Molecular Beacons: A Novel DNA Probe for Nucleic Acid and Protein Studies

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Abstract: A new concept has been introduced for molecular beacon DNA molecules. Molecular beacons are a new class of oligonucleotides that can report the presence of specific nucleic acids in both homogeneous solutions and at the liquid–solid interface. They emit an intense fluorescent signal only when hybridized to their target DNA or RNA molecules. Biotinylated molecular beacons have been designed and used for the development of ultrasensitive DNA sensors and for DNA molecular interaction studies at a solid–liquid interface. Molecular beacons have also been used to study protein–DNA interactions. They have provided a variety of exciting opportunities in DNA/RNA/protein studies.

Keywords: DNA recognition · DNA hybridization · fluorescence spectroscopy · molecular beacons · proteins · RNA

Introduction

Molecular beacons are a new class of DNA probes. Since first developed in 1996,[1] they have been providing a variety of exciting opportunities in DNA/RNA studies. Molecular beacons are single-stranded oligonucleotides that possess a stem-and-loop structure (Figure 1). The loop portion of the molecular beacons can report the presence of a specific complementary nucleic acid. The stem has five to seven base pairs which are complementary. A fluorophore and a quencher are linked to the two ends of the stem. The stem keeps these two moieties in close proximity, causing the fluorescence of the fluorophore to be quenched by energy transfer. When the probe encounters a target DNA molecule, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, leading to the restoration of fluorescence. The conformational state of a molecular beacon is thus directly reported by its fluorescence: in the closed state, the molecular beacon is not fluorescent; in the open state, when the fluorophore and the quencher molecules are apart, it emits intense fluorescence. Different molecular beacons can be designed by choosing loop sequence and size. Also, the quencher and the fluorophores can be changed according to the problem studied.[2] Recently, a biotinylated ssDNA molecular beacon has been designed for DNA hybridization studies at a liquid–solid interface and for the development of a ultrasensitive DNA biosensor.[3] Molecular beacons are useful in situations where it is either not possible or not desirable to isolate the probe–target hybrids from an excess of the hybridization probes, such as real-time monitoring of DNA/RNA amplification reactions and the detection of RNA within living cells.[4–10] Molecular beacons have shown many advantages over other DNA probes. These include the extremely high selectivity with single base pair mismatch identification capability, the excellent capability of studying biological process in real time and in vivo, and avoiding the inconvenience caused by using DNA intercalation reagents or by labeling the target molecules or using competitive assays.

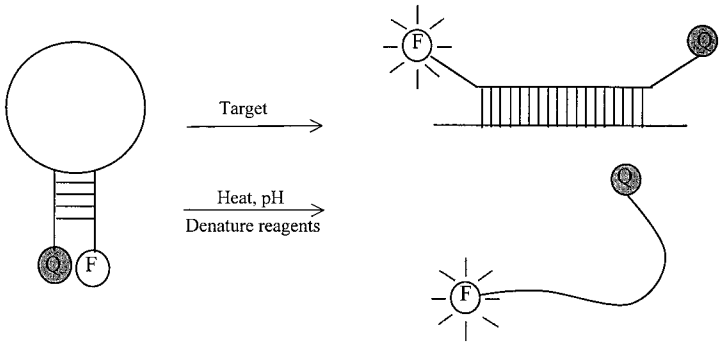

Figure 1. Mechanism of operation of molecular beacons. On their own, these molecules are non-fluorescent, because the stem hybrids keep the fluorophores close to the quenchers. Molecular beacons emit intense fluorescence only when the stems are apart through hybridization of DNA molecules with sequences complementary to their loop sequences or through unwinding the stem hybrids by increasing temperature or using denaturing reagents.

Molecular Beacon Synthesis

Molecular beacon preparation: The molecular beacon sequence can be chosen for specific targets and for specific

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applications. Molecular beacons are synthesized by using dimethylaminophenylazobenzoic acid controlled pore glass (DABCYL-CPG) as the starting materials. DABCYL, a nonfluorescent molecule, serves as a universal quencher for many fluorophores in molecular beacons.[2] There are four important steps in this synthesis.[1, 3] 1) A CPG solid support is derivatized with DABCYL and used to start the synthesis at the 3′-end. The rest of the nucleotides are added sequentially by using standard cyanoethylphosphoramidite chemistry. 2) A primary amine group at the 5′-end is linked to the phosphodiester bond by a six-carbon spacer arm. There is a trityl protecting group at the ultimate 5′-end that protects the amine group. 3) The oligonucleotide is hydrolyzed and removed from the CPG, then purified by reversed-phase HPLC. 4) The purified oligonucleotide is removed from the trityl group and labeled with a fluorophore. After labeling, the excess dye is removed by gel filtration chromatography on Sephadex G-25. The oligonucleotide is then purified again by reversed-phase HPLC. The main peak from the HPLC run is collected. The synthesized molecular beacon is characterized by UV and by mass spectra.

Design of a biotinylated ssDNA molecular beacon for surface immobilization: A biotinylated ssDNA molecular beacon with tetramethylrhodamine (TMR) as the fluorophore and DABCYL as the quencher has been designed and synthesized recently.[3, 11] There are five important considerations in the design of biotinylated ssDNA molecular beacons:

1) The functional group used for surface immobilization: One of the most common ways for biomolecule immobilization onto a solid surface is through biotin–avidin binding.[12] Since the 5′- and 3′-ends of a molecular beacon are linked to a fluorophore and a quencher, respectively, adding a biotin functional group to one base in a molecular beacon is the easiest strategy for the attachment of the molecular beacon to a surface.
2) The position for biotin binding: It is highly desirable to link biotin to the quencher side of the stem to minimize the effects biotin might have on the fluorescence of the molecular beacon, quenching, and hybridization.
3) The length of the stem and the sequence: Most molecular beacon studies[1, 2, 5–8] indicate that an excellent balance is achieved with 15 to 25 base sequences together with a 5–7-base-pair stem. A 16-base loop sequence has been designed. The 6-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 loop of the molecular beacon.
4) A spacer between biotin and the sequence: 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.
5) The fluorophores: When an ultratrace amount of molecular beacon is used or the molecular beacons are immobilized on a surface, photobleaching of the fluorophore will become a severe problem. A rhodamine dye is used for the molecular beacon as it has high quantum yields and is photostable in fluorescence detection. In addition, fluorescence intensity of rhodamine is independent on the pH used in a sample matrix. A biotinylated molecular beacon, 5′-(TMR) GCA CGT CCA TGC CCA GGA AGG AAC G(Biotin dT)G C(DABCYL)-3′, has thus been designed for surface immobilization. The newly synthesized molecular beacon has been characterized by mass spectra and its structure has been confirmed by molecular weight. The biotinylated ssDNA molecular beacon has been successfully immobilized on a silica surface or at the tip of an optical fiber probe.[3, 11, 13]

Hybridization Studies of Molecular Beacon

Hybridization study in solution: Molecular beacons have been used for DNA hybridization in solution using fluorescence measurements performed on a spectrofluorometer. Four sample solutions were used: 0.1 μM molecular beacon with a fivefold molar excess of one of the following four solutions: its complementary DNA, a one-base mismatched DNA, a two-base mismatched DNA, or a non-complementary DNA. The solutions were incubated for 15 min in a hybridization buffer (20 mM Tris-HCl, 50 mM KCl, and 5 mM $MgCl_2$, pH 8.0). Emission fluorescence at 580 nm was recorded at room temperature with excitation at 530 nm from a xenon lamp in the spectrometer. The hybridization of the molecular beacon with its complementary DNA inside the solution has shown a more than 10 times fluorescence enhancement. 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 enhancement factor of the molecular beacon after hybridization is high enough for a variety of applications. Hybridization dynamics of the biotinylated molecular beacon has also been investigated, and similar results to that of a molecular beacon without biotin attached have been obtained.[3]

There is an obvious difference in the fluorescence enhancement when different DNA molecules hybridize with the molecular beacon. Compared with the complementary DNA, the one-base mismatched DNA resulted in a much lower fluorescence restoration, while the two-base mismatched DNA and the non-complementary DNA had no fluorescence restoration (Figure 2). This clearly demonstrates the excellent

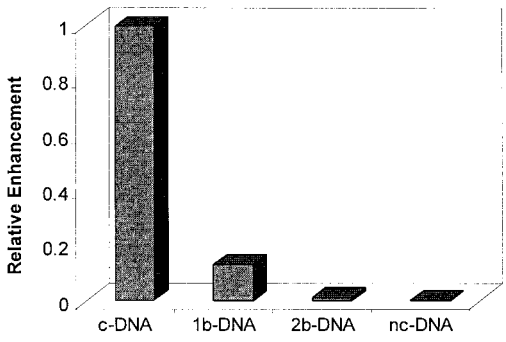

Figure 2. The relative fluorescence enhancement of the molecular beacon when hybridized with different DNA molecules: c-DNA: complementary DNA; 1b-DNA: one-base mismatched complementary DNA; 2b-DNA: two-base mismatched complementary DNA; nc-DNA: non-complementary DNA.

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selectivity of molecular beacons in the identification of its target DNA molecules. The specificity of molecular beacons is higher than that for a linear probe due to the existence of the molecular beacon's hairpin structure.[2] Tyagi and Kramer[1] reported that there was no fluorescence in the presence of a one-base mismatched oligonucleotide. The discrimination in the molecular beacon hybridization between perfectly matched and single-base mismatched DNA relies on the stability of the newly formed DNA duplexes. The length of the hybridization sequence, GC contents, the location of mismatch bases in the sequence, and the hybridization temperature all have strong effects upon duplex stability.[1, 2] Therefore, high specificity with one-base mismatch DNA identification capability is obtainable by using a carefully designed molecular beacon probe and the optimized hybridization conditions.

Hybridization of immobilized molecular beacons: The newly synthesized biotinylated molecular beacons can be immobilized on a silica surface through the avidin-biotin binding.[3, 12] A clean silica coverslip was treated and then incubated in avidin solution (0.1 mg mL^{-1}, 10 mM phosphate buffer, pH 7.0) for 12 h. The physically adsorbed avidin was stabilized by cross linking in 1 % glutaraldehyde solution for 1 h, followed by incubating in 1M Tris/HCl (pH 7.5) for 3 h. The avidin-coated coverslip was washed by the phosphate buffer and dried under nitrogen. A drop of a biotinylated molecular beacon solution (1×10^{-6} M in the buffer) was added to the avidin-coated coverslip. The avidin–biotin binding was quick and usually only took a few minutes. After immobilization, the coverslip was then washed by the hybridization buffer to remove any unbound molecular beacon. The binding process is quick and efficient.

An immobilized molecular beacon has been hybridized with its complementary DNA. Fluorescence signal monitoring was achieved with a highly efficient optical detection setup.[14] The system consists of a fluorescent microscope, an intensified CCD (ICCD), an Ar^+ laser (515 nm laser beam), an optical fiber probe for light transmission to the microscope stage, and a prism to generate an evanescent wave to excite the molecular beacon immobilized on the silica plate surface. The results from real-time measurements of the hybridization dynamics (Figure 3) indicated that the biotinylated molecular beacon is efficient for DNA hybridization. We have also carried out molecular beacon probe testing with different concentrations of complementary DNA molecules. The results show a good linear relationship between the initial hybridization rate and the concentration of the complementary DNA.[11] The molecular beacon immobilized plate can be used to detect target DNA molecules in the subnanomolar range.

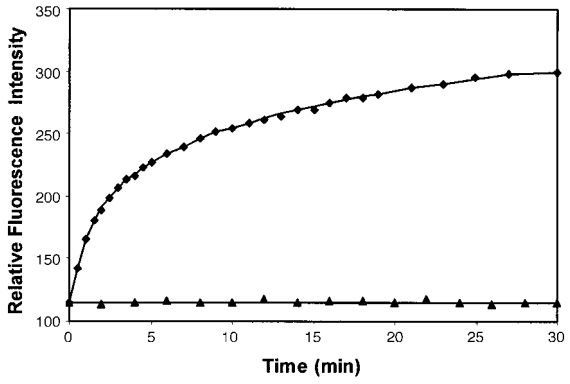

Figure 3. Immobilized molecular beacon hybridization kinetics study. Real-time measurements of the hybridization dynamics of an immobilized molecular beacon were obtained with target DNA molecules (◆) and non-complementary DNA molecules (▲).

Molecular Beacon Biosensors

Ultrasensitive fiber optical DNA sensors: An immobilized molecular beacon can be used for the preparation of a variety of optical fiber DNA sensors such as a submicrometer sensor and a fiber optic evanescent wave sensor.[13] Coupled with highly sensitive optical imaging and detection methodologies,[14] molecular beacon DNA optical fiber sensors have been developed with fast response time and ultrasensitivity. The microscopic optical fiber probe is fabricated by using either pulling or etching technologies.[15, 16] The fiber optic evanescent wave sensor is made by exposing the core surface of an optical fiber through chemical etching. Evanescent wave generated on the core surface is used for fluorescence excitation in the longitudinal surface of the fiber. Biotinylated molecular beacons have been immobilized onto these two types of optical fiber probes. The preparation process is similar to the molecular beacon immobilization on a silica surface.[3, 11] The sensors are stable, reproducible, regenerable, and have remote detection capability. Both molecular beacon biosensors have been applied to detect specific mRNA sequences amplified by polymerase chain reaction.

Multiple analyte molecuar beacon sensor on a silica surface: The immobilization method for biotinylated molecular beacons enables us to develop molecular beacon probe arrays on a silica surface.[11] By using a CCD camera as a fluorescence detector, we can make use of the spatially resolved imaging capability of the CCD camera to detect multiple target oligonucleotides simultaneously. To demonstrate the feasibility, we have immobilized two different molecular beacon (MB) probes on a coverslip: MB1: 5′-(TMR) GCA CGT CCA TGC CCA GGA AGG AAC G(Biotin dT)G C(DABCYL)-3′ and MB2: 5′-(TMR) CCT AGC TCT AAA TCG CTA TGG TCG CGC(Biotin dT)AG G(DABCYL)-3′. Two small drops of MB1 and MB2 solutions were deposited on an avidin-coated coverslip by a pulled glass micropipette. The diameters of the spots were about 300 to 400 μm. After the immobilization of MB1 and MB2 onto the surface, the coverslip was washed and dried. The fluorescence intensities of the two spots were monitored by the ICCD/fluorescence microscope. When a solution of a complementary DNA molecule to MB1 was used, hybridization took place only at the MB1 spot (Figure 4). It was also true that only the fluorescence intensity of the MB2 spot was increased when the corresponding complementary DNA to MB2 was used. Therefore, different moecular beacon probes can be immobilized on the same silica plate and maintain their selectivity for the detection of target DNA molecules.

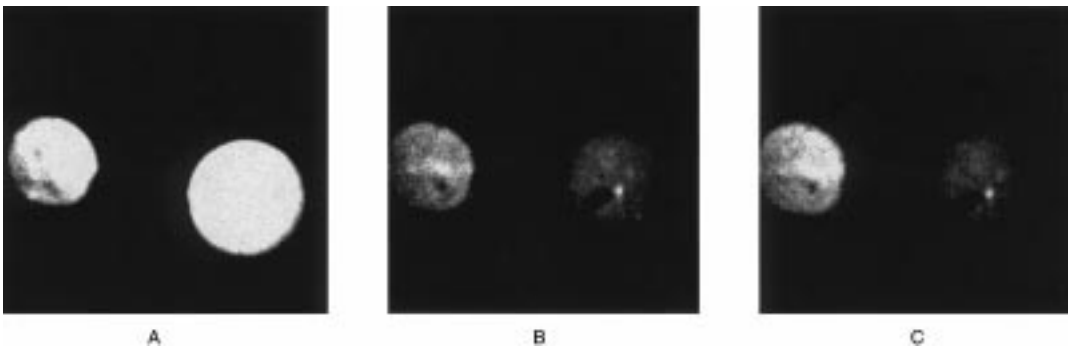

Figure 4. Optical image of two immobilized molecular beacon sensor areas on a silica surface (A); fluorescence images of the molecular beacon sensor areas after adding MB1's complementary DNA for 1 min (B) and 5 min (C).

Molecular Beacon Applications

There have been a variety of applications of molecular beacons in DNA/RNA studies, including sensitive monitoring of polymerase chain reactions, real-time detection of DNA–RNA hybridization in living cells, DNA mutation analysis. Here are a few examples.

Real-time monitoring of polymerase chain reaction (PCR): Molecular beacon probes are most suitable for real-time monitoring DNA/RNA amplification during the PCR.[1, 2] When the temperature is lowered to allow annealing of primers, the molecular beacon will not be fluorescent due to their stem hybrids. However, some of the molecules of the molecular beacon will bind to the target amplicons to generate fluorescence. The PCR reaction can only be monitored at lower temperature, since the molecular beacon itself dissociates at higher temperature where molecular beacons do not interfere with polymerization. Therefore, hybridization of molecular beacons to their targets takes place only in the annealing step of every cycle, and the resulting fluorescence directly indicates the concentration of the amplicon in the PCR process. The assay is fast, sensitive, and nonradioacticive. The measurement is carried out in sealed PCR tubes, thus avoiding carryover contamination. Rapid detection of the PCR has also been achieved by using surface immobilized PNA–DNA molecular beacon probes.[8] Amplicons can be detected by adding a PCR product in a micrometer-well containing the previously immobilized probe, and reading the generated fluorescence. No further transfers or washing steps are involved.

RNA detection in living cells: Demonstrating hybridization between an antisense oligodeoxynucleotide and its mRNA target and monitoring the changes of a specific messenger mRNA in living cells have long been a challenge in antisense research and in biological studies. The molecular beacon probe opens a way to solve these problems. A molecular beacon probe containing a 15-nucleotide-long antisense sequence for human basic fibroblast growth factor (bFGF) has been used to visualize the bFGF mRNA in the human trabecular cells.[9] The molecular beacon probe was carried by liposomes and was delivered into the cells. The real-time detection of molecular beacon/target mRNA hybridization in living cells has also been attempted by microinjecting the molecular beacon probes into K562 human leukemia cells.[6] Within 15 min, confocal microscopy revealed fluorescence in cells injected with target, but not the control sample. These studies suggest the possibility of real-time visualization and localization of oligonucleotide/mRNA interactions. The molecular beacon probe is expected to be useful in finding targetable mRNA sequences under physiologic conditions and in studying RNA processing, trafficking, and folding in living cells.

Genetic analysis: Molecular beacon probes have a high selectivity with one-base mismatch identification capability.[1] Combining with amplification techniques for DNA/RNA, they are becoming promising probes for genetic analysis. A method called "spectral genotyping" has been developed for the detection of genetic mutations.[4] In this method, alleles were identified by fluorescent colors generated in sealed amplication tubes. Two molecular beacon probes with different fluorophores were used: one specific for a wild-type allele and another for a mutant allele. The fluorescence measured at two different wavelengths during the amplification indicated whether the sample contained the homozygous wild-types, homozygous mutants, or heterozygotes. The alleles of β-chemokine receptor 5 gene that determined susceptibility to the HIV-1 were distinguished by this method. HIV disease progression has also been investigated.[5] Molecular beacons have also been used to detect a point mutation related to cardiovascular disease and neural tube defects,[6] to analyze an 81-bp region of the gene for mutations that confer resistance to the antibiotic rifampin.[10]

Protein studies: Recently, molecular beacons have been used for studying the protein–DNA interaction.[17] The interaction between single-stranded DNA binding (SSB) protein and molecular beacon DNA molecules has resulted in significant molecular beacon fluorescence restoration. The fluorescence enhancement brought about by SSB protein and by complementary DNA is very comparable. The molar ratio of the binding between SSB protein and the molecular beacon is 1:1 with an SSB protein–molecular beacon binding constant of around $2 \times 10^7\,\text{M}^{-1}$. The detection limit of SSB is $2 \times 10^{-10}\,\text{M}$

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when a conventional spectrometer with a xenon lamp is used. Preliminary results also show that there are significant differences in molecular beacon binding affinity by different proteins, which will constitute the basis for highly selective bioassay of a variety of proteins.

Future Outlook

Molecular beacons hold great promise in studies in genetics, disease mechanisms, and in disease diagnostics. Efforts are being made to explore their applications in different areas. More research is expected for the mutation detection for a variety of diseases and for RNA monitoring in living cells. This will be expedited with the development of molecular beacon biosensors and multiple molecular beacon sensors/molecular beacon chips. By improving the immobilization and localization techniques, a silica chip with an array of many molecular beacon probes with specific loop sequences can be fabricated. Each probe will only occupy a few pixels in a CCD image. The hybridization information for a sample containing multiple DNA targets can be obtained simultaneously in one single analysis. This multiple-analyte molecular beacon sensor will provide an easy and fast way for DNA analysis and disease diagnosis even on a single cell level. Molecular beacons will also be useful for studies of protein–DNA/RNA interactions by affording high sensitivity, speed, and convenience. They open the way to using easily obtainable and designer DNA molecules for genomics and proteomics and for new drug development. The kinetic study during the conformational change of the molecular beacon itself and during the hybridization is another interesting area for the understanding of the molecular mechanism of molecular beacons and for the better design of molecular beacon probes. Molecular beacons are expected to be capable of functioning as highly specific, highly sensitive recognition and signaling elements in state-of-the-art biological detection strategies for biomolecules.

Acknowledgements

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