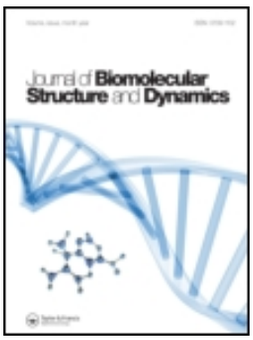

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Molecular Beacons: Nucleic Acid Hybridization and Emerging Applications

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Abstract

Molecular beacons (MBs) are a novel class of nucleic acid probes that become fluorescent when bound to a complementary sequence. Because of this characteristic, coupled with the sequence specificity of nucleic acid hybridization and the sensitivity of fluorescence techniques, MBs are very useful probes for a variety of applications requiring the detection of DNA or RNA. We survey various applications of MBs, including the monitoring of DNA triplex formation, and describe recent developments in MB design that enhance their sensitivity.

Introduction

Molecular beacons are fluorescent labeled single-stranded nucleic acid probes that remain non-fluorescent or weakly fluorescent in a stable stem-loop configuration bringing a fluorophore in close proximity of a quencher molecule (Fig. 1A) (1). MBs become strongly fluorescent when the quencher moves away from the donor, usually upon sequence specific interaction with target nucleic acids or by thermal dissociation.

The loop of the MB structure contains the sequence complementary to the target; Watson-Crick base pairing of 5-6 complementary nucleotides flanking the loop stabilizes the hairpin structure. The fluorescent donor molecule (e.g. fluorescein) and the acceptor (normally the universal quencher Dabcyl) are attached to the 5'- or 3'-ends by linker molecules. The design of MBs has been described in detail; see www.molecular-beacons.org for a comprehensive tutorial.

MBs combine the exquisite sensitivity of fluorescence techniques and the specificity of nucleic acid sequence recognition and thus are suitable for detecting and analyzing very small quantities of the target *in vitro* and *in vivo*, facilitating specific targeting in cells and subsequent visualization by sensitive microscopic and spectroscopic techniques (1,2).

Initial applications of MBs were based on their sequence specific interaction with single stranded DNA, and included detection of amplified PCR products, mutational analysis, clinical diagnostics, genotyping, and allele discrimination (1-9). Subsequent studies on the hybridization of MBs with RNA and duplex DNA showed that these probes could be very useful for two major approaches of oligonucleotide therapeutics - anti-sense strategies that depend on translation suppression by forming DNA-RNA hybrids with targeted mRNA (2,10,11), and triplex DNA based antigene strategies that depend on the modulation of gene expression by forming triplex DNA in the promoter site of targeted genes (12). Further applications of MBs include the study of DNA-RNA hybridization in real time (2), protein-DNA interactions (13,14), and the kinetics of nucleic acid degradation in presence of nucleas-

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es (15). We present an overview of typical applications of MBs (see also Table 1), and focus on our use of MBs to study the kinetics of DNA triplex formation.

Table 1
Applications of Molecular beacons

Applications	References
Detection of PCR gene amplification products	(1,3,5,6)
Mutational analysis	(47-50)
Genotyping and allele discrimination	(3,18-20,51)
Clinical diagnosis	(6,8,9,21,22,52,53)
Oligonucleotide degradation studies	(15)
Analysis of DNA·RNA hybridization	(2,43,54,55)
Real time visualization of hybridization in cells	(2)
Analysis of Triplex DNA formation	(12)
Protein-DNA interactions	(13,14)

DNA·DNA hybridization

The formation of duplex DNA by hybridization of the loop sequence of the MB and its complementary sequence forms the basis of many applications of MBs. Opening of the hairpin structure of MBs by unwinding of the duplex stem precedes the hybridization process. The kinetics and thermodynamics of this transition have been studied in some detail (16,17). An activation energy of +32 kcal/ mol was estimated for the dissociation of the stem formed from CCCAA and its complementary sequence, with loops of varying lengths. The dissociation rate of the stem was not affected by the loop size, but decreased with ionic strength. Thermal dissociation studies using steady state fluorescence techniques showed that the enthalpy (ΔH) and entropy (ΔS) for the dissociation of a stem formed from CGCTC and its complementary target were 34 ± 1 kcal/mol and 104 ± 3 cal/mol•K respectively (17). The ΔH and ΔS for the dissociation of duplexes formed from a MB (fluorescein –5'-CGCTCCCA$_{11}$CCGAGCG-3-dabcyl') with perfectly matching sequence (GGT$_{11}$GG) were 84 ± 3 kcal/mol and 237 ± 9 cal/mol·K respectively (17). The corresponding values for the duplex with a single mismatch (5'-GGT$_5$GT$_5$GG-3') were 65 ± 3 kcal/mol and 185 ± 10 cal/mol·K, showing a decrease in free energy (ΔG^0_{25}) by 3.5 kcal/mol, and illustrating the very high sensitivity of MBs for the detection of DNA mismatches. The structured nature of MBs yields a higher sensitivity for detection of sequence mismatches over a wider range of temperatures than the corresponding linear probes (17).

Detection of PCR amplification products

Fluorescence based approaches for the detection of PCR products have enhanced the sensitivity of PCR assays significantly. In one of the earliest applications of MBs, PCR gene amplification products were quantitated by sequence specific hybridization of a MB to target DNA (1). A MB with a sequence complementary to the middle of an 84-nucleotide amplicon was used in a closed PCR reaction tube. Increasing amplicon concentrations during PCR yielded a fluorescence intensity enhancement proportional to the different initial concentrations of the target, allowing the monitoring of the PCR reaction in real time. Using MBs with different donor fluorophores emitting at different wavelengths, the technique has been extended to a multiplex assay system, enhancing the utility of the approach (3,6). MBs have also been used to identify amplicons generated in nucleic acid sequence based amplification (NASBA), the isothermal amplification method (5). Since PCR amplification precedes many assays, MBs could find increasing use in genetic and biochemical analyses such as genotyping and detection of single nucleotide polymorphisms.

Genotyping and Allele discrimination

The property of MBs to bind selectively to perfectly matching sequences has been used for genotyping of human alleles (3,18-20). MBs, each specific to a particular allele, and each labeled with a distinct fluorophore were used to identify different alleles in a multiplex assay (3). Only perfectly matching sequences exhibited the

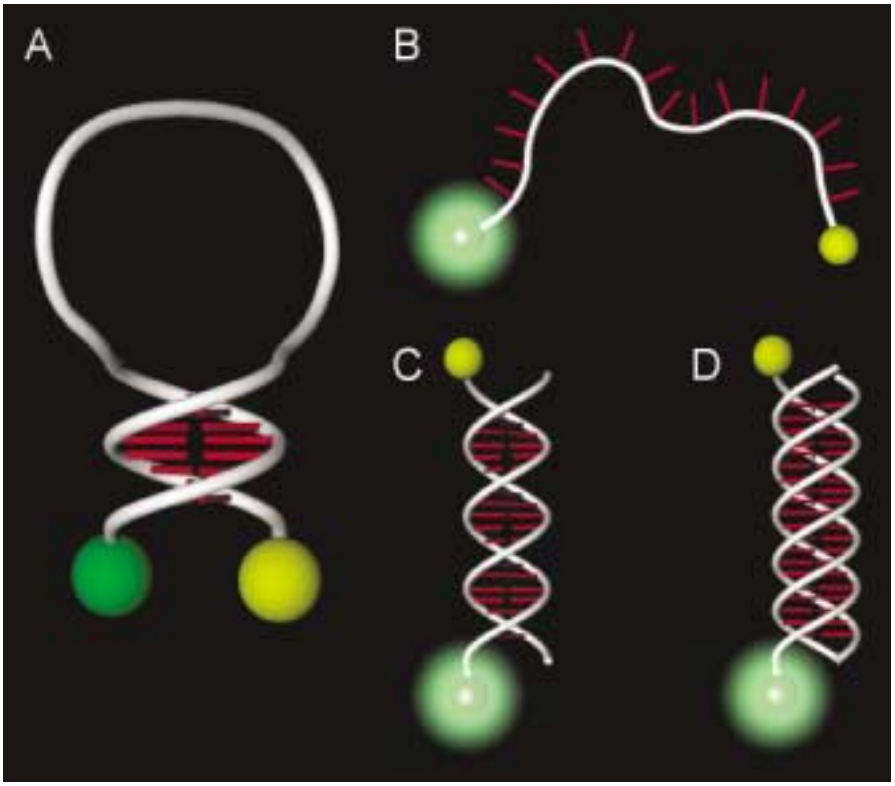

Figure 1: Schematic representation of molecular beacons. In its closed form (A), the proximity of the quencer (yellow) to the fluorophore (green) yeilds a non-flourescent molecule. The stem of the MB opens up by thermal dissociation (B) or by hybridazation with single stranded (c) or double stranded (d) nucleic acids, forming double helical or triple-helical structures, repectively.

characteristic fluorescence due to the opening of the MB stem, showing that this method is suitable and very specific for allele discrimination. Mutated alleles of the β-chemokine receptor (CCR5) with a 32 base deletion were differentiated from wild-type alleles using two MBs labeled by either fluorescein or tetramethyl rhodamine (18). These beacons were also used to identify the genotype of the human DNA samples CCR5 and could distinguish heterozygotes, homozygous wild type, and homozygous mutants. Wavelength shifting MBs have been used to distinguish the ten different genotypes that occur due to the presence of two closely spaced mutations in this gene (20). Tapp et al compared the ability of MBs and Taqman probes to distinguish the genotypes arising from three mutations in the human estrogen receptor gene, and found that these assays were comparable in distinguishing the genotypes with C→T and G→A transitions, while MBs performed better in distinguishing genotypes with C→G transversions (22).

Clinical Diagnosis

Coupled with PCR amplification of genes and the capability of real time detection of hybridization of MBs, methods have been devised to diagnose diseases very sensitively. Since the unbound MB probe is non-fluorescent, it is not necessary to separate the bound and unbound probes, making the diagnosis relatively simple. Vet et al. designed an exceedingly sensitive multiplex assay using four different MBs with four distinct fluorophores and loop sequences specific to HIV-1, HIV-II, and human lymphotropic viruses types I and II (6). This assay was able to identify 10 copies of a rare retrovirus in 100,000 copies of an abundant virus, and could be performed in a 96 well format in less than 3 hrs. By amplifying a part of the *gag* gene sequence of the genome of all currently known HIV-I subtypes (A, B, C and the circulating recombinant forms AE and AG) subtype specific MBs with multiple fluorophores were used to distinguish the different forms (9). However, this assay was not sensitive enough for clinical use. Manganelli et al. used a similar approach to study the transcript levels of the putative sigma factor genes in *Mycobacterium tuberculosis* (21), and to determine the RNA expression levels after changing external parameters like heat shock or low aeration. MBs were also used for the identification of the opportunistic fungal pathogen *Candida dubliniensis* (22).

DNA·RNA hybridization

The sequence specific binding of anti-sense oligonucleotides to targeted mRNA as a means to control translation is a major strategy used in oligonucleotide therapeutics. This approach has been found effective against various neoplasmic and viral diseases (23-25). MBs with embedded loop sequences complementary to the target mRNA can be used as anti-sense drugs that report the MB-target hybridization in real time. Hairpin structured anti-sense oligonucleotides have been used earlier to study anti-HIV activity (26). These structured probes were found to have higher sensitivity against DNA degrading enzymes. Steady-state fluorescence studies of hybridization of a MB targeted to *vav* proto-oncogene with both a synthetic complementary DNA oligonucleotide and with total cellular mRNA from K562 cells have been reported (2). The spectral changes were comparable for both targets, suggesting similar hybridization with DNA and RNA. The same MB was microinjected into K562 human leukemia cells allowing real-time visualization of DNA-RNA hybridization using fluorescence microscopy (2). In the absence of target molecules, the beacons remain non-fluorescent in living cells; only upon expression of the specific mRNA do the beacons bind and fluoresce (15-60 fold increase relative to a control MB), serving as a sensitive monitor of time-dependent gene expression. This strategy has been developed as an alternative approach in microscopy to other biosensors such as green fluorescent protein (GFP) (2,4,27). Arnold et al used MBs to quantitate the expression levels of mRNA for bone morphogenic proteins (responsible for metastasis) in MCF-7 and MDA-MB-231 breast cancer cell lines after radiation treatment (28). MBs are also useful for studying the cellular uptake, stability, and binding of different oligonucleotides that are being developed as drug candidates and for locating target mRNA in the cell (5,10,15).

In vivo applications have special difficulties associated with them, including degradation of MBs yielding fragments having only fluorophore and no quencher, which may lead to false results in *in vivo* hybridization experiments. Moreover, it has been observed that some MBs open up immediately after entering the cell, probably due to the lower stability of the stem under cellular ionic conditions (29). A systematic study of the stability of MBs under various cellular conditions is required.

Analysis of triplex DNA formation

The success of triplex DNA based anti-gene strategies depends on the formation of stable triplex DNA, usually targeted to the promoter sites of proto-oncogenes (30-34). Triplex DNA is formed by the binding of the TFO along the major groove of a duplex DNA target by Hoogsteen or reverse Hoogsteen hydrogen bonds. Depending on the sequence and orientation of the TFO, three types of triplexes can be formed – the TC motif (T.A*T, C.G*C+), the GA motif (T.A*A, C.G*G), and the GT motif (T.A*T and CG*G) (33). Stabilization of the TFO along the major groove requires stringent conditions such as high ionic strength or the presence of cationic ligands (35,36). So far, analysis of triplex DNA using MBs has been attempted only with GA motifs. Further studies are required to assess the utility of this approach for the analysis of TC and GT motif triplexes.

Fluorescence based techniques are being used increasingly for the analysis of triplex DNA because of the higher sensitivity and ease of experimentation compared to other conventional methods like UV, CD, NMR spectroscopy and electrophoretic mobility shift assay (EMSA). Among the different fluorescence based approaches, fluorescence polarization and FRET have been shown to be very sensitive and useful in the evaluation of kinetic and thermodynamic parameters under equilibrium conditions, which is often compromised in methods like EMSA (37-39). In addition, fluorescence techniques are fast, non-radioactive and nondestructive in nature. MBs offer an alternative method for the detection of triplex DNA. The thermodynamics of triplex formation can be analyzed using approaches similar to those used for duplex formation (17). We have used this approach to study triplex DNA formation in the promoter site of the human c-Src

proto-oncogene. The beacon used for these studies had a 15-mer loop (5'-Fluorescein-CGCTCGAAGGAGGAAGGAGGGAGCG-Dabcyl-3') designed to bind specifically to a sequence in the left side of the TC1 tract of the c-*Src* gene. As shown in Figure 2, titration of the MB with the target duplex resulted in ~40 fold increase in fluorescence intensity, suggesting triplex DNA formation. Strand exchange was ruled out since the sequence of the TFO was not complementary to the pyrimidine strand of the duplex. There was no significant increase in fluorescence in the presence of a nonspecific duplex, demonstrating sequence specificity in the interaction of the MB with the target.

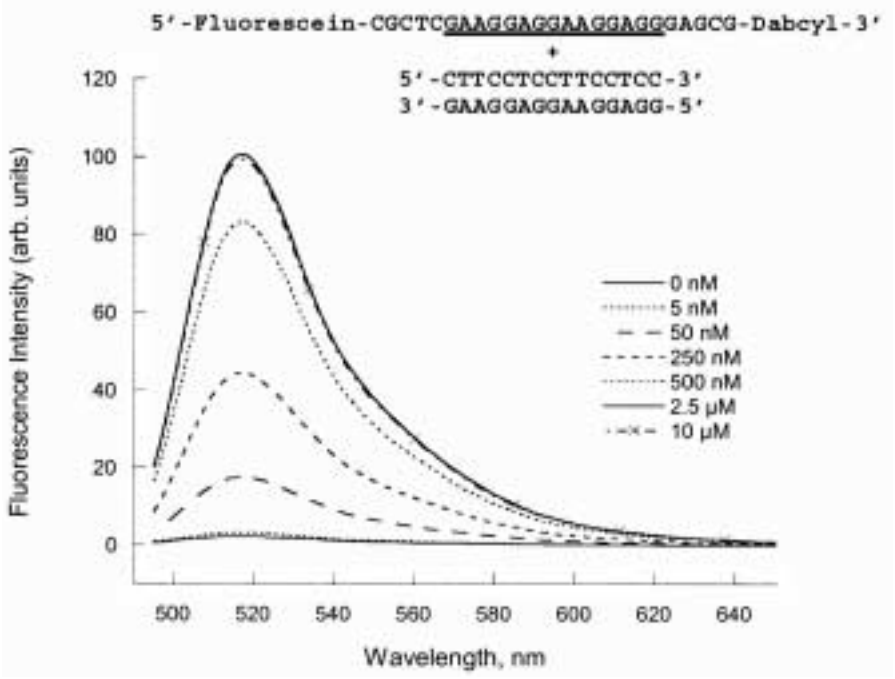

Figure 2: Fluorescence emission spectra of MB (50 nM) upon titration with target duplex. Conditions: 10mM Sodium Phosphate buffer, pH 7.3, 150 mM KCl.

The time-dependent enhancement of fluorescence intensity in the presence of the target duplex was used to analyze the kinetics of triplex formation. The pseudo first-order rate constants and second-order bimolecular rate constants for the triplex DNA stabilized by Na^+, K^+ and Mg^{2+} were on the order of 10^2 $s^{-1}$and 10^{-3} $M^{-1}s^{-1}$ respectively. The association curves for the binding of MB to target duplex in the presence of 1 mM $MgCl_2$, 150 mM KCl and 150 mM NaCl at 20 ºC are depicted in Figure 3. KCl, which is known to inhibit triplex DNA formation in some systems was found to be more efficient than NaCl in promoting triplex DNA formation between the MB and the target duplex used in this study. The thermodynamic analysis showed that the triplex DNA formed in the presence of KCl is entropically more favorable than that stabilized by NaCl, which may explain the higher association rate of triplex DNA formation in presence of KCl.

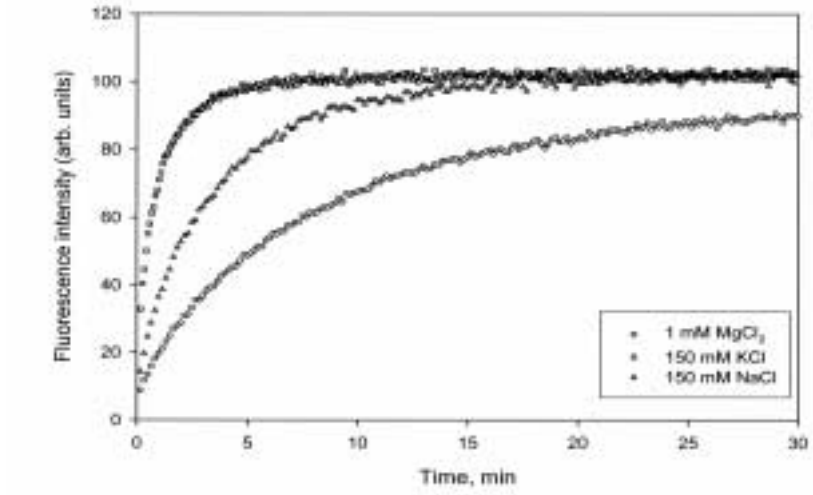

Figure 3: Kinetics of triplex DNA formation at 20 ºC in presence of 1 mM $MgCl_2$, 150 mM KCl and 150 mM NaCl. 5 µM duplex was added to 50 nM MB in 10 mM Na phosphate buffer, pH 7.3, containing salt as indicated, and the fluorescence intensity at 515 nm (λex = 485 nm) was recorded.

MBs are also useful in evaluating the thermodynamics of triplex DNA formation, as demonstrated recently in the analysis of binding of an MB with a 21-mer target sequence in the promoter site of cyclin–D1 stabilized by polyamine homologues (12). Triplex DNA formation resulted in an enhancement of beacon fluorescence by 12-fold. The variation of fluorescence intensity as a function of duplex concentration was analyzed for the determination of association constants, which were found to be ~ 10^8 M^{-1} in the presence of 1 mM spermine in 10 mM Na-cacodylate buffer,pH 7.3, wfΔ and increased ~ 10^{10} M^{-1} upon addition of 150 mM NaCl. The ΔH, ΔS and ΔG values calculated from the temperature dependence of the association constant also showed very stable triplex DNA formation in cyclin–D1 gene promoter site and the utility of using molecular beacons for such analyses.

The formation of triplex DNA requires the dissociation of a stable duplex stem. Thus triplex DNA formation occurs only when it is thermodynamically more favorable than stem stabilization. This may limit the use of MBs in triplex DNA formation studies when the triplex forming sequence is very small or under experimental conditions that stabilize the duplex stem more than the triplex configuration.

MBs as biosensors

With the human genome almost completely sequenced, fast and very sensitive methods for the analysis of genomic data are in great demand. Identifying and genotyping vast number of genetic polymorphisms in large populations pose a great challenge. MBs may be very useful molecules for these purposes, particularly for applications like detection of single nucleotide polymorphisms (SNPs). MB genotyping is emerging as a viable and cost effective method in addition to other fluorescence based assays like single base extension with fluorescence detection, homogeneous solution hybridization and oligonucleotide ligation assay (18,40,41). Tan et al. developed a MB based biosensor for the analysis of RNA and DNA using a biotinylated beacon surface immobilized on optical fibers (42,43). The enhancement of fluorescence after dipping in solutions containing gamma-actin mRNA amplified by PCR was analyzed and the concentration detection limit of this MB evanescent wave biosensor was found to be as low as 1.1 nM.

Recent developments in molecular beacons

Scorpion primers – Scorpion primers were developed to detect specifically the PCR amplification products in high throughput and automated methods. They have a basic MB design with a primer attached to the 3'-end. The heating and cooling (annealing) step after primer extension leads to binding of the loop to the sequence extended by PCR in a unimolecular mechanism. This binding leads to opening of the hairpin structure and a corresponding increase in fluorescence. Comparison with MB and Taqman polymerization methods showed that this is a superior method particularly under fast cycling conditions, under which hybridization is probably kinetically controlled (44,45). A major advantage of this method in PCR reactions is that scorpion primers have a PCR stopper that connects the loop and the 5' end, preventing the detection of non-specific PCR products. Development of a cystic fibrosis mutation detection assay using this method showed that scorpion primers are selective enough to detect single mutations and gave good sensitivity in all cases. Using scorpion primers with different fluorophores, this assay has been extended to a multiplex assay.

Wavelength shifting MBs – In a very recent development, Tyagi et al developed wavelength-shifting MBs that couple both the quenching and FRET mechanisms (19). In addition to the donor (termed as harvester fluorophore) and the quencher, another fluorophore that is a FRET acceptor (termed as emitter fluorophore) for the donor molecule, is attached through a spacer oligonucleotide. In the absence of target oligonucleotide, the molecule remains non-fluorescent due to the quenching of fluorescence as in the conventional MB. In the presence of a sequence specific target, the stem opens up, the quencher moves away, and the harvester fluorophore transfers energy to the emitter fluorophore which is in close enough proximity for

efficient FRET. A major advantage of this approach is that a single harvester fluorophore can be combined with different emitter fluorophores, allowing excitation at a single wavelength for different beacons targeted to bind to different sequences. This is particularly important for imaging applications where a single wavelength laser source is used. The application of this method has been demonstrated using wavelength shifting MBs with fluorescein as harvester fluorophore and 6-carboxyrhodamine 6G, tetramethylrhodamine or Texas red as the emitter fluorophore. Comparison of the hybridization to the targets of these wavelength-shifting MBs with conventional MBs showed significantly higher signal strength for the former showing the superiority of this approach. Its application in real time PCR and for the detection of two single nucleotide polymorphisms that occur very close to each other has also been demonstrated.

MBs with gold nanoparticles as quenchers – Recent developments in the assembly of hybrid materials composed of biomolecules and nonbiological, inorganic objects have been used to enhance the detection sensitivity of MBs further. Nanoparticles have been used as the quencher instead of the commonly used Dabcyl, whose quenching efficiency is lower for dyes emitting at longer wavelengths. Dubertret *et al.* developed a MB with a 1.4 Å gold nanoparticle that quenches fluorescein fluorescence by non-radiative energy transfer as much as 100 times more efficiently than Dabcyl (46). Comparison of the hybridization of MBs with gold nanoparticles and conventional MBs showed that the former are more sensitive for mismatch detection. These hybrid MBs are postulated to be more useful in detecting minute quantities of oligonucleotides in a pool of random sequences and for improved detection of single mismatches.

Summary

The applications described above show that MBs have become very important tools for a large number of biological applications. In the post genomic era, high throughput and low cost genetic analysis is expected to be crucial in the detection of various genetic diseases and to monitor the response to drug treatment. MBs with extremely high sensitivity and specificity will be very useful probes for many of these analyses. The possibility of using beacons in micro arrays to analyze a large number of samples simultaneously has already been demonstrated. With significant advances in fluorescence imaging techniques, MBs may find increasing applications in the study of localizing DNA and RNA, in understanding their dynamics in the cellular environment, and in tracking biochemical changes after blocking the transcription or translation with antisense oligonucleotides.

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