Designing a Novel Molecular Beacon for Surface-Immobilized DNA Hybridization Studies

Xiaohong Fang, Xiaojing Liu, Sheldon Schuster, and Weihong Tan*

Department of Chemistry and UF Brain Institute University of Florida, Gainesville, Florida 32601

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We have designed a biotinylated ssDNA molecular beacon for DNA hybridization studies at a solid interface. DNA hybridization and molecular interaction studies are major tools for the diagnosis of genetic disease, in which the clinical symptoms are linked to alterations in DNA. Identifying these mutations in human genome has become the focus of many research efforts. One recent new development is a novel class of oligonucleotide probes, molecular beacons (MBs). Molecular beacons, first developed by Tyagi and Kramer in 1996,[1] are single stranded oligonucleotide probes that possess a stem-and-loop structure. The loop portion of the molecule can report the presence of a specific complementary nucleic acid.[1−5] The five bases at the two ends of the MB are complementary to each other, forming the stem. A fluorophore and a quencher are linked to the two ends of the stem, as shown in Figure 1. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. When the probe encounters a target DNA molecule, it forms a hybrid that is longer and more stable than the stem, and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the MB undergoes a spontaneous conformational reorganization that forces the stem apart and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence. Therefore, at room temperature, the MBs emit an intense fluorescent signal only when hybridized to their target molecules.[1−8] The size of the loop and its content can be varied by designing different MBs. Also, the quencher and the fluorophores can be changed according to the problem studied. There have been a variety of applications of MBs,[1−8] including the real-time monitoring of polymerase chain reactions,[1] and even the investigation of HIV-1 disease progression.[4,5] MBs have extremely high selectivity with single base pair mismatch identification capability. They hold great promise for studies in genetics, disease mechanisms, and molecular interactions, for applications in disease diagnostics, and in new drug development.

It is expected that there will be many interesting applications for surface-immobilized molecular beacons. So far, MBs have only been used in a homogeneous liquid solution. This limits the applications of MBs in in vivo biomedical studies and in DNA biosensor development. To fully explore the potentials of MBs, we have designed a biotinylated ssDNA MB, shown in Figure 1, which is intended for immobilization onto a silica surface for a variety of applications. The MB has a total of 28 bases, of which 18 bases are the sequence of interest and 5 base pairs form the stem.

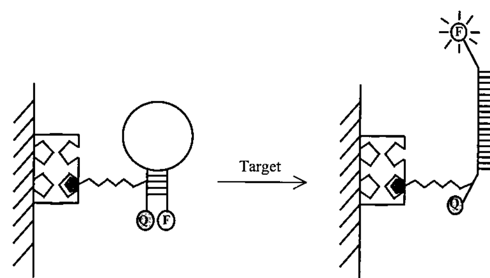

Figure 1. Schematic of the operation of a biotinylated MB immobilized on a solid surface. Biotin is added to the stem of the molecular beacon for surface immobilization with avidin. The MB is nonfluorescent since the stem hybrid keeps the fluorophore (F) close to the quencher (Q). When the probe sequence in the loop hybridizes with its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring the fluorescence of the fluorophore. In our MB design, TMR is used as the fluorophore and DABCYL as the quencher.

The biotinylated ssDNA molecular beacon has been synthesized with tetramethylrhodamine (TMR) as the fluorophore and DABCYL (dimethylaminoazobenzen aminoexal-3-acryinido) as the quencher. DABCYL, a nonfluorescent chromophore, serves as a universal quencher for any fluorophore in MBs.[2] There are five important considerations in MB design. First is the functional group for surface immobilization. One of the most common ways for biomolecule immobilization onto a solid surface is through biotin−avidin binding.[9,10] The biotin−avidin linkage to a surface is suitable for DNA hybridization. Since the 5′and 3′ends are linked to a fluorophore and a quencher, respectively, adding a biotin functional group to the MB is the easiest strategy to attach the MB to a surface. Second is the position for biotin binding. We tried different positions to link biotin: the loop sequence, the second base pair position of the fluorophore side of the stem, and the same position on the quencher side of the stem. We chose to link biotin to the quencher side of the stem to minimize the effects biotin might have on fluorescence, quenching, and hybridization of the MB. Third is the length of the stem and the loop sequence. Most MB studies[1−8] indicate that a 15−25 base sequence together with a 5 base pair stem is an excellent balance. We chose an 18 base sequence. The 5 base pair stem is strong enough to form the hairpin structure for efficient fluorescence quenching, while it is still weak enough to be dissociated when a complementary DNA hybridizes with the 18 base loop of the MB. Fourth is a spacer between biotin and the sequence. We used a biotin-dT to provide an easy access for target DNA molecules to efficiently interact with the loop sequence and an adequate separation to minimize potential interactions between avidin and the DNA sequence. Fifth is the fluorophores. So far most of the MB are based on fluorescein.[1] It is known that rhodamine dyes have higher quantum yields and are much more photostable than fluorescein in fluorescence detection. If bulky samples are used, photobleaching of fluorescein may not be critical. However, when an ultratrace amount of MB is used or the MBs are immobilized on a surface, photobleaching will become a more severe problem. This is the reason most of the single-molecule fluorescence detections have been carried out on rhodamine dye molecules.[11] In addition, fluorescence intensity of fluorescein is highly dependent on the pH used in a sample matrix. There is one

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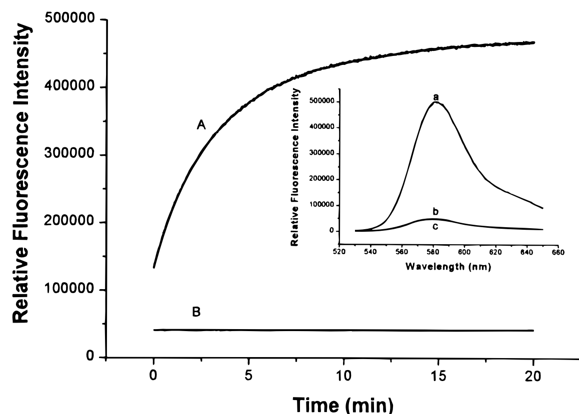

Figure 2. Biotinylated MB hybridization results obtained in solution for both target DNA (A) and noncomplementary DNA (B). Insert: fluorescence spectra of MB hybridization at room temperature with excitation at 515 nm with complementary DNA (a), with noncomplementary DNA (b), and with the buffer (c), 20 mM Tris-HCl, 50 mM KCl, and 5 mM $MgCl_2$, pH = 8.

potential disadvantage in using rhodamine dyes. The overlap between the emission spectrum of fluorescein and the absorption spectrum of DABCYL is better than that for rhodamine dyes.[2] We have thus designed the following MB for surface immobilization

5′-(_TMR) GCA CGT CCA TGC CCA GGA AGG AAC G(Biotin dT)G C(_DABCYL)-3′

The MB was synthesized using DABCYL-derived CPG (controlled pore glass) as the starting material. The synthesis was started at the 3′ end of the MB. The nucleotides were added sequentially, using standard cyanoethyl-phosphoramidite chemistry, including a biotin-dT residue that had biotin attached to the C5 carbon of the ring. It is this biotin which links to avidin on a solid surface. The final phosphoramide was 5′-amino modifier C6. This produced a primary amine group at the 5′ end which is linked to the phosphodiester bond by a six carbon spacer arm. This amino group was used for the labeling of TMR. The synthesized olignucleotide was purified by gel filtration chromatography and reverse phase HPLC. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to confirm the synthesis of the designed MB. The result showed only one product with a molecular weight of 10 085.7. The difference between the measured molecular weight and the calculated one, 10 076, is less than the typical error, 0.2%, in mass spectrum measurement.

The newly synthesized biotinylated MBs have been used for DNA hybridization in solution. Their hybridization properties were tested by means of fluorescence measurements performed on a SPEX Industries F-112A spectrophotometer. Three 50 nM MB solutions were prepared: the MB only, the MB and a 5-fold molar excess of its complementary DNA, and the MB and a 5-fold molar excess of a noncomplementary DNA. After incubation with their complementary DNA molecules for 20 min, the biotinylated MB hybridizing inside the solution shows a more than 10-times enhancement in fluorescence signal (Figure 2, insert). Theoretically, the enhancement could be as high as more than 200-fold with optimal design of the sequence and under optimal hybridization and optical detection conditions.[1] The solution with the noncomplementary DNA has no enhancement under the same conditions. The enhancement factor of the biotinylated MB after hybridization is high enough for a variety of applications. Hybridization dynamics of the biotinylated MB has also been investigated, and results similar to that of MB without biotin attached have been obtained (Figure 2). It is worth noting that the MB without biotin attached can only be used in homogeneous solution experiments.

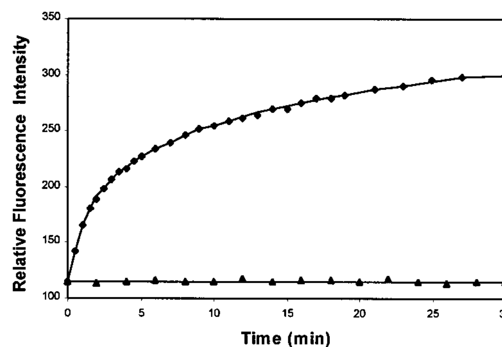

Figure 3. Immobilized MB hybridization kinetics study. Real-time measurements of the hybridization dynamics of immobilized MBs were obtained with target DNA molecules (♦) and noncomplementary DNA molecules (▲).

To immobilize the MB onto a solid silica surface, the surface first binds avidin by physical absorption. The physically adsorbed avidin are stabilized by a cross-linking agent, glutaraldehyde.[12] The avidin-conjugated glass surface then binds with the biotinylated ssDNA MB, creating a DNA molecule-covered surface.[13] The binding process is fast and efficient. Within a few minutes, an equilibrium coverage has been reached. Immobilized MB stay with the surface even after immersion inside buffer solution for a few days. There has not been any leakage of the immobilized MBs.

The immobilized MB has been hybridized with its complementary DNA. Fluorescence signal monitoring was achieved with a highly efficient setup for ultrasensitive optical detection. Excitation laser beam, 514 nm, was first directed to an optical fiber and was then coupled to a prism which was put on the stage of the microscope. Evanescent field was generated on the surface of the prism which was sandwiched with the MB immobilized silica plate glass, and used to excite the immobilized MB. Fluorescent signals thus produced were collected by an objective and directed to an intensified CCD. MB fluorescence intensities were monitored under different hybridization conditions. As shown in Figure 3, the newly synthesized MB is highly efficient in DNA hybridization after it is immobilized on the solid surface. We carried out MB probe testing with different concentrations of complementary DNA molecules, ranging from 5 to 600 nM. Our results indicate the MB-immobilized plate can be used to detect target DNA molecules in the subnanomolar range. In addition, preliminary experiments have shown that the immobilized DNA molecules on the plate can be regenerated after hybridization. Therefore, we will be able to reuse the plate multiple times for DNA detection and interaction studies.

In summary, we have designed a new MB for DNA immobilization and thus for DNA hybridization studies at a solid interface. The MB is a single-stranded biotinylated oligonucleotide probe that can detect the presence of specific nucleic acids in both homogeneous solutions and heterogeneous environments. It has been immobilized on a solid surface for hybridization study and has shown excellent sensitivity for DNA detection with subnanomolar concentration detection limit. Presently, we are using the new MB and a nanometer-scale optical biosensor technology[14] for the development of DNA sensors for single living cell monitoring and DNA molecular dynamic interaction studies. We expect MBs to be able to function as high-specificity, high-sensitivity recognition and signaling elements in state-of-the-art biological detection strategies for biomolecules.

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