Communication

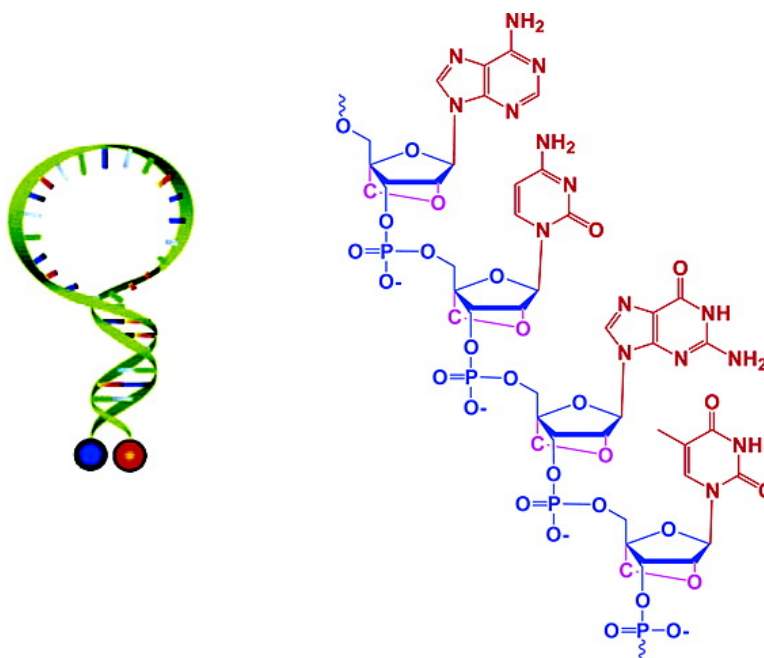# Locked Nucleic Acid Molecular Beacons

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Locked Nucleic Acid Molecular Beacons

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We have designed, synthesized, and investigated a novel molecular beacon (MB) using locked nucleic acid (LNA) bases. Quantitative studies of genomic information have driven a strong demand for advanced biomolecular recognition probes, which have high sensitivity and excellent selectivity. One such probe is the molecular beacon (MB),[1] a short hairpin oligonucleotide probe that binds to a specific oligonucleotide sequence and produces a fluorescence signal (Figure 1a). Due to their high signal-to-background ratio and molecular recognition selectivity, MBs have found broad applications in numerous in vitro hybridization assays.[2] However, when used in the detection and visualization of gene expression in living cells, MBs suffer from problems, such as endogenous nuclease degradation and nonspecific binding by DNA/RNA binding proteins, which result in a false positive signal and significantly limit the MB's general use in complex biological milieu.[3–5] To overcome these difficulties, MBs have been synthesized with nuclease-resistant backbone chemistries, such as negatively charged phosphorothioate,[6] 2'-O-methyl RNA bases,[7] and neutral peptide nucleic acids (PNAs).[8] However, these DNA analogues meet with problems, such as toxicity,[6] self-aggregation,[9] or nonspecific binding by DNA/RNA-binding proteins.

Aiming to design a probe for gene expression in living cells and other complex biological systems, we prepared a fully modified LNA-MB and compared its performance with an analogous MB prepared from DNA monomers (DNA-MB). Both beacons were synthesized with a 19-mer loop and a 6-mer stem, where Cy3 was the 5'-end reporter and DABCYL was the 3'-end quencher (Figure 1b, Supporting Information). We reasoned that a scaffold that differs as much as possible in the geometric and steric properties from ribose, but retains the repeating charge, would be the most likely to retain the desirable solubility and Watson-Crick base-pairing molecular recognition features of natural DNA, while avoiding binding to intracellular DNA/RNA-binding proteins. LNA[10–12] offers one possible implementation of this strategy. LNA contains one or more LNA nucleotide monomers with a bicyclic furanose unit locked in a RNA mimicking sugar conformation (Figure 1c). The methylene bridge connecting the 2'-oxygen of the ribose and the 4'-carbon endows LNA with many attractive properties,[10–12] such as high binding affinity, excellent base mismatch discrimination capability, and decreased susceptibility to nuclease digestion. All of these properties are highly advantageous for diagnostic and therapeutic applications.[13] Our results illustrate that these properties can be conferred to MB probes to make them more robust tools for bioapplications.

Melting temperature measurements were conducted to study the thermostability of both LNA-MB and DNA-MB. Interestingly, the LNA-MB did not open even at 95 °C, as indicated by the constant low fluorescence; while the DNA-MB lost its hairpin conformation
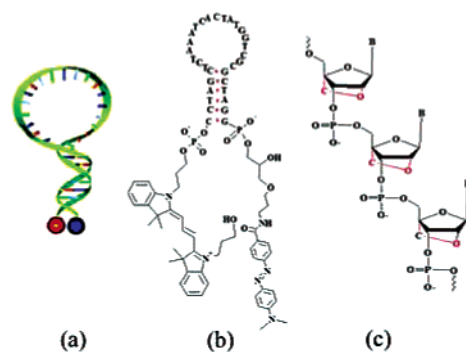

Figure 1. (a) Cartoon and (b) conformational structure of MB and (c) the LNA structure.
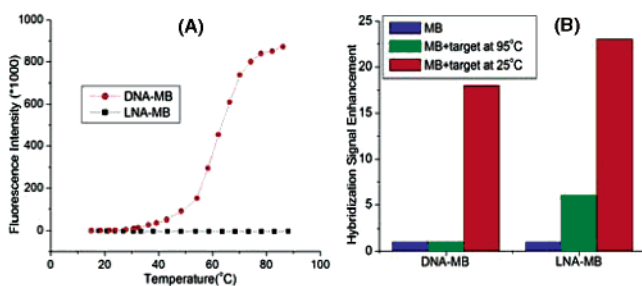

Figure 2. (A) Melting curves for both MBs. The buffer contains 40 mM Tris, 50 mM NaCl, and 5 mM MgCl₂. The temperature was brought to 15 °C and increased at 3 °C increments to 95 °C. (B) Signal enhancement of both MBs after hybridizing with the target at 25 and 95 °C. Final concentration ratio of MB:target = 1:10.

at temperatures above 60 °C (Figure 2A). This observation can be attributed to a tighter binding of the LNA·LNA homoduplex in the stem. To eliminate the concerns that the lack of signal is because the reporter dye was not attached to the stem, the quencher covalently linked to the reporter, or cross-linking of LNA-MB stem, we further added the target to the solution. Figure 2B shows the signal enhancement after hybridizing both MBs with the target at 25 and 95 °C, respectively. LNA-MB not only functioned well under room temperature but also hybridized with the target at 95 °C, implying its exceptionally strong affinity for the target. Such tight binding makes LNA-MB a more efficient molecular probe for in vivo mRNA monitoring as it could bind highly structured mRNA sequences more effectively.

The LNA-MB also displays a selectivity superior to that of DNA-MB. Perfectly matched and single central base mismatched targets were used to compare the single nucleotide polymorphism (SNP) detection capability of both MBs. The short and long dotted lines in Figure 3A display the response difference of both MBs, respectively, to perfectly matched and single base mismatched targets. LNA-MB shows an enhanced discriminatory power, outperforming the DNA-MB. Since our purpose was to compare
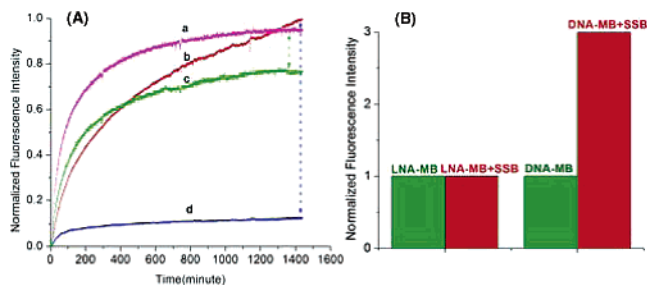
Figure 3. (A) Normalized hybridization kinetics of DNA-MB with (trace a) perfect matched target (PM), (trace c) single base mismatched target (MM), and those of LNA-MB with (trace b) PM and (trace d) MM at 25 °C. Final concentration ratio of MB:target = 1:1. Hybridization buffer: 40 mM Tris, 50 mM NaCl. (B) Response of both MBs to single-stranded DNA binding protein SSB. Final concentration ratio of MB:SSB = 1:5.

the selectivity of both beacons, the experiment was not carried out under optimized stringent conditions to achieve the best SNP performance.[14] By carefully optimizing the assay conditions and fine-tuning the stem and loop lengths, one can achieve better SNP selectivity. It is also worth noting that the hybridization kinetics of this LNA-MB are relatively slow, which might be due to the high energy barrier of opening the LNA stem. By carefully adjusting the stem length, the number of LNA monomers, and the sequence compositions, etc., it is possible to improve the binding kinetics. Indeed, we have found that by shortening the stem length to 3-mer the hybridization proceeded much faster, whereas maintaining the signal-to-background ratio.

In performing intracellular imaging and quantitation of gene expression, degradation of unmodified MBs by endogenous nucleases or structure interruption of MBs by nucleic acid binding proteins result in fluorescence signals unrelated to probe/target hybridization, which significantly compromise detection sensitivity. Consequently, earlier attempts to use MBs for gene detection in living cells have yielded uncertain results.[1,15−17] Our results show that the DNA-MB was degraded rapidly with the addition of DNAase I endonuclease, while the LNA-MB was not degraded under the identical conditions, even after incubation for 24 h (Supporting Information). This remarkable stability will extend the use of MB in monitoring gene expression for longer periods of time. Another striking property of the LNA-MB comes from its resistance to nonspecific protein binding. Normal DNA-MBs are subject to nonspecific binding by proteins, such as the ubiquitous single-stranded DNA binding protein (SSB).[18] Such binding causes a MB to open up and give a false positive signal, as shown in Figure 3B. Interestingly, our results show that the LNA-MB had no response to the addition of excess SSB. Although nuclease-resistant MBs have already been synthesized,[6−8] this is the first report that LNA-MB has no binding with SSB, which makes it well suited to monitor mRNA analytes in vivo since the native biological environment abounds with various proteins.

To illustrate the advantages of the LNA-MB over the DNA-MB, ratiometric gene expression experiments[17] were performed utilizing both MBs in an intracellular environment. The results show that the LNA-MB possesses a significantly lower background than the same concentration of DNA-MB delivered into the cancer cells (Figure 4). Within a single cell containing no complementary target, the LNA-MB showed no fluorescence increase over a period of an hour, while the DNA-MB exhibited a dramatic increase in signal after 30 min due to structure degradation. After binding with the synthetic complement, the completely open conformation of each beacon generates similar levels of fluorescence inside of the cells (Supporting Information). The longer lifetime and extremely low
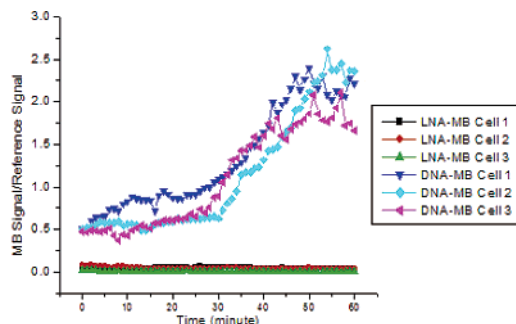

Figure 4. Background signal of both MBs as a function of time after being injected into cells.

background of the LNA-MB suggest that it would make an excellent probe for gene expression analysis at the single cell level.

In summary, engineering MB with an LNA backbone generates a novel LNA-MB with three distinguished features critically important for biological applications, whereas the normal DNA-MB suffers from limitations. First of all, high thermostability of such an LNA-MB probe, combined with the excellent affinity for a complementary sequence, makes this probe especially useful under stringent experimental conditions. Second, the selectivity of a MB can be further enhanced with an LNA backbone, which is of great significance in SNP analysis. Third, LNA-MB resists not only nuclease digestion but also, as first reported here, the binding of SSB, which reduces a false positive signal for MB applications in complex biological environments. As MBs have been widely used in many areas of research, our studies on LNA-MB will provide highly valuable information in improving the performance of MBs. Currently, intensive research using LNA-MBs for in vivo monitoring of multiple genes is being conducted in our lab.

Supporting Information Available: MB and target DNA sequences, cellular experiments, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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