Selective and sensitive turn-on detection of adenosine triphosphate and thrombin based on bifunctional fluorescent oligonucleotide probe

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A bifunctional fluorescent oligonucleotide probe for small molecules and protein detection has been developed based on turn on fluorescence response via the target induced structure-switching of molecular beacon. The two loops of this molecular beacon are designed in such a manner that they consist of thrombin (Tmb) aptamer sequence and adenosine triphosphate (ATP) aptamer sequence, respectively, which are utilized to sense thrombin and ATP. The oligonucleotide forms double stem-loops in the absence of targets, yielding no fluorescence emission because of the FRET from the excited fluorophore to the proximal quencher. Upon addition of the target, the ATP or Tmb, its specific interaction with loop sequence of the hairpin structure induce the separation of reporter fluorophore and the fluorescence quencher of the molecular beacon, resulting in an increase of fluorescence response. Hence, the separate analysis of ATP and Tmb could be realized through only one designed molecular beacon. The detection limits were estimated to be 25 nM for ATP and 12 nM for Tmb, respectively. The results of this study should substantially broaden the perspective for future development of oligonucleotide probe for analysis of other analytes.

1. Introduction

Aptamers, selected from combinatorial libraries using systemic evolution of ligands by exponential enrichment, are artificial oligonucleotides (DNA or RNA) that can bind with high affinity and specificity to a wide range of target molecules, including drugs, proteins and other organic or inorganic molecules (Ellington and Szostak, 1990; Ellington and Gold, 1990). Compared with natural receptors such as antibodies and enzymes, aptamers have several unprecedented advantages that make them ideal biosensing elements (Jayasena, 1999; Breake, 1997). Unlike antibodies, however, aptamers can be selected under nonphysiological conditions and for targets that are either too small or too toxic for antibodies to elicit effective immune responses (Liu et al., 2009; Liu and Lu, 2004). In addition, aptamers, once selected, can be synthesized with high reproducibility and purity, and are readily available from commercial sources. Also, aptamers often undergo significant conformational changes upon target binding. This offers high flexibility in design of novel biosensors with high detection sensitivity and selectivity (Reinemann et al., 2009; Mukhopadhyay, 2005; Nilsen-Hamilton, 2005; Jhaeri et al., 2000; Bayrac et al., 2011).

Molecular beacons (MB) are dual-labeled oligonucleotide probes with a reporter fluorophore at one end and a quencher at the opposite end (Tyagi and Kramer, 1996). They are designed with a target-specific hybridization domain positioned centrally between two short self-complementary segments. In the absence of target, the beacons produced no fluorescence emission because of the fluorescence resonance energy transfer (FRET) from the excited fluorophore to the proximal quencher (Stryer, 1978). The target DNA can cause a spontaneous conformational reorganization of molecular beacon that forces the stem apart, and incur the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence.

The conformation switching mechanism has also been fully explored for the development of aptamer-based fluorescent sensor toward DNA (Kuhn et al., 2002), proteins (Bang and Cho, 2005), metal ions (Wang and Liu, 2008a, b), small molecules (Wang et al., 2007), virus (Liming and Bhagwat, 2004), etc. However, these sensors are mainly directed at the detection of only single target and often invalid for other interestingly coexisted target molecule. Thus, there is an ever-growing demand to screen multiple targets with only sensing system. Recently, Wang groups developed a colorimetric oligonucleotide probe for the separate colorimetric analysis of two different analytes, target DNA and protein (Zhang et al., 2011). However, some drawbacks such as turn-off signal, relatively low sensitivity and narrow...
linear range still existed. Therefore, a more sensitive and selective but simpler sensor for turn-on signal multiple targets detection is required. Therefore, it would be interesting and significant to develop a bifunctional oligonucleotide probe for small molecules and protein detection. Due to the high sensitivity and versatility, fluorescence are most commonly used for the development of sensors or biosensors toward detection of various targets (Tsourkas et al., 2003; Kuhn et al., 2002; Nitin and Bao, 2008; Qian et al., 2008). Herein, taking advantages of aptamers and MB, we design a bifunctional fluorescent oligonucleotide probe for the detection of adenosine triphosphate (ATP) and thrombin (Tmb). The MB contains two hairpin loops that serve as the sensing elements, and a fluorophore at one end and a quencher at the opposite end, as the reporter (Fig. 1). The oligonucleotide forms double stem–loops in the absence of targets, yielding no fluorescence emission because of the FRET from the excited fluorophore to the proximal quencher. In the presence of targets, the loops of the hairpin structures are specifically bound by target molecules which causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence. The two loops of MB consist of the sequences that are used as the aptamers for target ATP and Tmb, respectively. In this way, the separate analysis of ATP and Tmb could be realized through only one designed molecular beacon.

2. Results and discussion

2.1. Principle

To demonstrate the feasibility of our approach, the fluorescence intensity of the oligonucleotide probe was tested in different conditions. As shown in Fig. S1, curve a is the fluorescent emission spectra of MB in the absence of ATP. The MB is now in a double stem–loops configuration and the fluorophore is in close proximity with the quencher. Thus, the molecular beacon is in the “dark” state. With increasing of the ATP concentration (curve b to d, Fig. S1), the fluorescence intensity of the oligonucleotide probe becomes more and more strong. It is undoubtedly attributed to the fact that ATP combines with one loop of the hairpin structures, which causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence.

2.2. Fluorescent analysis of ATP

For the ATP detection using the bifunctional oligonucleotide probe, different concentrations of ATP was mixed with the oligonucleotide probe solution. It was found that the quenched fluorescence got recovered, with increasing the concentration of ATP (Fig. 2A), which suggests that the designed oligonucleotide probe could be used for ATP sensing. The fluorescence intensity of the oligonucleotide probe induced by ATP was monitored. As depicted in Fig. 2(A), in the absence of ATP, there is no fluorescence emission at 520 nm. With increasing the concentration of ATP, the fluorescence intensity gradually increases. As shown in Fig. 2(B), the fluorescence intensity increased linearly over the ATP concentration range from

![Fig. 1. Schematic representation of the bifunctional oligonucleotide probe for fluorescent analysis of ATP or Tmb.](image)

![Fig. 2. (A) Fluorescence spectra of the oligonucleotide probe in response to various concentrations of ATP: 0, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 1.5 μM and 2 μM. (B) The linear relationship of the fluorescence change of the oligonucleotide probe and ATP concentration ranging from 50 nM to 2 μM. Excitation: 480 nm, emission: 520 nm.](image)
5.0 \times 10^{-8} \text{M} to 2.0 \times 10^{-5} \text{M}, with the linear correlation coefficient of 0.996. The detection limit was estimated to be 25 nM based on S/N of 3, and the sensitivity is comparable or better than that in the reported ATP assays (Wang et al., 2005; Wang and Liu, 2008a, b; Zhou et al., 2011; Zuo et al., 2007, 2009).

2.3. Selectivity of the oligonucleotide probe for ATP analysis

To test the selectivity of the oligonucleotide probe for ATP analysis, ATP analogues, such as guanosine triphosphate (GTP), cytidine triphosphate (CTP) and uridine triphosphate (UTP) were investigated. As shown in Fig. S2, only ATP makes the restoration of fluorescence obviously. In the presence of other ATP analogues, there is nearly no fluorescence, implying that other ATP analogues cannot interact with ATP aptamer sequence and thus not interfere with the detection of ATP. These results indicate that ATP can bind to its aptamer specifically and the bifunctional oligonucleotide probe shows a high selectivity toward ATP.

2.4. Fluorescent analysis of Tmb

To quantitatively detect Tmb using our oligonucleotide probe, fluorescent emission spectra of the oligonucleotide probe was monitored in the absence and presence of different concentrations of Tmb. As shown in Fig. 3(A), with the increasing concentration of Tmb, the fluorescence intensity of the oligonucleotide probe gradually increases. The Tmb can combine with another loop of the hairpin structures, which causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence. As shown in Fig. 3(B), the fluorescence intensity increased linearly over the Tmb concentration range from 2.0 \times 10^{-8} \text{M} to 5.0 \times 10^{-5} \text{M}, with the linear correlation coefficient of 0.999. The detection limit was estimated to be 12 nM based on S/N of 3, the sensitivity is comparable or better than that in the reported Tmb assays (Bini et al., 2007; He et al., 2007; Tennico et al., 2010; Wei et al., 2007; Yan et al., 2011; Sun et al., 2012).

2.5. Selectivity of the oligonucleotide probe for Tmb analysis

The selectivity of our approach to detect Tmb was evaluated in comparison with bovine serum albumin (BSA) and lysozyme (Fig. S3). In the presence of Tmb, the fluorescence intensity was remarkably increased. However, almost no fluorescence changes were observed when these two substances were mixed with fluorescent oligonucleotide probe, respectively. All these results suggested that Tmb binds to its aptamer specifically and the bifunctional oligonucleotide probe shows a high selectivity toward Tmb.

3. Conclusions

In summary, we developed a bifunctional oligonucleotide probe with the two loops consisted of ATP and Tmb aptamer sequence, respectively, for the separate analysis of two different analytes, ATP and Tmb. Through this detection strategy, the detection limits were estimated to be 25 nM for ATP and 12 nM for Tmb, respectively. Also the bifunctional oligonucleotide probe showed a high selectivity toward ATP and Tmb over their corresponding analogues. The results of this study should substantially broaden the perspective for future development of oligonucleotide probe toward analysis of other analytes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.10.007.

References
