

Hybrid Molecular Probe for Nucleic Acid Analysis in Biological Samples

Chaoyong James Yang, Karen Martinez, Hui Lin, and Weihong Tan*

Center for Research at Bio/Nano Interface, Department of Chemistry and UF Genetics Institute, Shands Cancer Center, University of Florida, Gainesville, Florida 32611

Received March 17, 2006; E-mail: tan@chem.ufl.edu

We have designed and synthesized a hybrid molecular probe for nucleic acid monitoring with high sensitivity and fast hybridization kinetics. This probe has the advantage of no false signals caused by the complex sample matrixes such as living cells. Full deciphering of life processes demands the study of biochemical events within living cells.¹ Although current RNA analysis techniques, including *in situ* hybridization, northern analysis, RT-PCR and microarray technology, have become powerful and indispensable tools in gene expression studies, they reveal little dynamic information on RNA synthesis, transportation, and localization in living cells. To elucidate these important molecular events, RNA has to be observed in real-time and in its native environment. GFP-fused RNA binding proteins² have been successfully used for *in vivo* RNA tagging, but they require reconstruction of transcription. Nucleic acid staining approach³ is simple but lacks specificity. Ideally, a RNA tagging molecular probe should be able to bind target RNA selectively and generate a distinguishable signal with high sensitivity. Molecular beacons (MBs)⁴ are promising in living cell nucleic acid monitoring. MB, a hairpin structure probe, is a dual-labeled oligonucleotide that only fluoresces in the presence of target sequences. The property of detection-without-separation makes MBs ideal probes for living cell monitoring. Several attempts have been reported using MBs to monitor RNA in living cells with various degrees of success.^{5–10} However, when used in living cells, MBs generate false positive signals due to nuclease degradation, protein binding, or thermodynamic fluctuations. False negative signals also exist as a result of sticky-end pairing between hybridized MBs.¹¹ Similar to MBs, quenched auto-ligation (QUAL) probes^{12,13} were designed to be highly sequence specific for nucleic acids and only fluoresce upon target binding. Unfortunately, QUAL probes suffer low temporal resolution due to the slow chemical reaction involved, and false signals result from the hydrolysis of the quencher.

To meet the demand for sensitive and selective monitoring of RNA *in vivo* and to overcome limitations of existing molecular probes, we have developed a new nucleic acid probe, called hybrid molecular probe (HMP). The probe consists of two single-stranded DNA (SS-DNA) sequences tethered to two ends of a poly(ethylene glycol) (PEG) polymer chain (Figure 1). The two SS-DNA sequences, typically 12–25 bases in length, are complementary to adjacent areas of a target sequence in such a way that hybridization of the probe with the target brings the 5' and 3' ends of the probe in close proximity. Depending on the functional moieties labeled on the ends of the probe, the distance change between the probe termini could be exploited by a variety of signaling approaches, including fluorescence resonance energy transfer (FRET),¹⁴ surface enhanced Raman scattering, and excimer light switching.¹⁵ In the case of FRET, a donor fluorophore is attached to one end of the probe and an acceptor fluorophore to the other. The two fluorophores will be away from each other in unbound probes due to the random coil structure of these two DNA strands. Excited upon donor excitation, only the donor gives fluorescence emission. When a target-binding event brings the donor and the acceptor in proximity,

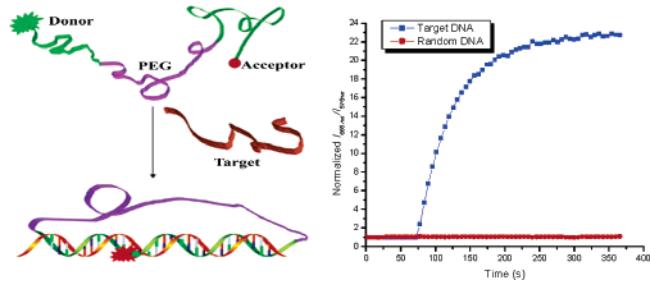


Figure 1. (Left) Schematic presentation of the working principle of a HMP. (Right) Hybridization of HMPTBL to its target (GCTCATCAGCAA AATGAGGGAGGAGTACCCAGACAG) and control (TCTGTGTAATCA ACTGGGAGAATGTAAC TGAC TAGC) in 20 mM Tris-HCl buffer (50 mM NaCl, 5 mM MgCl₂, pH 7.5).

FRET occurs, resulting in quenching of the donor fluorescence and the enhancement of the acceptor fluorescence.

To demonstrate the working principle, the following sequence, HMPTBL, was synthesized to target *Aplysia* tubulin mRNA(516–551): Cy5-CTC ATT TTG CTG ATG AGC-(X)₁₆-CTG TCT GGG TAC TCC TCC-FAM, where X stands for a PEG synthesizing monomer unit (Glen Research). Several criteria were considered when selecting a donor/acceptor pair for the probe for a good signal-to-background ratio. First, the emission spectrum of the donor should overlap with the absorption spectrum of the acceptor. Larger overlap results in higher FRET efficiency. Second, absorption of the acceptor at the donor excitation is negligible, which allows the acceptor to fluoresce only via the FRET process instead of direct excitation. Finally, their fluorescence emission spectra should be completely separated; hence, emission from the donor will not interfere with the acceptor signal. Many organic dye pairs and nanoparticles¹⁶ can be used for FRET. FAM and Cy5 were chosen in this study. Figure 1 (right) shows the response of 300 nM HMPTBL to 300 nM of its target DNA. When excited at 488 nm, the probe emitted strongly around 515 nm (FAM), in the absence of target DNA, with negligible Cy5 emission at 655 nm. Upon addition of the target, fluorescence of FAM decreased and emission of Cy5 increased. No significant signal change was observed from the probe when the same concentration of a control sequence was added, confirming good selectivity of the probe. Data shown in Figure 1 reveals two important advantages of the HMP, detection-without-separation and ratiometric measurement. The Cy5/FAM signal remains low until the probe binds to its target. This “light-up” signaling approach allows the detection of the target sequence without the need to remove unbound probe, a very desirable property for living cell studies or any sealed-tube detection applications. Another advantage of this probe is that it enables ratiometric measurements. By taking the intensity ratio of the Cy5 over the FAM emission, one could effectively eliminate signal fluctuation and minimize the impact of environmental quenching on the accuracy of measurement.

PEG was used in the probe design because of its facile synthesis, controllable length, nontoxicity, and good water solubility. The

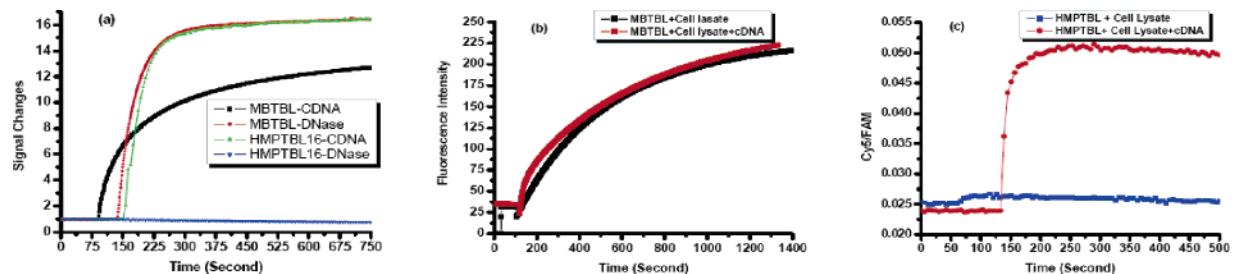


Figure 2. Responses of MB and HMP to non-specific interactions. (a) Responses of 300 nM HMPTBL and MBTBL to 300 nM target cDNA and 3 μ g/ml DNase; and the response of MBTBL and HMPTBL to cell lysate w/ and w/o cDNA; (b) MB cannot differentiate cDNA and cell lysate; (c) HMP can.

highly flexible PEG chain should not interfere with DNA hybridization as long as the linker length is appropriate. The linker in HMP tethers two DNA sequences together, helping them bind to one target molecule instead of to two different target molecules. This tethering also facilitates the hybridization of the probes to the target, as one binding probe will bring the other close to the target for hybridization. Such a synergistic effect also results in a stronger probe/target binding. Indeed, the melting temperature of the HMP/target duplex was found to be about 20 $^{\circ}$ C higher than that of a hybrid of the target with two separate probes without a linker. The HMP also showed a larger linear dynamic response to its target compared with the two separate probes without a linker.¹⁷ This nonproportional response from the latter is because the two separate probes tend to bind to two target sequences when the target is in excess. Another function of the PEG linker is to provide a scaffold for insertion of functional moieties such as biotins for immobilization of HMP, large molecular tags to prevent the probe from nucleus sequestering, or cell membrane penetrating peptides for probe delivery purposes.

The selectivities of the HMP and MB were compared under different conditions. MB has excellent selectivity, especially for single-base mismatched discrimination. An MB named MBTBL targeting the same target sequence of HMPTBL was prepared. It was found that the HMP had a higher signal-to-background (S/B) ratio upon target binding, while the MB had a slightly better single-base discrimination capability. The single-base discrimination capability of HMP could be improved by introducing a hairpin structure into one of the DNA strands.

The interaction of MB and HMP with proteins was investigated. Figure 2a compares the response of MB and HMP to target DNA and nucleases. No false positive was observed when nuclease was added to the HMP solution. In contrast, digestion of MB by nucleases caused an intense false positive signal that was indistinguishable from a true target binding response. Similar results were observed when DNA binding proteins were added to the probe solutions. Both probes interact with proteins, but only MB gives a signal change as the FRET pairs in a MB are separated by the interaction; HMP does not because the FRET pairs have not been brought together. This is critically important when the probes are used in an intracellular environment.

The performance of both HMP and MB were further tested with human cancer cell (CEM) lysate. Blast results against human genome precluded a positive response of probes for the CEM cell lysate. However, MBTBL responded immediately after the addition of cell lysate. With no target sequence in the biological sample, this false positive response could only result from nuclease digestion or nonspecific protein binding. Conversely, the HMPTBL did not give any signal change when cell lysate was added. As a result, MB failed to differentiate cell lysate containing cDNA from cell lysate without cDNA (Figure 2b), while HMP did differentiate these two cellular samples (Figure 2c). Another advantage of the HMP over MB is that it responds to a target sequence faster, as shown

in Figure 2. Unlike HMP, MB has to overcome the energy barrier for dehybridizing the self-complementary stem before hybridizing to its target sequence, which slows down the hybridization.

In conclusion, the HMP developed in this paper responds specifically to its complementary sequence. It allows a rapid detection of a nucleic acid target in a complex sample matrix. This new probe is similar to MB in the following aspects: (1) Both are light-up probes; (2) both can detect unlabeled target without separation; and (3) both are very sensitive and selective in monitoring nucleic acid targets. Compared to MBs, however, this new probe has its own advantages. First, it is easier to design and synthesize. Not every MB designed and synthesized based on target sequence can detect its target,¹⁸ but the HMP can. Second, HMP does not generate any false-positive signals upon digestion by nuclease, binding by proteins, forming complexes by sticky-end pairing, or by other factors. HMP is capable of selectively detecting targets from cellular samples. In addition, the signal generation in HMP can be easily made with ratiometric measurement, minimizing the effect of system fluctuations. Preliminary applications of HMP in monitoring the expression and trafficking of mRNA in single living cells and in developing DNA/mRNA biosensors and biochips are underway.

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Supporting Information Available: (1) Synthesis/optimization of HMP; (2) HMP for surface hybridization study; (3) comparison of HMP and MB. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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