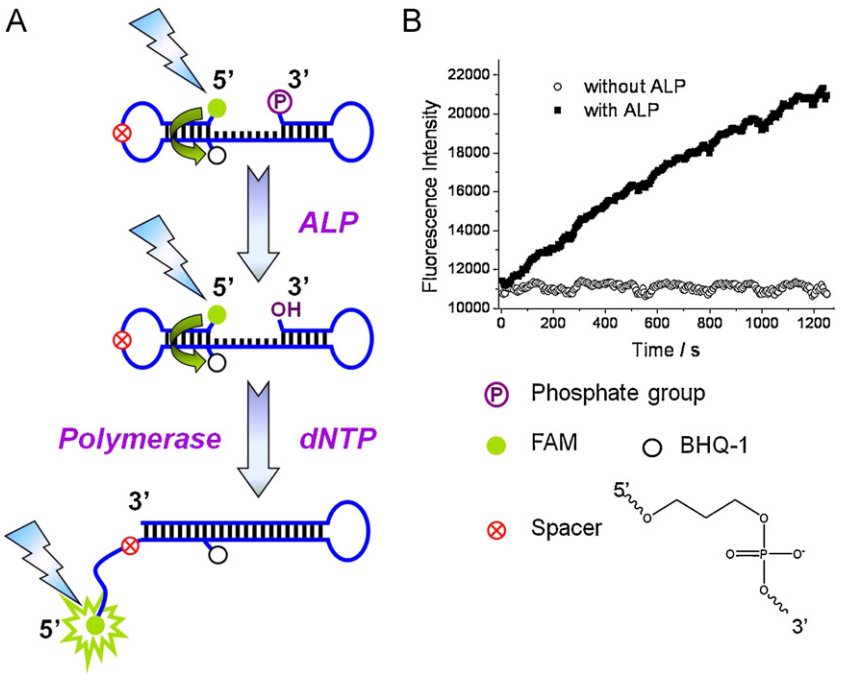

Fig. 1. (A) Principle of detecting the phosphatase activities by using the MB transformer. (B) Time courses of the ALP assay in the presence of appropriate concentrations of KF^- and dNTP.

resulting 3′-OH is immediately extended by the DNA polymerase and dNTPs, which instantly opens the hybridized structure at the 5′-end and emits strong fluorescence.

In previously reported methods (Ma et al., 2007), the substrate was a short oligonucleotide primer hybridized to half of the loop of the MB's hairpin structure. The formed complex was relatively unstable and showed high fluorescence background. The later developed system overcame above drawback (Song et al., 2010), however, a negative-readout format was employed which was unfavorable for the analysis of complex samples. Moreover, in both of above methods, MB was only used as a signal reporter. A separate substrate for phosphatase was added to serve as a primer or a template. In the present design, two functional elements were integrated into one molecule, thus greatly simplified the detection system.

Another innovative point of the new probe was that a C3 carbon spacer was inserted into the loop as a blocker. According to previous work, photo-induced electron transfer (PET) between natural bases and the fluorophore may result in notable fluorescence quenching (Knemeyer et al., 2000; Seidel, 1996). Based on this property, single labeled fluorescence probes with stacked guanines as the quencher have been developed and successfully used to detect DNA mutation and to monitor the DNA polymerization and phosphorylation processes (Knemeyer et al., 2000; Song et al., 2009; Song and Zhao, 2009). In the present work, an initial quenching state of the probe was designed to establish a positive readout system for monitoring of the dephosphorylation reaction. For this purpose, a quencher was labeled at the complementary base of the 5′-end since the PET efficiency between the fluorophore and the stacked guanines in the middle of the strand was much lower than those at the end of the strand. For the same reason, the extension reaction should not be allowed to form fully complementary strand to the 5′-end so as to avoid the loss of fluorescence after the reaction. Therefore, we modified the loop by inserting a C3 carbon spacer which can efficiently block the extension process and ensure high fluorescence intensity of the probe at the end of the reaction (Cradic et al., 2004).

In the application of MBs to analyze real biological samples, a major concern is the false-positive signals (FPSs) caused by non-specific nucleases in the sample matrix. Though many efforts have been made to modify the MBs so as to avoid degradation by nucleases (Chen et al, 2009; Kim et al., 2007a,b; Petersen et al., 2004), most of them also impeded the use of MBs in monitoring of the reactions between proteins and natural bases. In an attempt to solve this problem, we proposed an alternative strategy in this work. Under the optimized experimental conditions, the novel detection system shows distinct advantages in simplicity, rapidity and sensitivity. It has been successfully applied to quantify the phosphatase activity in the extract of Arabidopsis tissue and shows great potential in high-throughput analysis of phosphatases in other biological samples without any complex pretreatment procedures.

2. Experimental

2.1. Chemicals and apparatus

The DNA nucleotides including all modifications were synthesized by Sangon Co. (Beijing, China) and purified by HPLC and tested with 16% PAGE with 7 M urea in 1× TBE buffer. Their structures were confirmed by mass spectrometry. The exonuclease-deficient Klenow fragment DNA polymerase (KF^-), dNTP and reaction buffer were all purchased from New England Biolabs Inc. (MA, USA). The buffer contains 10 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$ and

Table 1
Sequences of the fluorescent oligonucleotide probes and the analogue inhibitor used in this study.

Probe name	Sequences (5′ → 3′)
MB transformer	5′-(FAM)*ACCCGG*TA(C3 carbon spacer)-AG*CCGGG*-(BHQ1)TGACATAAC*CGCACC*-TAAAG*GGTGCG*-(PO_4)-3′
Analogue inhibitor	5′-*ACCCGG*TAAAG*CCGGGT*GACATAAC-*CGCACC*TAAAG*GGTGCG*-3′

FAM is carboxyfluorescein; BHQ-1 is Black Hole Quencher 1. The self-complementary parts are underlined and shown in italic.