RNA Detection

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Highly Sensitive and Robust Linear Probe for Detection of mRNA in Cells**

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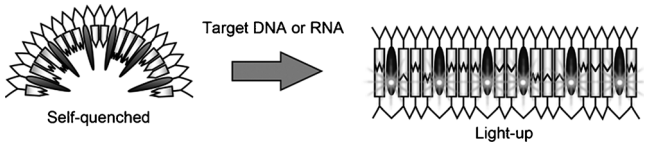
Abstract: *A stemless linear probe was designed that robustly detects mRNA in cells with high sensitivity. The probe is modified at some positions with base surrogates prepared from* D*-threoninol, with anthraquinone moieties near the 5′- and 3′-termini, and with perylene moieties. Even in cell lysate that involves various proteins and enzymes, background emission was very low. When the probe was hybridized with RNA, chromophores are intercalated between the base pairs, resulting in a remarkable light-up signal. The signal-to-background ratio was as high as 1600 under our standard buffer conditions. In the HeLa cell lysate, the linear probe had sufficient signal-to-background ratio (S/B = 40) for reliable mRNA detection. No degradation was observed after a 24 h incubation in HeLa cell lysate. In cells, a probe designed to target DsRed resulted in distinct blue fluorescence only in cells transfected with plasmid encoding DsRed; no fluorescence was observed in control cells.*

Selective labeling is one of the most important tools used to visualize biomolecules, including nucleic acids, proteins, enzymes, and saccharides in cells, during basic research and to diagnose pathogenic tissues.[1,2] Recent investigations have revealed that noncoding RNAs play important roles at both transcriptional and posttranscriptional levels to regulate gene expression.[3] Accordingly, expression of both coding and noncoding RNAs is of interest. Various fluorescent probes have been developed using DNA or peptide nucleic acid (PNA) backbones to detect target DNA and RNA in a sequence-specific manner,[4–6] and most are potentially applicable to the fluorescent monitoring of RNA in living cells. These probes are usually optimized in buffer solution, but living cells contain nucleases, which rapidly degrade these probes, and the cellular milieu may quench or otherwise limit the sensitivity of these probes.

One solution for providing robustness to oligonucleotide probes is the use of modified nucleotides or totally artificial nucleotides. 2′-*O*-Methyl ribonucleotide (2′-OMe), a commercially available modified ribonucleotide that is found in cellular RNAs,[7] has been conventionally utilized to provide nuclease resistance.[8] Replacing natural deoxyribonucleotides in the probe with 2′-OMe prevents rapid digestion in cells.[5e] Similarly, PNA-based probes are intrinsically insensitive to nuclease.[6d] In addition to nuclease resistance, probes may nonspecifically bind to biomacromolecules in the cell, resulting in background emission or a false-positive signal.[9] This is especially an issue with PNA probes due to the hydrophobic nature of these molecules.[10]

Recently, our group has developed new linear probes modified at multiple positions with perylene moieties on D-threoninol scaffolds as illustrated in Scheme 1.[11] In a single-stranded state, the flexibility of the linear probe facilitates self-quenching of the weakly interacting perylenes. Hybridization with the target DNA results in intercalation of perylene between base pairs and a strong fluorescent signal. As the number of introduced perylene increases, both quenching efficiency in the absence of the target and fluorescence intensity in its presence increase. Unlike conventional probes that involve a single fluorophore, emission intensity of this probe is about four-fold higher than monomeric perylene with high quantum yield upon hybridization with target. Unlike probes designed to form a hairpin structure, these probes are linear and facilitate rapid hybridization with the target DNA. One of the advantages of this linear probe design is that base surrogates that can be introduced at any position and in any number.[12] The base surrogates impart nuclease resistance. In this study, we designed and validated linear probes modified with base surrogates prepared from D-threoninol, with anthraquinone moieties near the 5′- and 3′-termini, and with perylene moieties. We show that this type of probe can detect mRNA in cells.



Scheme 1. Illustration of the linear probe.

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In this study, we designed a fluorescent probe targeting a region of the mRNA transcribed from endogenous DsRed-encoding plasmid, shown in Figure 1. We first prepared a linear probe with perylene having ethylene linkers ($\mathbf{E_2}$)

Nt: 5' -CACGCCCTTGAACTTC- 3'
$1E_2$: 5' -CACGCCC$\mathbf{E_2}$TTGAACTTC- 3'
$5E_2$-2: 5' -CAC$\mathbf{E_2}$GC$\mathbf{E_2}$CC$\mathbf{E_2}$TT$\mathbf{E_2}$GA$\mathbf{E_2}$ACTTC- 3'
s$5E_2$-2: 5' - C$\mathbf{E_2}$GC$\mathbf{E_2}$CC$\mathbf{E_2}$TT$\mathbf{E_2}$GA$\mathbf{E_2}$A - 3'
$8E_2$-2: 5' -C$\mathbf{E_2}$AC$\mathbf{E_2}$GC$\mathbf{E_2}$CC$\mathbf{E_2}$TT$\mathbf{E_2}$GA$\mathbf{E_2}$AC$\mathbf{E_2}$TT$\mathbf{E_2}$C-3'
$6E_2$-2: 5'- C$\mathbf{E_2}$AC$\mathbf{E_2}$GC$\mathbf{E_2}$CCTTGA$\mathbf{E_2}$AC$\mathbf{E_2}$TT$\mathbf{E_2}$C -3'
$6E_2$-3a: 5'- $\mathbf{E_2}$CAC$\mathbf{E_2}$GCC$\mathbf{E_2}$CTT$\mathbf{E_2}$GAA$\mathbf{E_2}$CTT$\mathbf{E_2}$C -3'
$6E_2$-3b: 5'- C$\mathbf{E_2}$ACG$\mathbf{E_2}$CCC$\mathbf{E_2}$TT$\mathbf{E_2}$GAA$\mathbf{E_2}$CTT$\mathbf{E_2}$C -3'

2Q-$4E_2$: 5'- C**Q**ACGC$\mathbf{E_2}$CC$\mathbf{E_2}$TT$\mathbf{E_2}$GA$\mathbf{E_2}$ACTT**Q**C -3'

Ds-ORN: 3'- GUGCGGGAACUUGAAG-5'

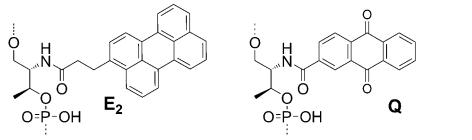

Figure 1. Sequences of the probes, substrate RNA, and chemical structures of the perylene and anthraquinone linked to D-threoninol scaffolds.

that was more suitable for RNA detection than for DNA detection.[11,13] Five $\mathbf{E_2}$ residues were intermittently introduced into a 16-nt long oligonucleotide (**$5E_2$-2**). As a control, a probe with a single $\mathbf{E_2}$ residue (**$1E_2$**) was also prepared. Figure 2 shows the fluorescent emission spectra of **$5E_2$-2**

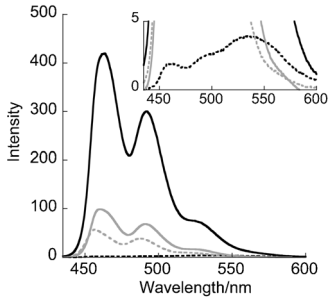

Figure 2. Fluorescence emission spectra of **$1E_2$** (gray) and **$5E_2$-2** (black) in the presence (solid) and absence (dotted) of target **Ds-ORN**. Inset shows magnified spectra. Solution conditions were 1.0 μM probe, 2.0 μM **Ds-ORN**, 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, 20 °C. Quantum yield of the probe/**Ds-ORN** was listed in Table S1.

(black lines) and **$1E_2$** (gray lines) in the presence (solid line) and absence (dotted line) of chemically synthesized target **Ds-ORN** in 100 mM NaCl, 10 mM phosphate buffer, pH 7.0 at 20 °C. Corresponding UV/Vis spectra of **$5E_2$-2** are shown in Figure S1. In the single-stranded state, incorporation of five $\mathbf{E_2}$ remarkably suppressed fluorescence compared with monomeric perylene in **$1E_2$** due to the static contact among these residues (Figure S1).[14] Emission intensity of **$5E_2$-2** was only 1.9, whereas that of **$1E_2$** was 52.3 at 460 nm. Hybridization with the target **Ds-ORN** remarkably enhanced the emission. In the presence of **Ds-ORN** the sample containing **$5E_2$-2** was four-fold brighter than **$1E_2$** because each perylene was separated by intervening base pairs (Figure 2); this is similar to previously described enhancements.[15] The signal-to-background (S/B) ratio of **$5E_2$-2** was 217 under these conditions. A probe containing perylene with a butylene linker that was optimized for DNA detection did not detect RNA under these conditions (Figure S2).

Next, we incubated these probes with HeLa lysate at 37 °C and analyzed them by PAGE to evaluate their resistance to nuclease degradation. **$1E_2$** was immediately digested by the nuclease present in the lysate, whereas **$5E_2$-2** had moderate resistance due to multiply incorporated base surrogates (Figure S3). This enzyme resistance was not sufficient because a cleaved band appeared after 15 min, and the full-length **$5E_2$-2** had completely disappeared after 2 h. Interestingly, digestion seemed to stop at the seventh band from the top. We hypothesized that exonuclease cleaved from both termini and stopped at one nucleotide before the $\mathbf{E_2}$ residue. To prove this hypothesis, we designed **s$5E_2$-2** corresponding to the postulated final product of digested **$5E_2$-2**. As shown in Figure S3c, **s$5E_2$-2** was not degraded even after a 24 h incubation, strongly indicating that it was the final digestion product of **$5E_2$-2**. Thus, a single surrogate at each end protects the probe from nuclease digestion. We thought that 1) introduction of the surrogate at the termini or one nucleotide inside each terminus and 2) further introduction of the surrogates intermittently at inner positions of the sequence should provide sufficient enzyme resistance.

Next, we designed several linear probes that were resistant to nuclease degradation and that had high S/B ratios. **$8E_2$-2** contained eight $\mathbf{E_2}$ residues with two nucleotides between each $\mathbf{E_2}$ residues. Background emission was remarkably suppressed in the single-stranded state due to the efficient self-quenching of perylenes (Figure 3a, red line).

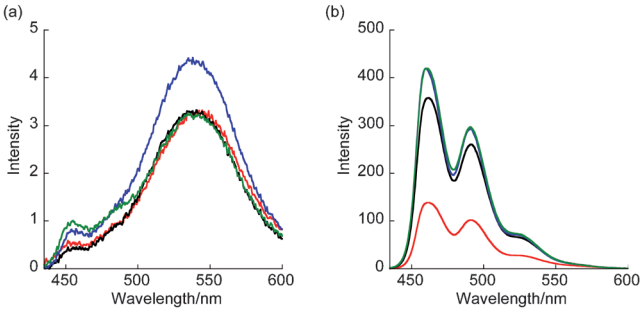

Figure 3. Fluorescent emission spectra of **$8E_2$-2** (red), **$6E_2$-2** (black), **$6E_2$-3a** (blue), and **$6E_2$-3b** (green) a) in the absence and b) presence of target **Ds-ORN**. Solution conditions were 1.0 μM probe, 2.0 μM **Ds-ORN**, 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, 20 °C. Quantum yields of the probe/**Ds-ORN** were listed in Table S1.

This probe was also resistant to degradation by enzymes in HeLa lysate (Figure 4a). However, emission intensity in the presence of the target RNA was similar to that of **$1E_2$**. We first hypothesized that insufficient light-up was due to the low T_m of **$8E_2$-2/Ds-ORN** (Table S1). Hence we designed probes with six $\mathbf{E_2}$s: **$6E_2$-2, $6E_2$-3a**, and **$6E_2$-3b. $6E_2$-2**, which lacked the two $\mathbf{E_2}$ residues in the central portion compared with **$8E_2$-2**, gave almost the same background emission spectrum as **$8E_2$-2**

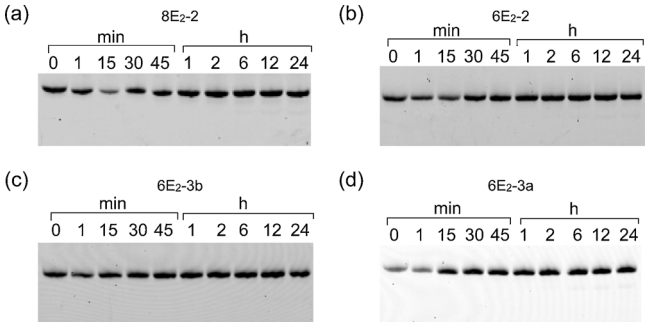

Figure 4. Nuclease resistance of a) **8E$_2$-2**, b) **6E$_2$-2**, c) **6E$_2$-3b**, and d) **6E$_2$-3a**. Probes were incubated with HeLa lysate at 37 °C.

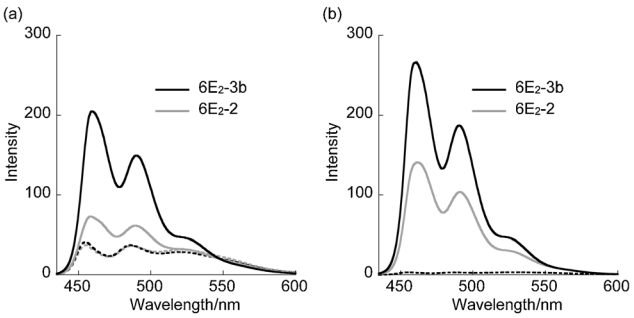

Figure 5. Fluorescence emission spectra of **6E$_2$-2** (gray line) and **6E$_2$-3b** (black line) in a) HeLa lysate or b) buffer. Solid and dotted lines indicate in the presence and absence of **Ds-ORN**, respectively. Solution conditions were 1.0 μM probe, 2.0 μM **Ds-ORN**, 37 °C. Quantum yields and brightnesses of **6E$_2$-2** and **6E$_2$-3b** in the presence of **Ds-ORN** are 0.066, 0.21, 0.90×10^4, and $3.2\times10^4\ M^{-1}cm^{-1}$, respectively.

(Figure 3a, black line). This result indicates that six consecutive nucleotides inserted within the central portion of the probe did not affect self-quenching as long as the **E$_2$** residues were separated by two intervening nucleotides. Similarly, **6E$_2$-3a** and **6E$_2$-3b** with three nucleotides between each **E$_2$** residues efficiently self-quenched. The background intensities of these probes at 460 nm, corresponding to monomeric emission, were slightly higher than those of **8E$_2$-2** and **6E$_2$-2**. This supports our previous conclusion that fewer intervening nucleotides were advantageous for self-quenching. Background emission corresponding to excimer at around 550 nm was almost the same for all tested probes with the exception of **6E$_2$-3a** with six **E$_2$** residues equally separated by three nucleotides. As expected, all the probes with six **E$_2$**s showed remarkable light-up in the presence of target **Ds-ORN** (Figure 3b) with S/B ratios more than 500 (Table S1). Emission intensity of **6E$_2$-3b** was the same as that of **6E$_2$-3**; both had higher emission intensities than that of **6E$_2$-2**. Emission intensity was not necessarily correlated with T_m, which were in the order of **6E$_2$-3a** > **6E$_2$-3b** > **6E$_2$-2**. The additional base pair in **6E$_2$-3a** and **6E$_2$-3b** compared to the other probes may have resulted in stronger intensity. The T_ms of these duplexes were higher than 45 °C. Nevertheless, emission intensities of **6E$_2$-3a** and **6E$_2$-3b** were almost the same below 40 °C, whereas the intensity of **6E$_2$-2** significantly decreased with the temperature even below 40 °C (Figure S4). Although direct contact among the perylenes was inhibited by intervening base pairs, excitonic interaction, which is inversely proportional to the cube of the distance between the chromophores,[16] might result in some quenching. In addition to the decreased T_m, excitonic interaction might have resulted in the relatively low intensity of **8E$_2$-2/Ds-ORN**. We also evaluated the stability of probes involving six **E$_2$**s in HeLa extract; all were resistant to nuclease degradation (Figure 4). **6E$_2$-3a**, with a surrogate at the 5′ terminus was not digested at all. This demonstrated that 5′ terminal modification protects the probe from exonuclease. Hence, high S/B ratios, high emission intensities, and nuclease resistance were attained with six **E$_2$**s introduced intermittently into 16-nt long DNA probe.

We then evaluated the fluorescent properties of **6E$_2$-2** and **6E$_2$-3b** at 37 °C in the cell lysate. In striking contrast to data obtained in buffered solution, background emissions of both probes in the absence of the target increased in HeLa lysate (Figure 5, dotted lines). We hypothesize that hydrophobic perylene moieties were weakly bound by protein in the extract, inhibiting quenching. Emission increased when target RNA was added to the lysate (Figure 5, solid lines), but intensities were lower than those in the buffer. The decreased intensities observed in lysate compared to buffer are likely due to degradation of the target RNA as spectroscopic measurement was started after 5 min of incubation. In fact, the emission intensity decreased with time in the lysate (Figure S5). The apparent S/B ratios for **6E$_2$-2** and **6E$_2$-3b** at $t = 0$ were 2.3 and 6.0, respectively, at 37 °C in the lysate.

To promote quenching in the presence of the cell extract, anthraquinone (**Q** residue), an excellent quencher of perylene,[4f,13] was incorporated into the probe via D-threoninol. To avoid quenching of perylene emission in the duplex state, four nucleotides were inserted between the **Q** and **E$_2$** residues. The probe **2Q-4E$_2$** had little background emission (Figure S6a, dotted line); the emission intensity was also far lower than that of the linear probes without **Q**. Hybridization with the target RNA resulted in an emission intensity (Figure S6a, solid line) similar to intensities of **6E$_2$-2** and **6E$_2$-3b**. These results show that **Q** residues that are separated by four nucleotides from **E$_2$** had little background emission in a single-stranded state and did not quench at all in the duplex. The S/B ratio for **2Q-4E$_2$** was 1600. This may be the most sensitive linear probe ever reported. We also examined sequence-specificity of **2Q-4E$_2$** as well as **6E$_2$-3b**, and found that the introduction of mismatch lowered the emission intensity (Tables S2 and S3).[11] Thus, these linear probes kept sufficient sequence-specificity although many surrogates were tethered. Since both termini were protected by the base surrogates, this probe was resistant to nuclease present in HeLa lysate (Figure S6b). Then, we evaluated the fluorescent property of **2Q-4E$_2$** in lysate. Although the background intensity was higher in the HeLa lysate than in buffer (Figure 6), it was only around 6. In the presence of target RNA in the lysate, the intensity was even higher than that of **6E$_2$-2** or **6E$_2$-3b** (Figure 6a, solid line). The intensity of the signal due to the **2Q-4E$_2$/Ds-ORN** duplex gradually decreased, likely due to nuclease digestion of the target RNA (Figure S5). The apparent S/B ratio of **2Q-4E$_2$** was

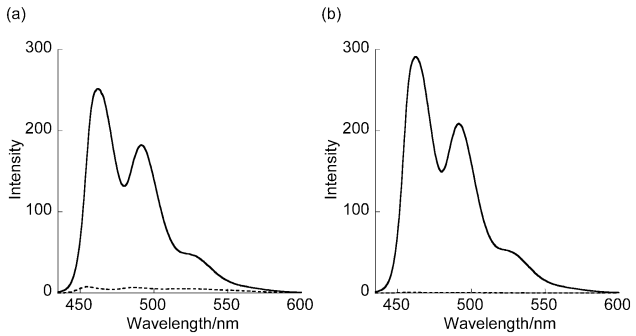

Figure 6. Fluorescence emission spectra of **2Q-4E$_2$** in a) the HeLa lysate and b) buffer. Solid and dotted lines indicate the presence and absence of **Ds-ORN**, respectively. Solution conditions were 1.0 μM probe, 2.0 μM **Ds-ORN**, 37 °C. Quantum yield and brightness of **2Q-4E$_2$** in the presence of **Ds-ORN** are 0.26 and $3.3\times10^4\ M^{-1}\,cm^{-1}$, respectively.

approximately 40 in the lysate at 37 °C, suggesting that this probe should be suitable for use in the living cells.

The most robust linear probe of those tested, **2Q-4E$_2$**, was evaluated in HeLa cells. Here, we transfected plasmid encoding DsRed to the cell, in which DsRed was highly expressed.[17] For delivery of the probe into cells, we used the transfection reagent Lipofectamine. Images were taken 16 h after the probe was transfected into cells to examine the nuclease resistivity in living cells. **2Q-4E$_2$** did not emit light when a plasmid for expression of the target DsRed was not transfected to the cell (Figure 7, top panels).

As expected, no fluorescence was observed when a plasmid for expression of a nontarget, eGFP, was transfected into cells (Figure 7, bottom panels). The same pictures are also obtained in the other cells (Figure S8). Tucker et al. reported that chemical transfection reagents result in entrapment of

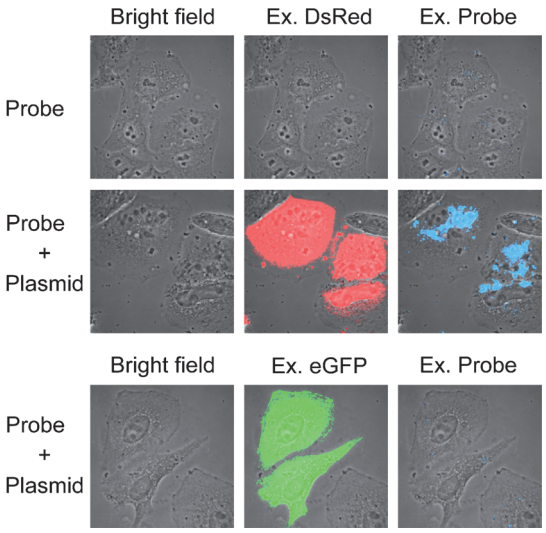

Figure 7. Visualization of mRNA transcribed from DsRed encoding plasmid by **2Q-4E$_2$** using confocal microscopy. DsRed: excitation at 543 nm, collected emission at 555–655 nm. eGFP: excitation at 488 nm, collected emission at 500–600 nm. Probe: excitation at 405 nm, collected emission at 425–475 nm. Magnified images are shown in Figure S7.

DNA in endocytotic vesicles, and DNAs are degraded.[18] We did not observe background emission from perylene, suggesting that the probe was resistant to degradation even in endocytotic vesicles. The results shown in Figure 7 clearly demonstrate that **2Q-4E$_2$** bound to the target after 16 h incubation in living cells.[19] Since anthraquinone is a potent singlet oxygen sensitizer, other quenchers such as azobenzene derivatives might be more suitable in cells.[4f] We also evaluated **6E$_2$-2** and **6E$_2$-3b** in cells (Figure S9). Background emission of these probes in the absence of DsRed seemed slightly higher than that of **2Q-4E$_2$**, probably due to weak binding to proteins in the cells. These probes also emitted blue fluorescence upon cotransfection with DsRed-encoding plasmid, demonstrating these probes were functional in the living cells. This indicates that lysate conditions are not completely reflective of actual cellular conditions.

In conclusion, we have developed a novel linear probe by incorporating perylenes and anthraquinones as fluorophore and quencher into natural DNA. The probe had sufficiently high resistance to enzymatic degradation and affinity to reliably detect target RNA in lysate and in living cells. The robustness of this probe design will allow detection of both DNA and RNA under highly contaminated conditions.

Keywords: D-threoninol scaffold · oligonucleotides · perylene · probes · RNA monitoring

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[19] Similar fluorescent image was also observed when the linear probe was applied to a fluorescence in situ hybridization (FISH) probe (Figure S10).

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