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Small-molecule fluorescent probes for specific RNA targets†

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A method was developed that uses small molecules as fluorescent probes to detect specific mRNAs. In this approach, the fluorescence of fluorophore–quencher conjugates is restored by the binding of an mRNA aptamer tag to the quencher segment of the molecules. The method allows real-time detection of mRNA transcripts *in vitro*.

Real-time visualization of mRNAs in living cells would permit detailed analysis of mRNA dynamics during biological processes.[1] A number of powerful methods for visualizing RNAs have been developed, which allow real-time imaging of specific RNA targets. In one method, cells are transfected with molecular beacons, whose fluorescence intensity changes upon binding to endogenous mRNA targets.[2] Another method uses GFP-tagged proteins that bind to specific RNA tags.[3] Although these methods have provided valuable information about mRNA dynamics in living cells, methods based on nucleic acids or proteins have the respective drawbacks of poor cell permeability and a low signal-to-noise ratio. An alternative strategy using small molecules would avoid these problems.

Several groups have reported small molecule-based methods, in which a short RNA aptamer serves as a tag that binds to a non-fluorescent dye, whereupon the dye becomes fluorescent.[4] The advantages of this approach are that small molecules can be added to the sample at any time point, and co-expression of proteins that detect specific RNA tags is not required. However, the design of fluorogenic probes with desirable properties is challenging, and imaging of mRNA in live cells using small molecules has not previously been achieved.

In this study, we describe the development of another small-molecule-based method that is potentially useful for the future achievement of live cell imaging of RNA. A series of potential probes were synthesized by coupling a black hole quencher (BHQ1)[5] and a fluorophore (Fig. 1). BHQ1 was conjugated covalently with one of three fluorophores (Alexa594, Cy3, or fluorescein) *via* a PEG linker (Fig. 2; ESI†, Scheme 1). The length of the linker (<30 Å) is within the Förster distance, so that the fluorescence of the probe is quenched. BHQs have broad absorption spectra that span the entire visible region, and can be paired with all common reporter fluorescent dyes. In fact, all of the synthesized fluorophore–BHQ1 conjugates (compounds **2–4**) displayed lower fluorescence than the original fluorophore (ESI†, Fig. S1). We hypothesized that the fluorescence intensity of the probes might be restored by binding an aptamer to BHQ1, which would mask energy transfer from the fluorophore to BHQ1.

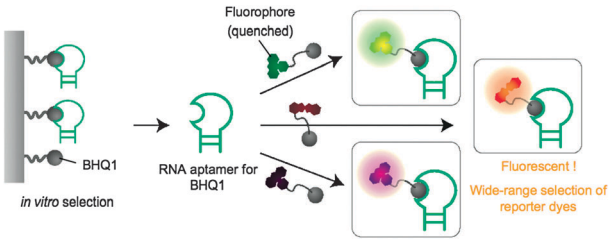

Fig. 1 Functional design of the fluorophore–BHQ1 conjugates.

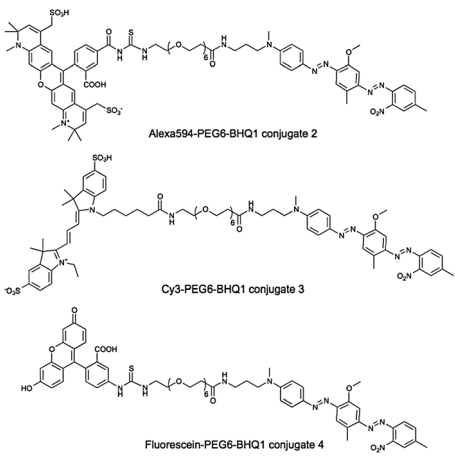

Fig. 2 Structures of fluorophore–BHQ1 conjugates **2–4**.

In vitro selection (SELEX)[6] was performed to isolate aptamers for BHQ1. We prepared an RNA library, containing a 60-base random region flanked by 20-base primer regions on the 5′ and 3′ ends. After 13 rounds of selection against BHQ1-immobilized agarose resins, we obtained four RNA sequences (A1–A4) that shared a conserved sequence of 17 nucleotides, and one RNA sequence (A5) that did not contain

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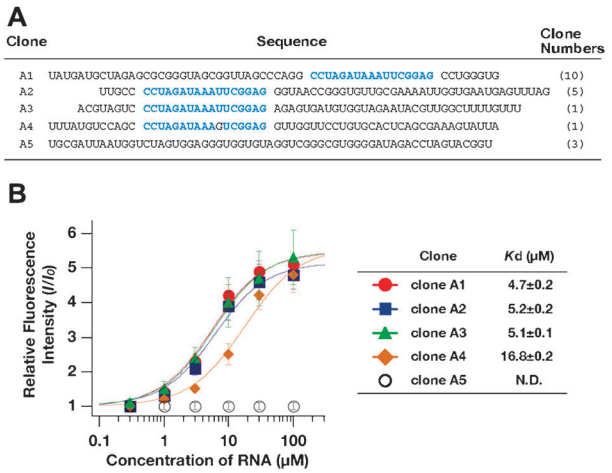

Fig. 3 (A) Nucleotide sequences of the N60 randomized region in RNA selected against BHQ1. The conserved sequences found in clones A1–A4 are shown in blue letters. (B) Fluorescence intensity changes following binding of clones A1–A4 to conjugate **2**.

the conserved sequence (Fig. 3A). The five RNAs were tested for their ability to restore fluorescence of conjugate **2** (Alexa594–BHQ1). A1 had the highest ability to restore fluorescence (4.5-fold increase at 3 μM), with an estimated K_d of 4.7 (Fig. 3B). Although A2 and A3 were less potent than A1, they had similar K_d values. A4, which contained a one-base mismatch in the conserved sequence (AUU *vs.* AGU), had much lower affinity than A1–A3, suggesting that the sequence of the conserved region is important in the recognition of BHQ1. In contrast to A1–A4, A5 failed to enhance the fluorescence intensity of conjugate **2**. Although the sequence of A5 was enriched in the final pool of BHQ1-binding RNA, A5 bound to the agarose resin and not to BHQ1 (ESI†, Fig. S2).

A1 was selected for further analysis. A1 restored the fluorescence intensities of all three fluorophore–BHQ1 conjugates (**2–4**) in a concentration-dependent manner (ESI†, Fig. S4). However, the three conjugates responded differently to A1. For example, the fluorescence intensity of conjugate **3** was enhanced 7.4-fold by 3 μM A1, while that of conjugate **4** was enhanced only 1.9-fold. Conjugates **2–4** had similar quenching efficiencies relative to the unquenched dyes (92%, 98%, and 98%, respectively, at 0.3 μM of each conjugate; ESI†, Fig. S1). Therefore, the different responses to A1 may be due to different dequenching efficiencies of the fluorophores: Alexa594 and Cy3 are better suited than fluorescein for RNA detection.

The aptamers A1–A4 were predicted to form a stem–loop, with the conserved sequence in the loop region (ESI†, Fig. S3). We examined the effects of disruption of the stem–loop structure on the binding affinity of A1 to conjugate **2**. A mutated version of A1 (A1m) was prepared by replacing the 3′-face of the stem GCCUGGG with CGGACCC (Fig. 4A), and subjected to fluorescence titration. The prevention of stem–loop formation caused a decrease in fluorescence restoration of conjugate **2** by A1 (Fig. 4B). Thus, we also prepared a short version of A1 (A1-sh), containing only the stem–loop region, and A1m-sh, its mutated version (Fig. 4A).

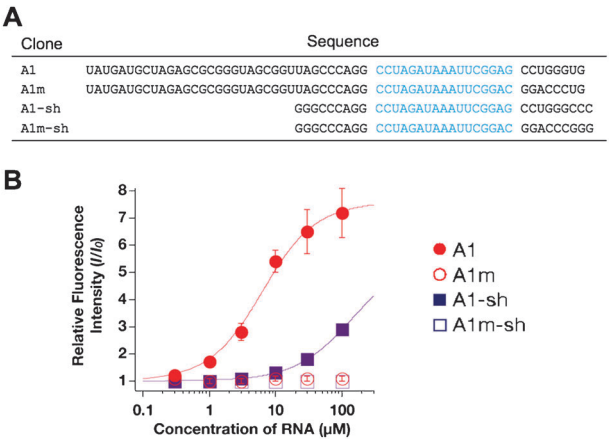

Fig. 4 (A) Nucleotide sequences of A1 and its mutated (A1m) and short (A1-sh and A1m-sh) versions. The conserved sequences are shown in blue letters. (B) Fluorescence intensity changes following binding of A1, A1m, A1-sh or A1m-sh to conjugate **2**.

A1-sh enhanced the fluorescence intensity of conjugate **2**, although to a lesser extent than parental A1 (Fig. 4B; K_d = 142.1 ± 10.8 μM). In contrast, A1m-sh failed to restore the fluorescence of conjugate **2**.

These results indicate that the stem–loop region of A1 is important in binding and masking BHQ1. Perhaps the conserved loop region interacts directly with BHQ1, and the non-conserved stem region may preorganize the loop for the interaction.

To determine if the conjugates could be used for real-time monitoring of transcript levels in a cell-free system, we prepared two DNA templates, each containing a T7 promoter and a downstream region encoding either A1 or A1m (Fig. 5A). The templates were transcribed by T7 RNA polymerase at 30 °C in the presence of conjugate **2**, and fluorescence was measured during the course of RNA synthesis. Fluorescence of conjugate **2** showed a time-dependent increase in response to the expression of transcripts of the A1 template (Fig. 5B); however, expression of A1m had little effect.

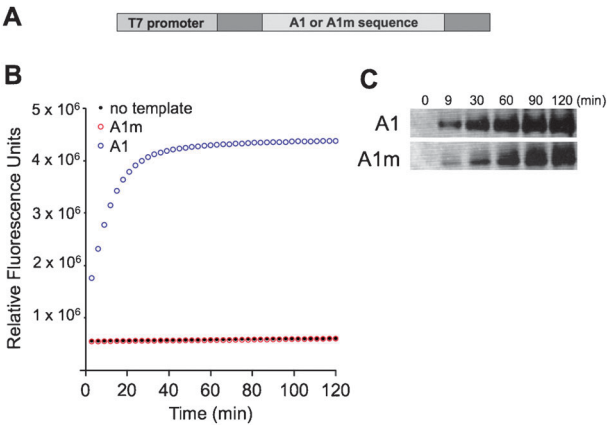

Fig. 5 (A) Schematic diagram of DNA templates. (B) Real-time monitoring of transcripts corresponding to A1 or A1m using conjugate **2**. (C) Time-dependent increase of transcripts synthesized by T7 RNA polymerase from DNA templates encoding A1 or A1m. Gels were stained with ethidium bromide.

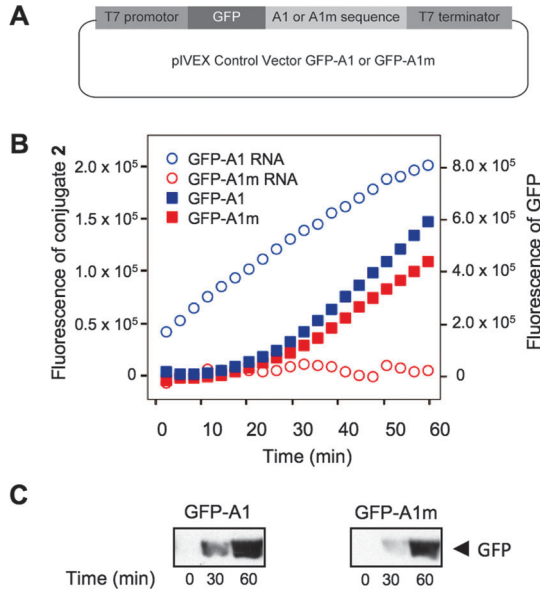

Fig. 6 (A) Schematic diagram of DNA templates for GFP expression. (B) mRNAs encoded by GFP-A1 (blue) or GFP-A1m (red), monitored by 610 nm fluorescence of conjugate **2** (open circles). Protein synthesis monitored by 495 nm fluorescence of GFP (filled squares). Data were normalized to fluorescence in a sample lacking the DNA template. (C) Western blot analyses of GFP expression by anti-His antibody. Loaded amounts of overall proteins are essentially the same (shown in Fig. S9, ESI†).

Similar results were obtained for conjugate **3** (ESI†, Fig. S5). Densitometric analysis of the transcript bands on a gel showed that amounts of A1 and A1m transcripts were similar at given time points (Fig. 5C; ESI†, Fig. S6). Thus, conjugate **2** can be used to monitor specific RNA transcripts in real-time in a cell-free system.

We next examined whether specific protein-coding mRNA transcripts could be detected in cell extracts. We used an expression vector encoding GFP with A1 or A1m in 3′ UTR as a reporter gene (Fig. 6A). The templates were transcribed and translated *in vitro* by a coupled transcription/translation reaction. The fluorescence intensities of conjugate **2** (610 nm) and GFP (495 nm) were simultaneously monitored during transcription and translation. The sample containing the GFP-A1 vector showed an immediate and time-dependent increase in fluorescence of conjugate **2**, while expression of GFP-A1m had little effect (Fig. 6B; ESI†, S7AB). The fluorescence of GFP emerged ~30 min later than that of conjugate **2**, due to the delay in protein synthesis from mRNA. Western blot analysis confirmed that GFP proteins were expressed equally from the A1 and A1m constructs (Fig. 6C; S7C, ESI†). These results demonstrate that conjugate **2** is capable of detecting a specific protein-coding mRNA in cell extracts.

In summary, the present study designed and demonstrated the usefulness of fluorophore–BHQ1 conjugates for real-time detection of specific RNA transcripts in cell-free systems. To apply the methodology to living cells, the quenching efficiency and chemical properties of the Cy3–BHQ1 conjugate need to be optimized by changing the linker length or the structure of the fluorophore. Aptamers with a lower K_d for BHQ1 are also needed for real-time detection of expression of low-abundance mRNAs. Optimization of the stem–loop structure of A1 may generate a short RNA aptamer with a K_d in the nM or pM range.

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