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Molecular Beacon DNA Probes and their Bioanalytical Applications

INTRODUCTION

In the post-genomic and proteomic era, a continued demand still exists for biomolecular recognition probes with high sensitivity and selectivity for use in quantitative studies of genomic and proteomic information. These are particularly important for disease diagnosis as well as drug discovery.¹⁻⁴ Since they were first reported in 1996,⁵ molecular beacons (MBs) have become a class of molecules widely used in chemistry, biology, biotechnology, and medical sciences for biomolecular recognition,^{3,4,6-8} due in part to their functionality and molecular specificity. In the traditional format, MBs act like switches that are normally closed, or “off”. Binding induces conformational changes that open the hairpin and as a result the fluorescence is turned “on”. More re-

cently, MBs have also been engineered to incorporate aptamers as the target-binding region of the probe. This has also allowed MB based protein detection assays.⁹⁻¹²

MOLECULAR BEACON FUNDAMENTALS AND SPECTROSCOPIC PRINCIPLES

Molecular beacons operate on the principal of DNA base pairing. MBs are synthetic DNA molecules that conform to a basic “stem-loop” or hairpin structure (Fig. 1). The DNA hybridization acts as the basis for target recognition and signal transduction in MB studies. The loop sequence (15–30-mer) is complementary to a target DNA, while the stem is a 5–7-mer sequence complementary to itself so that prior to binding target DNA sequences the structure remains in the closed state.

For signal transduction, resonance energy transfer (RET) is employed.¹³ Energy transfer is the transfer of the excited-state energy from the initial-

ly excited donor to an acceptor. Donor molecules typically emit at wavelengths that overlap with the adsorption spectrum of the acceptor. RET occurs without the appearance of a photon and is the result of long-range dipole–dipole interactions between the donor and acceptor. Energy transfer rates depend upon the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the quantum yield of the donor, the relative orientation of the donor and acceptor molecules, and the distance between the donor and the acceptor. The distance at which RET is 50% efficient is characterized as the Förster distance and is typically in the range of 20–60 Å.¹³ For MBs, a fluorescence moiety, or the donor, is covalently coupled to one end of the MB, and a quenching moiety, or the acceptor, to the other end. When the two moieties are brought together by the stem, the fluorophore and quencher are brought close enough

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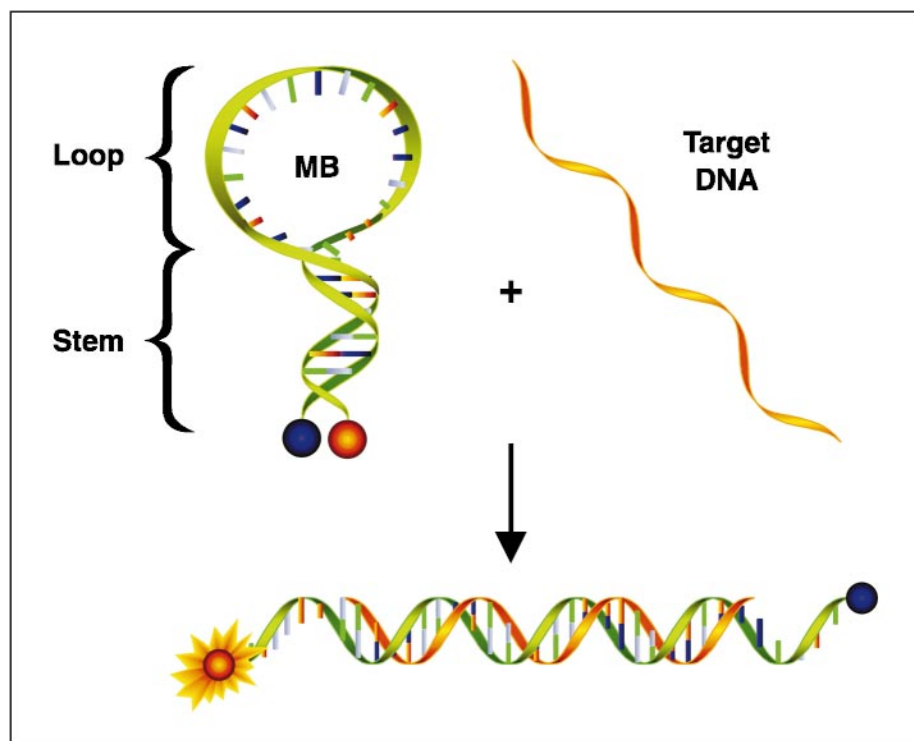


FIG. 1. Conformational structure of molecular beacon probes.^{5,15} Prior to hybridization with target nucleic acid sequences, the molecular beacon (MB) takes on a stem-loop structure which maintains the close proximity of the fluorophore (F) and quencher (Q) moieties. The resulting fluorescence is minimal due to static quenching of the fluorophore molecule. After introducing target nucleic acid sequences, the loop sequence of the molecular beacon hybridizes to the target and unstabilizes the stem hybrid. Consequently, the two moieties spatially separate and result in the restoration of the fluorescence.

to each other so that most of the absorbed energy is dissipated as heat and only a small amount of energy is emitted as light, a phenomenon sometimes referred to as static quenching.^{13–15} In the presence of target molecules, the loop region forms a hybrid that is longer and more stable than that of the stem. This forces the MB to undergo a spontaneous conformational change that forces the stem apart. With the quencher no longer positioned near the fluorophore, fluorescence is restored, thus signaling the binding of the MB to its target.

When designing MBs for particular applications, an important factor to consider is an appropriate fluorophore and quencher pair. Based on the instrumentation available for excitation and emission detection of the fluorophore, the choice of fluorophores can be reduced; however, with the growing availability of fluorophores and quenchers, the decision has become more difficult. Recently, a systematic study of various static quenching efficiencies for different fluorophore and quencher pairs has been described.¹⁶ In Table I the most efficient quenchers for several fluorophores that have been implemented in MB designs are shown. Matching the fluorophore with an effective quencher molecule can lead to substantial improvements in detection capabilities by reducing the background fluorescence from the MB in the absence of target DNA. Due to the static quenching that exists in MBs, it has also been shown that spectral overlap is less important for effective quenching to occur.¹⁶ One observation that further supports the static quenching mechanism of MBs is the alteration that is observed in the absorption characteristics of the fluorophore and quencher.¹⁵ The hairpin-loop structure of the MB brings the fluorophore and quencher sufficiently close to one another to perturb their electronic structure. Consequently, the nature of static quenching provides not only for improved quenching efficiencies, but also for the use of quenchers when a significant spectral overlap is not

TABLE I. Commonly used fluorophores and most efficient quencher molecules.^a

Donor fluorophore	E_{\max} (nm)	Quencher	A_{\max} (nm)
Alexa 350	441	BHQ-1	543
Coumarin	475	QSY-7	460
Cy2	507	BHQ-1	543
Alexa 488	517	BHQ-1	543
FAM	517	BHQ-1	543
Alexa 430	535	BHQ-2	579
Alexa 532	551	QSY-7	560
Cy3	564	BHQ-1	543
TMR	577	BHQ-1	543
Cy3.5	593	BHQ-1	543
Alexa 568	599	QSY-7	560
Texas Red	603	BHQ-1	543
Al4xa 633	645	BHQ-1	543
Cy5	663	BHQ-2	579
Cy5.5	687	BHQ-1	543

^a E_{\max} = emission maximum of the fluorophore, A_{\max} = absorption maximum of the quencher, BHQ-1 = Black Hole Quencher-1; BHQ-2 = Black Hole Quencher-2; Table adapted from Ref. 16.

present. An example of this is the case for Black Hole Quencher-1, which can often be used in MBs with near-infrared fluorophores even though its maximum absorbance is 534 nm.

Another concern when designing MBs for bioanalytical applications is the final nucleotide base positioned before the fluorophore in the DNA molecule. Several investigators have observed that nucleotides can quench the fluorescence of fluorophores.^{16–18} The results indicate that nucleotides do not quench as efficiently as the commonly used quencher moieties; however, the nucleotides exhibited a variable degree of quenching. Guanosine (G) is the most efficient, followed by adenosine (A), cytidine (C), and thymidine (T). The quenching properties of guanosine can be attributed to its electron-donating ability, which permits the charge transfer of nucleobase and the nearby fluorophore.¹⁸ As a result, care must be taken in stem sequence design to avoid the influence of neighboring nucleotides of the fluorophore in both strands of the hybrid as well as adverse affects the base pairs may have on the stability of the stem (which will be discussed later).¹⁶

While the basic function of MBs is similar for most of the bioanalytical methods that employ them, each MB is individually tailored to meet the needs of the application. A good understanding of how their fluorescence changes with temperature in the presence and absence of targets is critically important. The longer the loop sequence and the higher its GC content, the higher the melting temperature (or stability) of the probe–target hybrid will be. Consequently, the sensitivity and specificity of the probe for its target can be optimized for the application by adjusting the sequence to be more GC rich or deficient.¹⁹ It is important to note that the probe–target hybrid melting temperature can be adjusted independently from the melting temperature of the stem by selecting a target region of appropriate length. This alters the degree of constraint placed on the MB conformation and can

shift the equilibrium of the “on” versus “off” states, yielding higher sensitivities. Similarly, the stem can be tuned to provide more stability by adding GC content. It was found that increasing the length (or strength) of the stem of MBs increases the difference between the melting temperatures of perfectly complementary duplexes and mismatched duplexes. Thus, the presence of the stem offers a means of maximizing the specificity of the probe, without needing to alter experimental conditions. There is, however, a limitation on the length to which the stem can be extended. When the stems become too long, hybridization kinetics are slow and the probes tend to remain closed while bound to their targets, particularly at lower temperatures. This problem can be overcome by extending the probe sequence in such a manner that one arm of the stem also binds to the target.

Due to the unique structural and thermodynamic properties of MBs, these probes offer several advantages for biotechnology method development, one of which is their ability to differentiate between two target nucleic acid (NA) sequences that differ by as little as a single nucleotide.^{20,21} This degree of molecular specificity is very advantageous in a variety of bioanalytical applications, such as real-time polymerase chain reaction (PCR) monitoring,^{20,22} NA array development,^{22–24} and NA analysis.^{25–29} The origin of this discrimination is from the stem-loop structure and the design of the MB. By paying close attention to the GC content, melting temperatures, and experimental conditions, single mismatch determinations can be realized.¹⁵ In comparison to their linear probe counterparts, experiments have shown that the range of temperatures within which perfectly complementary NA targets form hybrids but mismatched NA targets do not is significantly wider for MBs than for the corresponding range of conventional linear probes.³⁰ Unfortunately, when designing probes for single base mismatch determinations, the stem acts as a counterweight to the loop, so

shorter loop sequences are employed to effectively distinguish the single base difference in the loop at the cost of probe stability. If, on the other hand, single nucleotide discrimination is not desired, longer and more stable probes can be chosen.

Another advantage to MB probes is their inherent signal transduction mechanism. Exploiting the structural change associated with target molecules binding to the MB has allowed for the ultrasensitive analysis of DNA, RNA, and proteins, as well as investigating single molecule interactions.³¹ The signal transduction of MBs also allows for the detection of target NA in situations where it is not possible or desirable to isolate the probe–target hybrids from an excess of the unhybridized probes, such as during real-time monitoring of PCR in sealed tubes or in the detection of mRNAs within living cells.^{20,32–36} These advantages make them attractive to biologists as well as chemists for bioanalytical applications; however, obtaining good working probes is occasionally a daunting task. To assist in this, one can utilize m-fold shareware software³⁷ as well as an Excel algorithm,³⁸ which will calculate potential structures and predict the top candidates for forming good stem-loop structures for any target of interest. Unfortunately, this algorithm is limited to 20-mer loop lengths and 5-mer stems, which can be limiting for certain applications.

BIOANALYTICAL APPLICATIONS OF MOLECULAR BEACONS

With the development of MB based assays, researchers have been able to conduct studies in areas that were previously not possible. These have included solution-based RNA/DNA interaction investigations^{14,20,39,40} and living systems measurements,^{32,34,35} as well as protein/DNA interaction studies.^{39–42} Figure 2 schematically depicts several homogenous solution assay designs that have enabled the fundamental studies of both MBs and their target molecules.

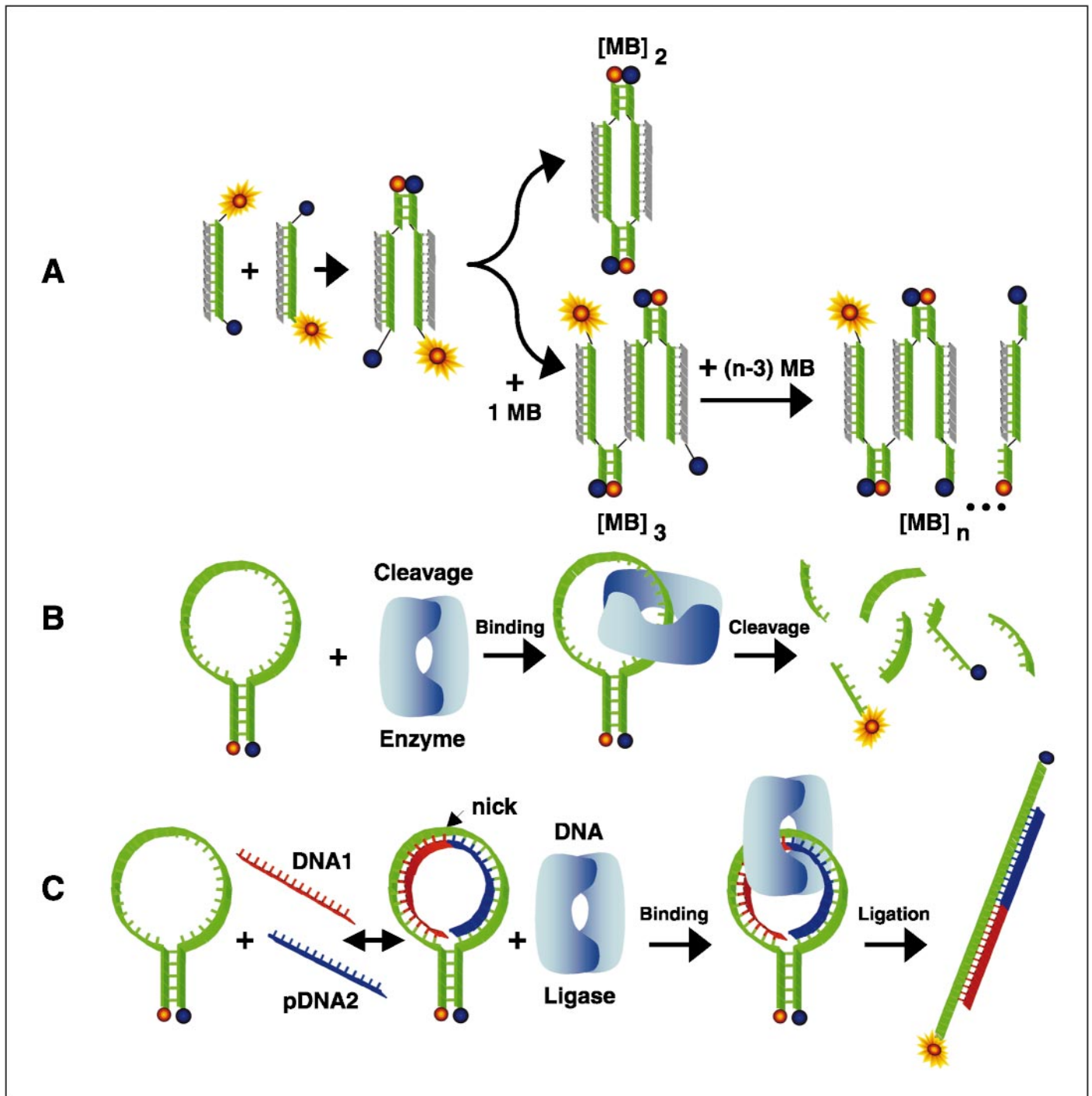


FIG. 2. A wide variety of sensitive assays can be developed based on the molecular beacon structural confirmation changes in the presence of both proteins and nucleic acids. (A) Molecular beacons can exhibit a substantial amount of intermolecular interactions as a result of sticky-end pairing of the MB stems in the presence of target nucleic acids. Two complementary sticky ends from two MB hybrids can pair to form a short double helix, leading to association of the two hybrids at one end. These two MB hybrids can form a closed structure, $[MB]_2$, by pairing the other two sticky ends, or polymerize into a multimolecular structure, $[MB]_n$ ($n > 3$), by pairing with more hybrids. With sticky-end pairing, F and Q are drawn together again, causing fluorescence quenching.^{5,15,52} (B) DNA cleaving enzymes, or nucleases, can also be monitored by using the ability of the enzymes to cut the molecular beacon into short sequences. Eventually, the enzyme cleaves the stem sequence and destabilizes the hybrid in such a manner as to restore the fluorescence of the fluorophore.^{54,55} (C) Ligase activity can also be monitored, where the molecular beacon is designed to be complementary to the ligated product.⁶⁴ Initially, DNA1 and pDNA2 (5' phosphorylated DNA) can individually bind to the probe, creating

IN VITRO RNA/DNA MONITORING

One common use for MBs has been for real-time monitoring of DNA/RNA amplification during PCR.^{5,15} Since the non-hybridized MB has minimal fluorescence, the increasing fluorescence signal at each cycle is representative of the increasing concentration of the amplified sequence. Detection limits with this method can be as few as 10 genomic copies of an individual sequence.⁴³ Recent studies have demonstrated that real-time PCR monitoring with MBs is comparable or preferred to other methods. For example, MBs have achieved more reliable genotyping results than TaqMan probes, especially for GC-rich sequences.⁴⁴

Molecular beacons have also provided an important tool for DNA sticky-end pairing analysis. DNA sticky-end pairing (SEP) plays an important role in cellular processes and various biotechnologies⁴⁵⁻⁴⁷ and has been studied with different methods including ultracentrifugation, gel electrophoresis, electron microscopy, and atomic force microscopy.⁴⁸⁻⁵¹ However, these methods need product separation, DNA ligation, and surface deposition, all of which tend to shift the reaction equilibrium of SEP. In terms of MBs, SEP is defined by the intermolecular interaction between the stems of two MBs once target DNA is present (Fig. 2A). A real-time assay based on strategically designed MBs has provided researchers with a simple, convenient, and highly sensitive analysis tool for characterizing DNA SEP.⁵² The result of intermolecular sticky-end pairing causes a severe loss in the fluorescent signal in MB DNA probes during hybridization (Fig. 3). Such assays allow for the observation and quantitation of DNA SEP as

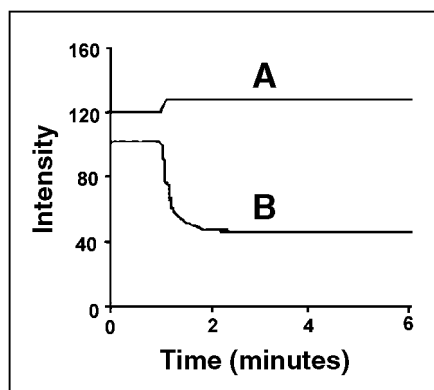


FIG. 3. Fluorescence determinations of DNA sticky-end pairing.⁵² (A) A MB-target DNA hybrid was prepared in 1 mM MgCl₂ buffer, and SEP was triggered by increasing Mg²⁺ concentration to 5 mM. The fluorescence consequently decreases due to the intermolecular interactions that can occur under these conditions. (B) A MB without target DNA was run as a control and no decrease was observed. Upon increasing the Mg²⁺ concentration, a slight increase in signal is observed due to an increased stability of the target-MB hybrid.

well as the study of various biophysical processes that involve DNA sticky-end pairing, from nonhomologous end-joining to DNA self-assembly. Consequently, one must also take SEP into account when developing MB for conventional assays. Another important finding was that higher analytical sensitivity can be achieved by designing the MB to incorporate one side of the MB stems for sequence specificity and reducing the SEP phenomena.

In addition, the cutting of DNA into shorter pieces, or DNA cleavage,⁵³⁻⁵⁵ has been studied using MB probes as “molecular break lights” for monitoring and studying enediynes.⁵⁴ In this case, the fluorescence intensity of the MB increases upon cleavage by enediyne when an ap-

propriate cleavage site is integrated into the stem or loop of the molecule. Similarly, nuclease activity can be sensitively monitored and detected (Fig. 2B).^{53,55} Nuclease is a general term for enzymes that catalyze the hydrolysis of nucleic acid by cleaving chains of nucleotides into smaller units. Homogeneous formats (with no wash or separation steps) incorporating a microplate-based quenching fluorescence assay are possible using the MB technology, which could have an impact in pharmaceutical development. These assays are based on the unquenching of the MB through nonspecific cleavage of the DNA molecule. Increased sensitivities and detection limits (~3 orders) have been observed in comparison to the traditional methods. Given the generality, simplicity, speed, and sensitivity of such approaches to monitor DNA cleavage mechanisms, the methodology could be adapted to a high-throughput format and become a new method for modern drug discovery to screen for novel protein-based or small molecule-derived DNA cleavage agents.

While DNA cleavage is detectable using MBs, studying the joining of DNA fragments by their ends with a phosphodiester bond, or ligation, is also possible. Nucleic acid ligation is a vital process in the repair, replication, and recombination of nucleic acids.⁵⁶⁻⁶¹ Traditionally, ligation is assayed by denaturing gel electrophoresis and autoradiography, which are not sensitive and are complex and discontinuous.^{62,63} For DNA ligase analysis, MBs are designed in such a way that its sequence is complementary to the product of the ligation process (Fig. 2C).⁶⁴ Since MBs can be used for real-time monitoring of DNA hybridization, this assay allows for real-time monitor-

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two adjacent bases that are not connected by a phosphodiester bond, or a nick. As a result of the two sequences binding to the MB, the fluorescence slightly increases. Once the nick is formed, the DNA ligase can bind and join the two target sequences. The full complementary strand then has the ability to fully restore the fluorescence of the MB. (Yellow circle = quenched fluorophore, Red circle = fluorescing fluorophore, and Blue circle = quencher.)

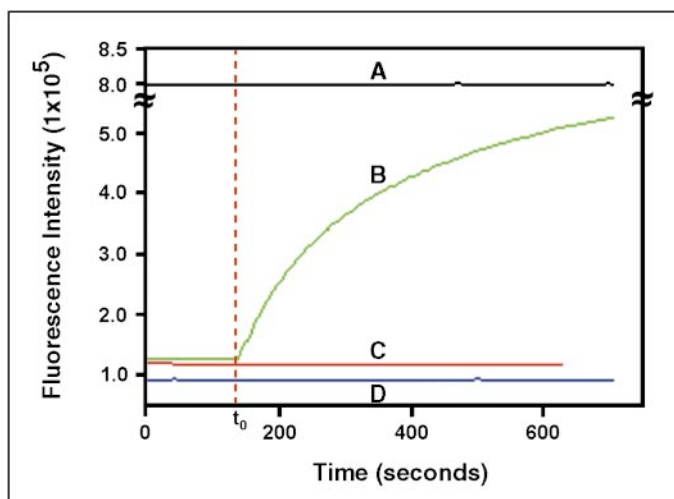


FIG. 4. Real-time fluorescence scans of DNA ligase activity.⁶⁴ As a result of the ligation process and the production of target DNA, the fluorescence of the MB increases with time. (A) A time scan of fluorescence intensity of MB with full length target DNA; (B) MB with DNA1 and pDNA2 (phosphorylated at the 5' end); upon introduction of T4 DNA ligase the two DNAs are ligated, form the full complementary target, and fully open the MB; (C) MB with DNA1 and DNA2 (non-phosphorylated); due to the lack of the phosphodiester bond formation of the MB remains partially open because of partial binding of DNA1 and DNA2 to the MB; (D) MB itself; a slightly lower fluorescence due to the ability of DNA and DNA2 or pDNA2 to bind and partially open the MB. t_0 is the time when T4 DNA ligase is added into the MB-oligo solution.

ing of the ligation reaction and its kinetics in homogeneous solutions (Fig. 4). After ligation of the two oligonucleotide sequences, which together make the full complement of the loop, the MB can form stable hybrid complexes. The major advantages of this MB-based ligation assay are its speed, simplicity, sensitivity, specificity, and convenience and the ability to perform real-time monitoring of homogeneous solutions; however, the ligated product must be known.

LIVING SYSTEMS STUDIES

The ability to detect and quantitate changes in gene expression, especially in real-time and with a degree of sensitivity suitable to monitor minor changes at a single cell level, will have considerable value in functional genomics. RNA analysis within a living cell has proven to be a tremendous challenge to biologists. With the rise of functional genomics, our fundamental understanding of RNA synthesis, transport pathways, and subcellular localization is in-

creasingly important. Traditional RNA analysis methods such as *in situ* hybridization are generally performed with fixed and pretreated cells or tissues.⁶⁵ Using fixed samples limits the ability to monitor dynamic cellular events such as synthesis and transport, and may not even be truly representative of living samples or even single cells. With MBs, on the other hand, real-time measurements of mRNA *in vivo* and *in vitro* have allowed new directions to be investigated.

Molecular beacons have been reported for the detection of basic fibroblast growth factor (bFGF) mRNA in human trabecular cells,⁶⁶ human c-fos mRNA in transfected Cos7 cells,³⁶ and β -actin mRNA in PTK2 cells and in K562 human leukemia cells.^{34,35} Successful implementation of MBs for mRNA detection depends on a combination of rational sequence design, efficient probe insertion into the cell, and optimized fluorescence imaging conditions. It is also important to choose an appropriate portion of the mRNA

target sequence that will be accessible to the probe and a loop sequence that will be non-complementary to other possible interfering sequences. This is often accomplished using software to predict secondary structures of the mRNA sequences, the NCBI Gene Bank, and intuitive engineering. Synthesizing several MBs for a single mRNA is frequently done. It is expected that not all of the MBs will yield fluorescence enhancements when testing *in vitro*. Computer programs that predict secondary structures of DNA are also useful for optimizing the MB design. Once a MB has been found to yield an appropriate response, the MB can be inserted into the cell by means of liposome delivery or by direct microinjection. Both of these methods have been demonstrated to maintain the physiological and structural integrity of the cells.

Recently, strategic MB and experimental design resulted in the visualization, distribution, and trafficking of mRNA in *Drosophila melanogaster* oocytes.³² Nuclease resistant MB were microinjected into the oocyte cells to track *oskar* mRNA, which is known to migrate to the poles of oocytes. The idea of using two MB beacons simultaneously to hybridize adjacent regions of the same target, resulting in fluorescence resonance energy transfer (FRET), has also been investigated.³² In FRET, the donor fluorophore transfers its excited-state energy to an acceptor fluorophore, rather than a quencher. FRET results in the appearance of a photon from the emission of the acceptor fluorophore. This is in contrast to the static quenching mechanism, where no photon is released. In this example, the dual MB approach positions the two MBs in such a way that the donor and acceptor fluorophore are adjacent to each other in the presence of target molecules. When no target is present, the two MBs are independent of each other and rely on RET to provide quenching of the fluorophore, and then, by binding to the target, the two fluorophores can energy transfer through the FRET mechanism. This scheme can reduce

false-positive signals in detecting target nucleic acids. The detection of a FRET signal also leads to an increased signal-to-background ratio compared with that seen in single MB assays and enables discrimination between fluorescence due to specific probe–target hybridization and a variety of possible false-positive events. One major limitation to such an approach is that one needs twice as much mRNA sequence available for hybridization, which considerably limits the chances for obtaining functional MBs.

BIOSENSING APPLICATIONS

While solution applications of MBs have been widely applied, their use in biosensor development has become popular as well. Various applications of surface immobilized MB⁹ sensors have been described, including ultrasensitive fiber-optical DNA sensors based on evanescent wave detection⁶⁷ and miniaturized hybridization assays developed with positional separation in fiber-optic bundles⁶⁸ and microarrays.^{22–24} The typical configuration of MB biosensors includes the immobilization of the hairpin onto a surface. For reporting binding events, the MBs are still equipped with a fluorophore and quencher moiety. One of the major concerns in the immobilization of MB probes is the impact it has on the “on” versus “off” ratios. The dynamics of the DNA hybridization process on a liquid–solid interface has been investigated previously.³¹ Theoretically, the formation of a DNA duplex is a multi-step reaction, and each base-pairing step has a different reaction constant. Analysis of the hybridization process is complicated in this mode. A much simpler mode, all-or-none mode (or “on” and “off”), an approximation that only considers the initial and final states of a reaction, has been proposed.⁶⁹ This mechanism has been found to be sufficient to explain most of the kinetic experimental data and is widely accepted for DNA hybridization studies. The all-or-none mode is also supported by the results of a thermodynamic study of bulk

MB hybridization.³⁰ In the case of immobilized MBs, two major types of kinetics (fast and slow) have been observed during their hybridization using single molecule detection, the majority being a fast dynamics process.³¹ Additionally, decreased signal-to-background ratios have been seen for immobilized molecule beacons. This is most likely attributed in part to the fact that the solid surface adds additional strain to the confirmation, which can destabilize the stem hybrid and result in more “on” molecules than in a homogeneous assay. Efforts have been made to improve this situation through the use of longer spacer arms to further isolate the MBs from the solid surface.⁷⁰

Using commercially available aldehyde-coated glass slides, the detection of *Francisella tularensis* has been accomplished with amine functionalized MB in a microarray format.⁷¹ Cy3 and Black Hole Quencher 2 were used for the fluorophore and quencher pair. The array demonstrated characteristic MB specificity toward complementary oligonucleotide sequences, single nucleotide mismatch sequences, and multiple nucleotide mismatches. It was also determined that if the only measurement parameter is the induced fluorescence upon interaction of a MB with a target, the molecular discrimination is highly dependent on number and location of target mismatches, suggesting at least in part that fluorescence amplitude upon hybridization is target dependent.

In human disease diagnostics, gene expression studies, and gene profiling, the collection of rare DNA/mRNA molecules in complex matrices is very important. As a result, MB immobilized magnetic nanoparticles, or genomagnetic nanocaptors (GMNC), have been developed to collect, separate, and detect trace amounts of DNA/RNA molecules with one single-base difference.⁷² The GMNC was constructed by conjugating MB DNA probes onto magnetic nanoparticle surfaces through avidin–biotin interactions. The excellent ability to differentiate

single-base-mismatched DNA/mRNA samples was accomplished by combining the exceptional specificity of MBs with the separation power of magnetic nanoparticles, and real-time monitoring of the collected gene products (Fig. 5). The GMNC was demonstrated to work well for artificial and cancer cell samples. This newly developed technique could be useful for a variety of sample sources in forensic, medical, and biotechnological fields.

While the majority of surface-immobilized probes to date incorporate a single dye-based quencher molecule into the signal transduction mechanism and the material to which the probe is attached serves only a passive role, some attention has been paid to developing alternative approaches. Gold offers particularly attractive molecular characteristics and has been extensively used in biotechnology and nanotechnology for signal transduction purposes.⁷³ At the core of these approaches is a gold surface that has the ability to quench fluorescent molecules. At separation distances between fluorophore and metal surfaces within the order of the emitted wavelength, an oscillatory behavior of fluorescence lifetime with distance is observed.⁷⁴ This is ascribed to constructive and destructive interference effects between the waves directly emitted from the fluorescent molecule and those reflected by the metal surface, which acts like a mirror. However, for distances smaller than about 100 Å, radiationless energy transfer to the metal surface dominates the lifetime of the excited molecule.⁷⁵ At these short distances, a reduction of the excited-state lifetime by several orders of magnitude occurs. Concomitantly, quenching of fluorescence and broadening of the emission spectra can be observed.⁷⁶ These phenomena can be exploited to build sensing devices in which the fluorophore is in close proximity to the metal surface in the absence of analyte but separates from the surface upon exposure to the analyte. By immobilizing a fluorophore-functionalized hairpin onto a gold sur-

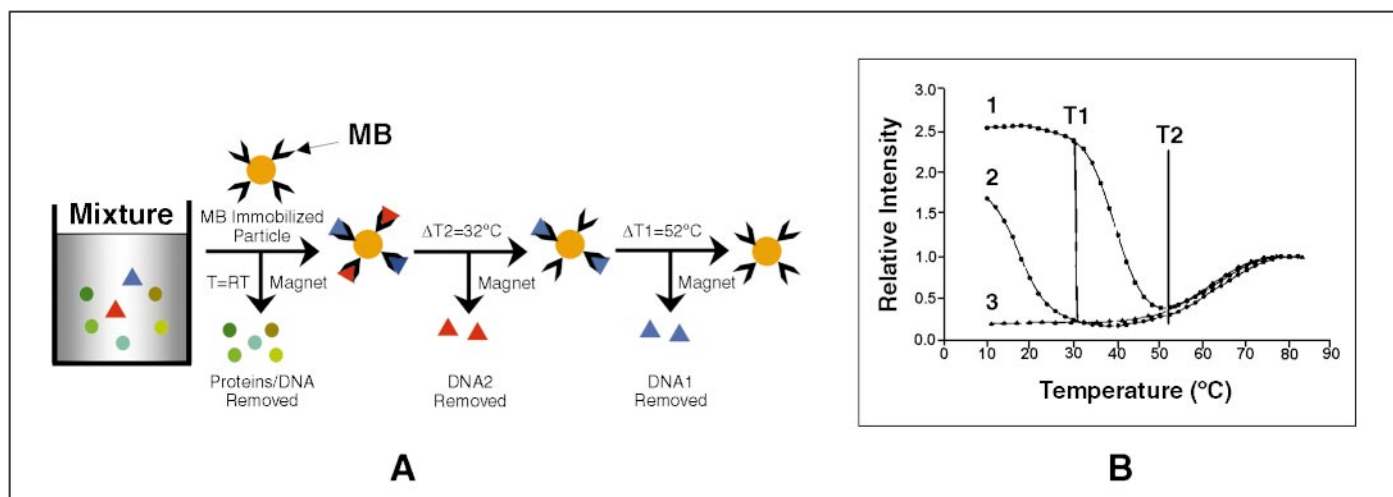


Fig. 5. Isolation and detection of two single-base-mismatched DNA molecules using MB probes.⁷² (A) Schematic diagram of the working principle of the genomagentic nanocapturer for separation and collection of gene products with a single-base difference in sequence. The stability of single-base-mismatched hybrids with the molecular beacon are significantly different to allow for separations to be done at three distinct temperatures. At room temperature (RT), the GMNC can extract the two NA targets from a complex mixture. At 32 °C, the GMNC will release the single-base-mismatched sequence. At 52 °C, the GMNC will release the perfect complementary target. The molecular beacon can also be used to monitor the amount of target present at RT by incorporating a fluorophore and quencher molecule. (B) The melting temperature profile for the MB (not immobilized on a GMNC surface) and its target duplexes in buffer solutions. The solution contained 0.6 μM DNA1 and 0.1 μM MB for (1) and 0.6 μM DNA2 (single-base mismatched) and 0.1 μM MB for (2). (3) is for molecular beacon only in solution. The single-base-mismatched sequence melts, or unhybridizes, at a much lower temperature, which can allow for the isolation of the perfect complementary sequence in a homogeneous solution using magnetic nanoparticles.

face, in the absence of target, the fluorescence is quenched due to the close proximity of the fluorophore to the gold surface.⁷⁷ When targets bind to the MB probes, the fluorophore separates from the gold film and restores the fluorescence. As a result, fluorophore-tagged DNA hairpins attached to gold films can function as highly sensitive and selective sensors for oligonucleotides. With improvements, these methods are promising in the areas of microarray technology due to surface-enhancement capabilities of roughened metal substrates.^{78,79}

PROTEIN DETECTION

Although MBs were originally designed to bind and recognize specific nucleic acids, these probes can also lead to increased fluorescence upon binding to certain proteins. Since the binding of proteins to DNA or RNA molecules can readily disturb the conformation of the nucleic acid, it is expected that this binding would result in spatial separation of the MB

fluorophore and quencher. The protein recognition ability of MBs was first recognized with an *E. coli* single-stranded DNA binding protein (SSB).^{42,78} SSB is a 75.6 kDa tetrameric protein that acts as an accessory protein in DNA replication, recombination, and repair. A tetramethylrhodamine/dimethylamino-phenylazobenzoic acid (TAMRA/DABCYL) MB was used, and SSB concentrations as low as 20 nM could be detected using a conventional fluorescence spectrophotometer. Monitoring fluorescence over time shows that the SSB–MB interaction is rapid, reaching equilibrium within 10 seconds, which is much more rapid than with its complementary DNA (Fig. 6). The MB-based SSB assay is not, however, particularly specific. SSB leads to a fluorescence enhancement nearly equal to that of the complementary DNA, but other proteins can also bind with the MB and cause fluorescence changes. For example, histone and RecA proteins have both been dem-

onstrated to bind with MBs and augment fluorescence.⁴² These results demonstrate that while MBs are sensitive and somewhat selective to DNA-binding proteins, they are not specific enough to be capable of distinguishing a particular protein. In order to apply MBs for real-time, specific detection of proteins, a more selective protein recognition mechanism is required.

The combined sensitive signal transduction mechanism of MBs with the specific protein binding capability of aptamers results in a novel class of analytical probes termed molecular beacon aptamers (MBAs).^{11,80} Aptamers are typically single stranded DNA or RNA molecules, generally 25–60 nucleotides in length, that have been selected in a process termed SELEX (systematic evolution of ligands by exponential amplification) from a combinatorial library by their ability to bind specific molecular targets, such as a protein or metabolite.^{81–83} As an alternative to antibodies, aptamers have

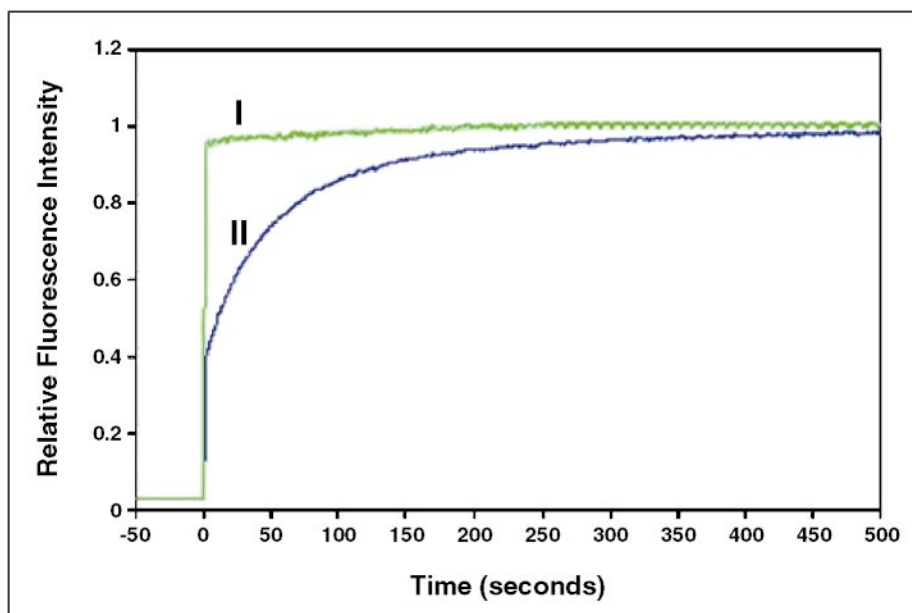


FIG. 6. Relative fluorescence intensity time scans of the binding of the molecular beacon to *E. coli* single-stranded DNA binding (SSB) protein (I) and target DNA (II).⁴² The result indicates that SSB proteins bind to the molecular beacon and result in a much faster restoration of fluorescence than that of target DNA. The SSB and target DNA are both in a 4:1 molar excess versus MB.

several inherent advantages. Since they consist of a short, single strand of DNA or RNA, they are much easier and cheaper to synthesize and have a much longer shelf life. The

selection process mimics natural selection, so it is possible, in theory, to develop a highly specific aptamer for virtually any target molecule with high affinity similar to those of

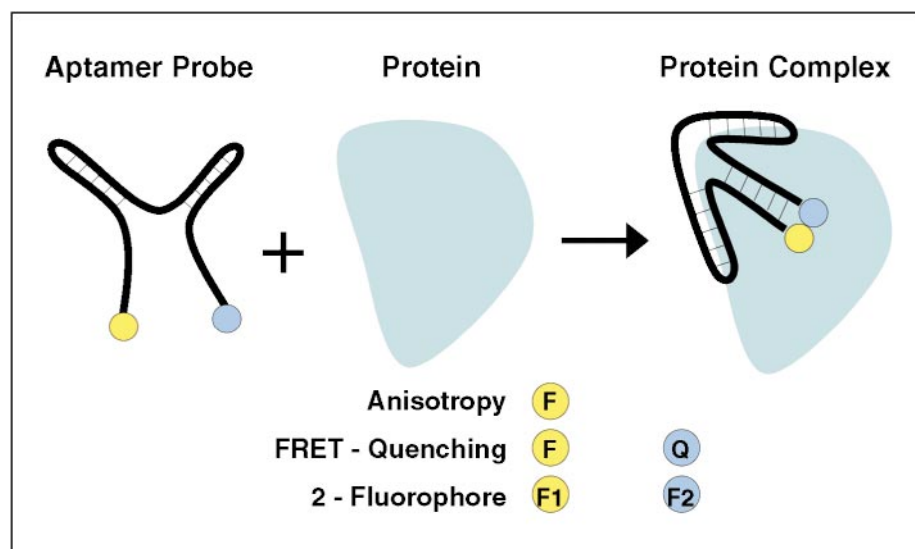


FIG. 7. Engineering a molecular aptamer beacon for protein detection. By strategic placement of single or double fluorophores (F) and a quencher (Q), the aptamer can be transformed into fluorescence anisotropy, quenching, or a FRET two-fluorophore molecular aptamer beacon. Positioning the labels at either end of the aptamer sequence minimizes interference with aptamer-protein binding interactions.

monoclonal antibodies. In comparison to engineering antibodies for particular applications, the incorporation of site-specific labels or coupling sites into an aptamer is typically a trivial process. Since the aptamer itself lacks any means of signal transduction upon binding a protein, this ease of molecular labeling is crucial for their development into fluorescence probes for specific protein detection.

The MBAs developed to date can be described by three general classifications: RET fluorophore/quencher pairs, FRET donor/acceptor pairs, and single labeled anisotropy probes (Fig. 7). Utilizing RET between fluorophore and quencher moieties for signal transduction of protein binding is one of the more popular designs. Depending on the relative positions of the fluorophore and quencher before and after protein binding, these probes can result in either enhanced or reduced fluorescence upon binding. In general, these RET-based MBAs are the most sensitive form of aptamer probes. However, designing optimized probes is not always a trivial process. A maximum change in fluorescence upon binding is often achieved by gaining knowledge about the conformations of the free aptamer and the aptamer-protein complex. Ideally, when this structural information is available, one can strategically incorporate the fluorophore and quencher moieties on the aptamer probe in such a way that the transition from bound to unbound conformations causes a dramatic change in their relative positions. However, structural information is not a necessity for designing workable aptamer beacons, but a higher probability exists that the probe sensitivity will not be optimal.

An example of a RET based quenching MBA is the one developed using the known and well-studied thrombin aptamer.⁴² The first reported aptamer for thrombin contains a 15-nucleotide consensus sequence.⁸⁴ When bound to thrombin, the aptamer exists primarily in its quadruplex form containing two G-quartet structures, but in free solu-

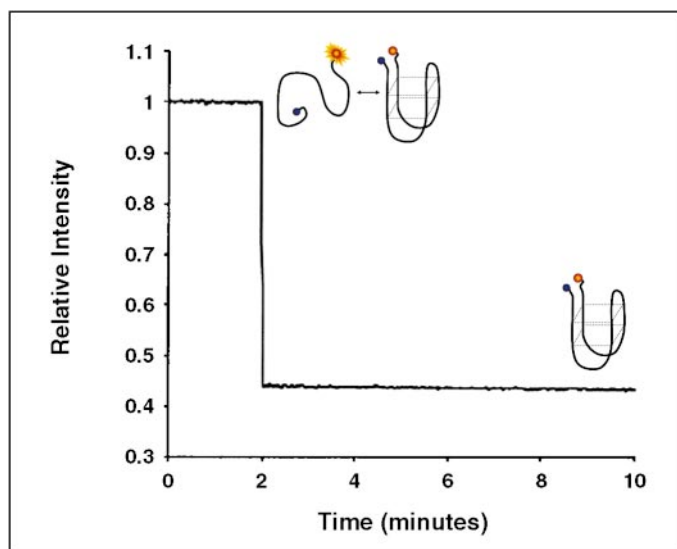


Fig. 8. Time course of fluorescence response before and after the addition of thrombin into a solution of the thrombin MBA.¹¹ The thrombin was added in 3-fold molar excess versus the MB. Prior to protein binding, an equilibrium exists between the random coil and G-quartet conformations. The G-quartet conformation will bind to thrombin, shifting the equilibrium and causing a loss in fluorescence signal.

tion, it can adopt either conformation, dependent in part on the ionic strength and temperature. This conformation shift provides the basis for a MBA. By labeling the two ends of the aptamer with a fluorophore and quencher pair and extending the aptamer by one base on each end, aptamer binding of thrombin would force the quencher adjacent to the fluorophore, resulting in a substantial decrease in fluorescence (Fig. 8).

A related class of MBAs is the two fluorophore FRET probes.¹¹ As with the analogous MB,⁸⁵ FRET between a donor (F1) and acceptor (F2) occurs when the two moieties are spatially close to one another. Binding of target protein can be detected by monitoring the fluorescence of F2 directly or preferably by the ratio of fluorescence of F2/F1. Ratiometric detection provides enhanced sensitivity, with reported detection limits in the low pM regime. All of the design considerations applicable to the quenching aptamer beacons are also crucial to this class of aptamer probes. Detailed structural information of the aptamer and aptamer-protein complex allows optimized positioning of the fluorescence donor and acceptor.

The thrombin aptamer quenching assay above has also been utilized in a two-fluorophore FRET based configuration.¹¹ While the quenching-type probe is suitable for homogeneous quantitation of target proteins in real time, it is difficult for monitoring proteins in living specimen. Decreasing fluorescent signals upon binding makes it hard to detect minute amounts of protein and to trace a target protein in living cells. The two-fluorophore approach also allows for the use of ratiometric imaging measurements. As a result, a greatly improved signal-to-background ratio and therefore much higher sensitivity in protein imaging are obtained.

The third class of molecular aptamer probes is based on monitoring the change in fluorescence anisotropy upon protein binding.^{80,86-88} Anisotropy probes are constructed by labeling an aptamer with a single fluorophore. When the labeled aptamer is bound with its target protein, the rotational motion of the fluorophore becomes much slower as a result of the larger molecular weight of the aptamer-protein complex. This results in a significant increase in fluorescence anisotropy that can be

monitored using plane-polarized light. While not strictly “molecular beacons” in that they do not utilize the FRET mechanism, aptamer anisotropy probes provide an alternative mechanism of signal transduction for aptamer-protein binding that results in “on” and “off” type signals much like those of MBs. One particular advantage of anisotropy probes is that they are more universal than the FRET-based probes. Since only a single fluorophore is utilized, structural details of the bound complex are not needed to design a working probe. Aptamer anisotropy probes also have an advantage versus analogous antibody probes as a result of their respective sizes. Since most aptamers are substantially smaller than antibodies, the relative increase in molecular weight and fluorescence anisotropy will be much larger upon binding with proteins for aptamer probes. However, the sensitivity of aptamer anisotropy probes is generally not as good as FRET-based MBAs due to a lack of sensitivity in the technique as well as the instrumentation used. By using polarized light, the excitation efficiency is dramatically reduced and the probe sensitivity is therefore reduced.

CONCLUSION

With the completion of the human genome project, there has been a rapid shift in focus from simply collecting and archiving genomic data to utilizing genetic analysis for prediction and discovery. The development and utilization of new quantitative tools for research across disciplinary interfaces will prove vital in achieving these objectives. Molecular beacons are ideally suited for and hold great promise in genomics and proteomics. More research is expected for MB applications in mutation detection for a variety of disease diagnostics and disease mechanism studies. Gene expression monitoring in living cells and tissues under different conditions with precise quantitation also provides an avenue for further investigation. The extraordinary target-specific capabil-

ity along with the availability of different fluorophore–quencher pairs makes MB probes extremely useful for multiple analyte applications as well. These properties also make MBs suited for use in the identification of genetic alleles or particular strains of infectious agents. With further development, MBAs are expected to be useful as intracellular protein recognition agents to probe proteins in different environments and to monitor protein–DNA/RNA interactions. All of these developments will open the possibility of using easily obtainable and designer DNA molecules for genomics and proteomics studies, for molecular diagnosis of diseases, and for new drug development.

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