Label-free visual detection of nucleic acids in biological samples with single-base mismatch detection capability†

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We have combined an allosteric molecular beacon for target recognition and guanine-rich DNAzyme for signal amplification to develop a new platform for visual detection of nucleic acids with single-base mismatch detection capability. The fully DNA-structured platform can undergo color change in response to target DNA/RNA, which enables sensitive and selective visual detection in biological samples.

The sensitive and selective detection of DNA and RNA targets is critically important in areas such as genetics, pathology, medical diagnosis, environmental monitoring, food safety, and bioterrorism. 1-3 Especially, practical applications have driven a strong demand for the sensitive and convenient methods to detect sequence-specific DNA/RNA in complex biological samples. Various strategies and technologies have been developed to identify unique DNA/RNA sequences, such as polymerase chain reaction, 4 DNA microarrays, 1 fluorescence, 2 electrochemistry, 3 and surface plasmon resonance. 5 While sensitive and specific, most of these methods require expensive reagents, costly equipment, tedious assay processes, and well-trained personnel, which limit the range of their practical use, especially for emerging point-of-care applications. Continuing efforts have been made to seek an ideal method that can realize sensitive, selective, fast, low-cost and easy-to-perform detection of nucleic acids in biological samples without any complicated pre-treatment or sophisticated instruments. In this regard, visual detection methods are highly attractive. Gold nanoparticle colorimetric biosensors have been developed in recent years due to their easy manipulation and visual readout. 6-8 However, this strategy suffers from time consuming (about 20–40 h for particle preparation and assembly) and the performance has been compromised by the poor stability of these particles in solution. Another widely used technique is the incorporation of a general enzymatic immunoassay with visual detection capability. Horseradish peroxidase (HRP) can be used as a conjugated label for an enzymatic assay to detect DNA. 9,10 Unfortunately, its modification is expensive: avidin-HRP must be formed firstly and bound to the biotinylated probe. Excess HRP must be removed to minimize the background signal. Thus, new strategies are highly desired to develop a sensitive, rapid, practical, and economical visual detection method.

DNAzyme, which is totally made of DNA, has also been reported processing enzymatic function. For example, an interesting DNAzyme with single-stranded guanine-rich structure has revealed peroxidase-like activity when forming a G-quartet complex with hemin. 11,12 This complex catalyzes the oxidation of 2,2′-azion-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS 2-) by H2O2 to produce the colored radical anion (ABTS +•-), resulting in a detectable color change. The use of DNAzyme as catalytic label for biosensing is attractive since the whole DNA structure can be easily synthesized with good reproducibility, eliminating the complicated protein-based labelling. 13 More recently, we have developed allosteric molecular beacons (aMBs), 14 whose working principle is based on allosteric effect to regulate the binding affinity of probes towards streptavidin (SA) microbeads. The aMBs achieved an ultrahigh signal-to-background ratio and can directly detect targets in complex biological samples without any sample pre-treatment. Herein, we combined the aMBs for molecular recognition and DNAzyme for signal amplification to develop a fully DNA-structured platform for sensitive and selective sequence-specific detection of nucleic acids with the naked eye in complex biological samples.

As shown in Scheme 1, the aMB 14 probe consists of an SA aptamer sequence (shown in pink), a target binding loop (cyan), and a short sequence (orange) that is complementary to part of the SA aptamer. A stable hairpin structure is formed by intramolecular hybridization between the SA aptamer and the short complementary sequence, temporarily disabling the probe’s ability to bind with SA microbeads. In the presence of target, however, the loop binds to the target, which, in turn, disrupts the hairpin structure to free the SA binding aptamer, thereby activating the probe’s binding affinity towards SA beads. The free unreacted aMBs can be easily separated from SA beads. Afterwards, the G-quartet probe (blue), which has a

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linker complementary to the stem of aMB (orange), is introduced. Consequently, the G-quartet probe will bind to SA beads with target-bound aMB probes, which will catalyze the color changing reaction with the addition of hemin and ABTS. In our design, no DNA labelling is needed. The target pre-enrichment and background reduction can be easily realized by microbead-based separation. The G-quartet DNAzyme can trigger a colorimetric enzymatic signal amplification, which ensures the visual detection with high sensitivity.

To prove the concept, we chose the β-actin-aMB as a model. β-actin plays an important role in cell division, morphogenesis, organelle positioning and vesicle trafficking. β-actin mRNA has been widely used as a standard control in studies of gene expression. Therefore, β-actin mRNA was taken as an experimental model for system investigation. The complementary DNA of β-actin mRNA was designed into the loop sequence of aMB, and linked with the SA aptamer on one side. On the other side, the short sequence complementary to partial SA aptamer was designed to form a hairpin structure. In order to achieve a high signal-to-background ratio, we first optimized the stem length of β-actin-aMB. Three sequences with different stem lengths have been designed and tested (see ESI†). According to our results, β-actin-aMB3 was chosen for further experiment, which has a stable hairpin structure to avoid leaking reaction and can response quickly and open completely upon target binding.

To investigate the feasibility, the aMB was first labelled with TMR dye in order for rapid flow cytometry analysis. As shown in Fig. 1A, upon target binding, a significant increase in the fluorescence intensity of microbeads was observed, indicating the probe design is successful and the first step of our principle works effectively. Afterwards, G-quartet DNAzyme was applied to demonstrate the visual detection. Non-labelled aMB probes were mixed with different concentrations of target RNA for hybridization, and then incubated with SA beads. After washing to eliminate unbound free aMB probes, SA beads hybridized with G-quartet DNAzyme. By incubating with hemin for 20 min, the G-quartet probe and hemin formed a catalytic complex, which can catalyze the oxidation of ABTS$^{-}\text{2-}$ by H$_2$O$_2$ to produce the ABTS$^{\text{3+}}$, therefore causing a detectable color change. In the absence of target, the binding affinity of the SA aptamer was strongly inhibited by the formation of hairpin structure, therefore no subsequent reactions and no color response. However, upon target binding, as shown in Fig. 1B, a significant color change could be easily observed by the naked eye and the intensity changes are corresponding to the increase of target concentrations. As low as 1 nM RNA can be distinguished by the naked eye. This result has proven the feasibility of our aMB-DNAzyme method for highly sensitive visual detection of nucleic acids.

As noted above, the target-bound probe is enriched on the surface of microbeads so that background noise from a matrix can be significantly eliminated by a simple washing step. Therefore, another advantage of our method is the ability to sensitively detect targets in complex biological samples. To demonstrate this advantage, HEK-293FT cell lysate was used as a model matrix. Cell lysate contains a large amount of auto-fluorescence species including proteins, proteases, organelles, as well as cell debris. The background signal from these species spans the visible spectrum and masks the probe signal, making homogeneous detection of biomolecules extremely difficult. In our experiment, β-actin-aMB was added to cell lysate for hybridization. Then, SA beads were introduced, incubated and collected. After the incubation with a G-quartet probe, hemin was introduced for enzymatic reaction. As shown in Fig. 1C, our visible method worked well in cell lysate. Compared with the blank sample and control probe RSV-aMB (targeting respiratory syncytial virus influenza A), only β-actin-aMB showed a distinct color change towards HEK-293FT cells which have a high expression of β-actin mRNA, confirming that the color change is indeed caused by the specific hybridization of target mRNA to the probe. These results demonstrate the feasibility of detecting target nucleic acids in a complex biological system with the naked eye.

We further applied our visual detection to evaluate the success of a plasmid transferring experiment. Classical methods for evaluating the transfer effect include RT-PCR and Western Blot. Unfortunately, both methods involve a complicated preparation process, expensive instruments and reagents, and tedious labor work.
Herein, a flag-aMB has been prepared to target mRNA of flag, a conventional label protein used in protein separation. Only when the HEK-293FT cells transfected with Plasmid flag-FGF19 (see ESI†), cell lysate can lead to a color change. If no transfection, the background color was the same as the blank (Fig. 2A). Meanwhile, we did Western Blot (see ESI†) to identify the transfection efficiency (Fig. 2B). Our results suggest that an aMB-DNAzyme method is able to visually confirm cell transfection at the mRNA level without the need of any instrument, dark room, expensive antibody and enzyme, or complicated process.

Single nucleotide polymorphism (SNP) represents the largest source of diversity in the human genome. Some of these variations have been directly linked to human diseases.16 Although sequencing is adequate for the initial discovery of SNP, simpler and faster methods are needed for routine clinical diagnostics and population study. A number of laboratories have confirmed the utility of using molecular beacons (MBs) for the detection of SNP.17 However, traditional MBs need expensive dual-labelling. Fluorophores often result in a high fluorescence background, and thus, a decrease in detection sensitivity.18 Our aMB-DNAzyme is a label-free method and no instrumentation is needed. Herein, a SNP-aMB was designed to identify a C to T transition at position 627 of the human chemokine receptor 2 genes. As shown in Fig. 3A, with the increase of mutation cDNA in the total cDNA quantity, color change intensity decreased proportionally. 30% mutation cDNA in the total cDNA can induce an obvious distinction. Using a UV/vis spectrometer, the percentage of mutation can be quantitatively evaluated and as low as 10% mutation can be distinguished (Fig. 3B). The result has proven the high selectivity of our visible method for qualitative and semi-quantitative SNP detection.

In conclusion, we have developed a new simple platform for visual detection of nucleic acids that combines the allosteric molecular beacons for sequence recognition and guanine-rich DNAzyme for signal amplification. Our visual detection method has several distinctive features. First of all, it is inexpensive and simple to prepare, since all of our probes are DNA structures without any labelling. Second, the method can directly detect targets in complicated biological samples without any sample pre-treatment. Third, this simple system is highly sensitive and is able to detect less than 1 nM target RNA with the naked eye. More importantly, it can be used for rapid visual confirmation of cell transfection at the mRNA level without the need of any instrument, dark room, expensive antibody and enzyme, or complicated process. Finally, the method is highly selective and can be used to visually detect a small fraction of single nucleotide mutation. Combining these unique properties, this sensitive, selective, simple, rapid, and sequence specific visual detection approach will find its wide applications for fundamental research, biotechnology, and biomedical diagnosis.

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Notes and references