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Cleavable DNA-protein hybrid molecular beacon: A novel efficient signal translator for sensitive fluorescence anisotropy bioassay

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ABSTRACT

Due to its unique features such as high sensitivity, homogeneous format, and independence on fluorescent intensity, fluorescence anisotropy (FA) assay has become a hotspot of study in oligonucleotide-based bioassays. However, until now most FA probes require carefully customized structure designs, and thus are neither generalizable for different sensing systems nor effective to obtain sufficient signal response. To address this issue, a cleavable DNA-protein hybrid molecular beacon was successfully engineered for signal amplified FA bioassay, via combining the unique stable structure of molecular beacon and the large molecular mass of streptavidin. Compared with single DNA strand probe or conventional molecular beacon, the DNA-protein hybrid molecular beacon exhibited a much higher FA value, which was potential to obtain high signal-background ratio in sensing process. As proof-of-principle, this novel DNA-protein hybrid molecular beacon was further applied for FA bioassay using DNAzyme-Pb^{2+} as a model sensing system. This FA assay approach could selectively detect as low as 0.5 nM Pb^{2+} in buffer solution, and also be successful for real samples analysis with good recovery values. Compatible with most of oligonucleotide probes' designs and enzyme-based signal amplification strategies, the molecular beacon can serve as a novel signal translator to expand the application prospect of FA technology in various bioassays.

1. Introdution

Fluorescence anisotropy (FA) is among the most popular techniques for bioassays, including food analysis, drug screening, toxins detection, clinical diagnosis and so on [1,2]. FA assay has been well known for its unique features, such as high accuracy and sensitivity, homogeneous format, real-time and automated high-throughput detection capability [3]. Insensitive to solution color and fluorescence fluctuation, FA assay is also a suitable method for detection of targets in various complex samples [4]. Because of oligonucleotides' (i.e. aptamers) various advantages including ease of synthesis and labeling, good stability, high binding affinity and selectivity, oligonucleotide-based FA assay is becoming the hotspot in FA assay research [5,6]. In this way, the oligonucleotide-based FA has been applied for the assays of numerous targets, such as cells [7], enzyme activity [8], proteins [9–11], oligonucleotides [12,13], small molecules [14–16], metal ions [17,18] and so on.

Because the FA value results from fluorophore's local motion and probe's global diffusion, it mainly relates to mass change after the probe's binding to its target. However, either the different secondary structures of functional oligonucleotides [4] or the low molecular weights of many targets, i.e. small molecules, may result in a weak signal response. Thus the direct binding approach via target-induced conformational change strategy is not easily generalizable for all oligonucleotide-based FA assays [19]. To meet this challenge, several bioassay approaches either using proteins [15,20] or nanomaterials [14,17] as signal amplification moieties or employing specific structure design strategies [19,21] have been adopted for oligonucleotide-based FA bioassay. However, there are also many problems to be solved. On one hand, the methods involving nanomaterials might not be compatible with signal amplification strategies using enzymes, because some nanomaterials often inhibit enzyme's activities [22,23]. On the other hand, customized probe's structures such as specific oligonucleotide end structure [21] or dsDNA polymers [24], are neither generalizable for different probes nor effective to obtain sufficient signal response. To facilitate further application of oligonucleotide-based FA bioassay, it is therefore very necessary to develop a novel and general probe design strategy that can not only obtain large FA signal response but also be effectively compatible with signal amplification strategies using enzymes.

Molecular beacon is well-known as hairpin structure, which

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has been frequently used as a unique design strategy for oligonucleotide-based probes [25]. Owning excellent sensitivity and selectivity, molecular beacon has been widely used for various applications, such as biosensor construction, biochip development, screening of single nucleotide polymorphisms, cell imaging and so on [26,27]. The specific thermodynamic stability of the MB's hairpin structure is beneficial for oligonucleotide-based FA bioassay, because it has been reported that the secondary structure of oligonucleotide probes greatly influenced the FA value [19,21]. In addition, the highly predicted stem helix structure and efficient intrinsic signal switching eliminate the need to carefully engineer the instability of probe's secondary structure [19,21], making molecular beacon as a more universal probe design for various oligonucleotide-based FA bioassays. Most importantly, because molecular beacon is compatible with most of the enzyme-based signal amplification strategies [28–31], molecular beacon hold great potential to construct a generalizable probe design strategy for various FA bioassays with high sensitivity.

Due to its high stability and affinity ($K_d = 10^{-15}$), exceptional specificity, and streptavidin's molecular mass ($M = 53{,}000$), biotin-streptavidin pairing has found wide applications in DNA nanotechnology [32–34]. Also in our previous aptamer-based FA assay researches based on DNA-protein hybrid nanowires or enzymatic cleavage [24,35], streptavidin has been successfully employed as a signal amplifier to lower the detection limit to one order of magnitude or more. However, the two strategies need both complicated structure designs and expensive labels in the middle position of DNA probes, thus not widely suitable for various FA bioassays. Herein, by engineering of a novel DNA-protein hybrid molecular beacon based on conventional molecular beacon structure together with streptavidin as a mass amplifier, we report the proof-of-principle of a novel probe design strategy for oligonucleotide-based FA bioassay. To demonstrate the compatibility of this novel DNA-protein hybrid molecular beacon with other enzyme-based signal amplification strategies, 8–17 DNAzyme and Pb^{2+} were employed as the catalytic moiety and model target molecule respectively. The proposed novel cleavable DNA-protein hybrid molecular beacon enabled to greatly improve the FA assay sensitivity via a double signal-amplified approach, with a detection limit as low as 0.5 nM for Pb^{2+}.

2. Experimental section

2.1. Materials and instrumentation

All DNA oligonucleotides were synthesized and HPLC-purified by Sangon Biotech Co., Ltd. (Shanghai, China). The fluorophore, 6-carboxyfluorescein (FAM), was attached to the 5′-end of the oligonucleotides, and the sequences of the oligonucleotides were listed in Table S1. Streptavidin was purchased from Sigma-Aldrich Company (Shanghai, China), while other chemical reagents were purchased from Shanghai Chemical Works (Shanghai, China). All solutions used Milli-Q water from a Millipore system (resistance $> 18\,M\,\Omega\,cm^{-1}$).

All the fluorescence measurements were carried out using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, France) at 25 °C via L-format configuration. Excitation and emission monochromators were set to 490 nm and 518 nm, respectively, with bandwidths of 10 nm.

2.2. Fluorescence anisotropy (FA) measurements

The oligonucleotide and streptavidin stock solutions were prepared in water and stored at −20 °C. The working buffer conditions for Pb^{2+} assay were 50 mM HEPES–NaOH, pH 7.3, 50 mM NaAc. Probes were prepared via slow annealing: MB (1 μM) or MB-biotin (1 μM) was mixed respectively with DNAzyme in 50 mM HEPES–NaOH buffer solution, heated to 95 °C and then slowly cooled down before use.

For kinetic FA studies, 90 μL working solution was added to a cuvette, and then its initial FA signal was recorded for 400 s with time interval of 5 s. Then, 10 μL of 1 μM Pb^{2+} was added using a microsyringe and stirred to mix the components thoroughly. The sample was incubated to continue the FA measurement until the signal reached an equilibrium. For the assay approach using conventional molecular beacon, the working solution contained 200 nM MB and 100 nM DNAzyme, while it was 200 nM MB-biotin, 100 nM DNAzyme and 20 μg/mL streptavidin for the approach using DNA-protein hybrid molecular beacon.

For studies of FA assay using conventional molecular beacon, 10 μL Pb^{2+} solution was mixed with 90 μL annealed mixture of MB (200 nM) and DNAzyme (150 nM). After equilibrating the resulting mixture at 25 °C for 20 min, the fluorescence anisotropy (FA) was recorded.

For studies of FA assay using DNA-protein hybrid molecular beacon, 10 μL of 150 μg/mL streptavidin was added to 80 μL annealed product of MB-biotin (200 nM) and DNAzyme (150 nM). Then 10 μL Pb^{2+} solution was mixed and equilibrated for another 20 min, finally followed by FA measurement. Three determinations were performed for each Pb^{2+} concentration to calculate standard deviation.

2.3. Selectivity of the FA measurements

The selectivity of the FA measurements was investigated using other metal ions, including Hg^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, and Ag^{+}. The competition experiments were carried out in the presence of Pb^{2+} and other metal ion. The final concentration of Pb^{2+} was 100 nM, whereas the concentration of all other metal ions was 1 μM. These experiments were performed for three times under the similar conditions mentioned above.

2.4. Gel electrophoresis measurement

2% agarose gels were prepared in 50 mL TBE buffer (1 ×) containing 1.5 μL ethidium bromide. A volume of 10 μL of different reaction products (various concentrations of Pb^{2+}, 200 nM MB-biotin, and 150 nM DNAzyme) was added to each lane via loading buffer (1 ×). The resulting agarose gels were run for 30 min (110 V), and finally visualized under UV light.

2.5. Determination of Pb^{2+} in real water samples

Samples of drinking water and river water were collected from purified water (Wahaha Co., Ltd., China) and Xiang River (Xiangtan, China), respectively. After simple filtration over Whatman #3 filter, 1 mL aliquots of the drinking water or the river water were spiked with standard Pb^{2+} solutions, and then stored at room temperature for use. Finally, these real water samples with various concentrations of Pb^{2+} were analyzed in a similar procedure described above.

3. Results and discussion

3.1. Design of DNA-protein hybrid molecular beacon

As dsDNA is more favorable for inhibiting fluorophore's local motion than ssDNA [21], extending the terminal stem of DNA stem-loop structure could promote a polarization process. Meanwhile, a unique feature of molecular beacon is that its