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Molecular Engineering of DNA: Molecular Beacons

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***M**olecular beacons (MBs) are specifically designed DNA hairpin structures that are widely used as fluorescent probes. Applications of MBs range from genetic screening, biosensor development, biochip construction, and the detection of single-nucleotide polymorphisms to mRNA monitoring in living cells. The inherent signal-transduction mechanism of MBs enables the analysis of target oligonucleotides without the separation of unbound probes. The MB stem–loop structure holds the fluorescence-donor and fluorescence-acceptor moieties in close proximity to one another, which results in resonant energy transfer. A spontaneous conformation change occurs upon hybridization to separate the two moieties and restore the fluorescence of the donor. Recent research has focused on the improvement of probe composition, intracellular gene quantitation, protein–DNA interaction studies, and protein recognition.*

1. Introduction

Nucleic acids, especially DNA bases, make an ideal framework for the molecular engineering of probes for a variety of unique applications in biochemistry and biomedicine. The advantages of nucleic acids lie in the simplicity of their synthesis, their suitability for structural modification, and their high selectivity and affinity. Specifically engineered DNA molecules can efficiently recognize target nucleic acids and other molecules, such as proteins and small molecules. In this Minireview, we summarize recent advances in the molecular engineering of DNA probes for a variety of applications, with a focus on molecular beacons (MBs). MBs are now widely used in areas such as genetic screening, biosensor development, biochip construction, the detection of single-nucleotide polymorphisms (SNPs), and messenger-RNA (mRNA) monitoring in living cells. This broad spectrum of applications can be attributed to the particular way in which these probes interact with DNA, RNA, and protein molecules. The unique stem–loop structure and fluorophore–quencher pair enable the detection of target nucleic acids to be reported in real time with excellent sensitivity and selectivity. Nevertheless, there are still many challenges associated with the development of MB probes, for example, in the design and engineering of MBs for optimal use in the intracellular monitoring of gene expression. Therefore, in addition to the design of MBs and their application in bioanalysis and biomedicine, we examine herein current approaches to the solution of specific problems encountered in the use of these DNA probes.

The discovery of the double-stranded structure of DNA in 1953[1] shed light on our understanding of the significant role that nucleic acids play in life processes.[2] The base pairing of nucleic acids, one of the strongest and most specific biomolecular recognition events, forms the foundation of almost all molecular probes for nucleic acids. Advances in molecular biology and the chemical synthesis of nucleic acids have

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benefited the development of nucleic acid probes, many types of which have been designed and applied in the fields of biology, medicine, and chemistry since the early 1960s.[3–5] Today, nucleic acid probes, in particular those based on DNA, are essential tools for exploring the biological processes of nucleic acid duplication, recombination, transcription, and expression. In the postgenomic era, there is a continuing demand for highly sensitive and selective DNA probes. Many kinds of DNA probes have been developed in recent years through various molecular-engineering strategies to meet this demand.

Molecular beacons, which were first described in 1996 by Tyagi and Kramer, are an excellent example of such DNA probes.[6] The targeted design of MBs appears simple, but is in reality quite sophisticated. Generally, MBs are DNA sequences composed of one target-recognition region of about 15–30 bases flanked by two short complementary stem sequences, which force the entire sequence to form a stem–loop structure in the absence of a target. The formation of the stem–loop structure brings the quencher and fluorophore, which are located at opposite ends of the MB, into close proximity, whereby fluorescence is quenched effectively. In the presence of a target DNA or RNA molecule, hybridization between the target and the loop sequence of the MB takes place, and the stronger intermolecular hybridization opens the weaker stem helix. The spatial separation of the fluorophore and quencher results in the restoration of fluorescence (Figure 1).[6–8] The thermodynamic stability of the hairpin structure, the highly efficient intrinsic signal switching, and the possibility of using a variety of fluorophores make MBs exceptional DNA probes with excellent sensitivity and selectivity, and real-time-detection capability. MBs are therefore used for a variety of applications,[6,9–25] such as DNA and RNA detection,[6,9–11] the monitoring of living systems,[12–15] the investigation of enzymatic processes,[16–18,27] the design of biosensors,[19,20] the study of protein–DNA interactions,[21–23] and the fabrication of biochips.[19,24,25] However, the scope of this Minireview is limited to fundamental aspects of MB design, recent applications of MBs in bioanalysis and the monitoring of intracellular processes, and a discussion of problems that still impede extensive application of MBs.

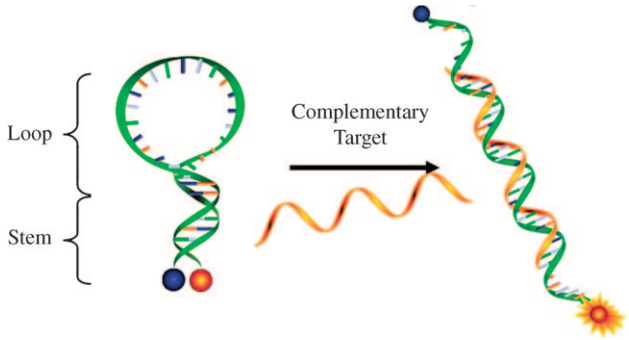

Figure 1. Working mechanism of a molecular beacon. The MB adopts a stem–loop structure and thus holds the fluorophore (orange) and quencher (blue) in close proximity. As a result, the fluorescence emission of the fluorophore is strongly suppressed (in the absence of a target). The target sequence hybridizes with the loop domain of the MB and forces the stem helix to open, whereupon fluorescence is restored because of the spatial separation of the fluorophore from the quencher.

2. Fundamentals and Design of Molecular Beacons

Despite numerous reports on the optimization of the structure of probes, on special base modifications, and on alternative methods for fluorescence generation, the design of MBs is based on two key factors, which determine their characteristics and functionality: a stem–loop structure and fluorescence resonance energy transfer (FRET).[6,8] Accordingly, in this section we explain briefly how the design of MB DNA probes is affected by signal transduction, thermodynamics, kinetics, and selectivity. Table 1 lists the various MB types discussed herein.

2.1. Energy Transfer and the Design of DNA Probes

Many nucleic acid probes, including adjacent probes[28–39] and TaqMan probes,[40,41] employ fluorescence resonance energy transfer (FRET) as their signal-transduction mechanism. These probes report the presence of target nucleic acids by various means. Adjacent probes, for example, are usually composed of two oligonucleotides designed to hybridize with target DNA or RNA at adjacent positions. TaqMan probes, which are complementary to the target sequence and labeled

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Table 1: Classes and properties of molecular beacons.

MB class	Properties
2-OMe-modified MBs	resistant to nuclease digestion; high affinity for the target; not substrates for RNase H; not resistant to single-strand-binding proteins (SSBs)
PNA-MBs	resistant to nuclease digestion; high affinity for the target; low solubility in aqueous media
LNA-MBs	resistant to nuclease digestion; higher affinity than most other MBs for the target; excellent structural stability; not substrates for RNase H; low background fluorescence in a living cell; depending on MB design, resistant to SSBs
QD-labeled MBs	bright signal upon hybridization; excitation of multiple QDs with a single wavelength for multiplex analysis
CP-linked MBs	bright signal upon hybridization; can be quenched with a single quencher
gold-NP MBs	excellent quenching efficiency; very good selectivity
SQ MBs	remarkable signal enhancement (320-fold with a triple-quencher assembly); simple purification

PNA = peptide nucleic acid, LNA = locked nucleic acid, QD = quantum dot, CP = conjugated polymer, NP = nanoparticle, SQ = superquencher.

with two fluorescent molecules, identify target DNA or RNA through a decrease in the FRET signal upon digestion of the probe through the 3′,5′ exonuclease activity of polymerase.[40,41] Owing to its convenience and the simple molecular interactions that lead to signal generation, FRET has proved an efficient signal-transduction mechanism for many studies of biomolecular interactions.

To construct MBs, a fluorescent moiety (donor), is coupled covalently to one end of the molecule, and a quenching moiety (acceptor) to the other end. When the two moieties are brought into immediate proximity by the stem helix, 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. In the presence of the target molecule, the loop region of the MB forms a hybrid helix that is longer and more stable than the stem helix. This interaction forces the MB to undergo a conformational change, which separates the stem helix. As the quencher is no longer positioned near the fluorophore, fluorescence is restored and signals the binding of the MB to its target.

MBs use different energy-transfer mechanisms for signal transduction. Essentially, two major categories exist: dynamic and static fluorescence quenching. Dynamic quenching includes Förster transfer (RET or FRET) and Dexter transfer (collisional quenching or electron-transfer quenching). Dynamic quenching, or RET, occurs without the release of a photon and is the result of long-range dipole–dipole interactions between the donor and the acceptor. In this case, energy-transfer rates depend upon the extent of spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, the quantum yield of the donor, the relative orientation of the donor and acceptor, and the distance between the donor and the acceptor. Specifically, the distance at which RET occurs with 50% efficiency is known as the Förster distance and is typically in the range of 20–70 Å. Static quenching requires the formation of ground-state complexes. The exact mechanism depends upon the stem sequence and the chromophores and linkers used.

For signal transduction, most MBs depend upon static or contact quenching (which was the original signaling mechanism of MBs).[11] The term of contact quenching describes the close proximity of a donor fluorophore to a nonfluorescent acceptor. Under these circumstances, the bulk of the transferred energy is not emitted as light, but through nonradiative processes, such as the release of heat.[26,42] This phenomenon has been exploited in the development of MBs through the use of nonfluorescent quenchers to further improve quenching efficiency and decrease background noise. The overall result is more-sensitive detection. Frequently used nonfluorescent quenchers include dabcyl (4-(dimethylaminoazo)benzene-4-carboxylic acid) and Black-Hole Quenchers BHQ1 and BHQ2. The typical static/contact quenching efficiency of these compounds lies between 85 and 97%. They can be paired with many common fluorophores;[26,42] dabcyl, for example, is often used in MBs with near-infrared fluorophores, even though its maximum absorption occurs at around 475 nm.

2.2. Thermodynamic Aspects

The hybridization of MBs with their targets can, in large part, be explained by the thermodynamics of the system. A simple all-or-nothing (or "on"/"off") model has been used as an approximation of the initial and final states of the reaction. This model was supported by experimental data, which showed that the melting temperature of a perfectly matched MB–target helix was 42 °C, whereas that of MB–target helices with a single mismatched base pair was between 28 and 31 °C. Thus, the model predicted the negligible effect of a mismatched base pair in the target DNA/RNA, but also predicted that the selectivity would be highest when the mutation point was positioned centrally within the loop region.[12,49,50]

A comparison of the thermodynamics of MBs and linear DNA or RNA probes further illustrates the relationship between melting temperature and selectivity, and explains why MBs are more selective than linear probes. First, linear DNA and RNA probes have two possible states or phases, that is, they can be free or bound to a target, whereas MBs have three possible states or phases: hybridized with a target, free in the stem–loop conformation, and free as a random coil.[48] The selectivity potential of a given probe is determined by the difference between the phase-transition temperatures of a perfect target–probe helix and a mismatched target–probe duplex. Detailed thermodynamic studies have shown that the free energy of MBs is lower in the stem–loop conformation (hairpin-structured MBs) than in the random coiled state.[9,10,12,47–49] These results suggest that the temperature difference causes the phase change from a duplex to a hairpin conformation before transition to the random-coil state, in which hybridization is most likely to occur. Thus, a longer or more stable stem helix should give more selective MBs, as the difference in the temperature of the phase transition between the perfect complementary helix and the mismatched duplex is increased.[48,49] Hence, it is the three-phase thermodynamic behavior of MBs (in contrast to linear DNA and RNA probes) that increases the difference in the transition temperatures between the perfectly matched helix and the mismatched duplex and thus causes the enhanced selectivity.

MB probes can be attached to surfaces and interfaces and thus used for the development of biosensors.[19,25,51,52] Their high sensitivity and excellent selectivity make MBs effective biosensor probes on gene chips and microarrays,[24] as well as for near-field optical and microfiber sensors.[20] To gain an understanding of the specificity of surface-immobilized MBs, the hybridization of MBs with target DNA (cDNA) was investigated at the single-molecule level by atomic force microscopy (AFM)[46] by measuring the force required to separate the MB from the target. The difference between the mean rupture force measured for a MB/cDNA hybrid and that measured for a MB/DNA hybrid with a single mismatched base pair was 0.50 ± 0.11 nN and thus considerably higher than the corresponding difference found for a linear DNA probe (0.21 ± 0.08 nN). These results show that immobilized MBs are much more specific probes than linear DNA probes for the detection of single mismatched bases.

2.3. Selectivity versus Kinetics

The excellent selectivity of MBs is a major advantage with respect to linear DNA and RNA probes. This high degree of selectivity results directly from the hairpin structure of MBs. The selectivity of MBs can be improved readily by optimizing their sequence.[49,53] For example, according to thermodynamic considerations, a longer or more stable stem helix should improve selectivity by increasing the difference between the phase-transition temperatures for the perfect complementary helix and mismatched duplexes,[48,49,59] as explained in Section 2.2. Therefore, the simplest way to improve the selectivity of MBs should be to elongate the stem or increase the G,C content in the stem region. Unfortunately, although a more stable stem helix increases the stability of the hairpin structure of the MB, it also decreases the rate of hybridization. Thus, the rate constant for the formation of a helix is about $10^4\,\text{M}^{-1}\,\text{s}^{-1}$ for linear DNA probes of 17–19 bases in length, whereas the rate constant for MBs decreases dramatically from about $5000\,\text{M}^{-1}\,\text{s}^{-1}$ to $40–300\,\text{M}^{-1}\,\text{s}^{-1}$ when the stem length is increased from four to six base pairs.[49] On the other hand, shorter stem sequences result in a higher fluorescence background and thus a decrease in the detection sensitivity. Therefore, a compromise must be found between the higher selectivity of longer stem sequences and the faster hybridization of shorter stem sequences. Usually, the stem sequence is about five to seven base pairs in length, and the loop contains between 15 and 25 bases. An alternative strategy is to use a longer stem sequence that is part of the target-binding sequence. In this way, higher hybridization rates are possible without sacrificing the specificity of the longer stem sequences.[50]

The relationship between selectivity and hybridization kinetics is also uniquely affected by another critical factor: the temperature difference. Therefore, temperature is an important external factor that should also be taken into account when designing MBs for certain applications. Higher temperatures drive free MBs from the hairpin structure to a random-coil state, in which fluorescence is partially restored. Consequently, applications with temperatures higher than 25 °C, such as in vivo imaging or real-time PCR detection, require MBs with more-stable stem regions for low background fluorescence to be observed.[6,13] At temperatures over 50 °C, the hairpin structure begins to melt into a random coil. They then cause such a high background signal that they are ineffective as MBs.[48,49] This problem can be overcome by synthesizing MBs from locked nucleic acids (LNAs), which have a bicyclic furanose unit locked in an RNA-mimicking sugar conformation (Figure 2).[54,55] Since LNA–DNA hybridization is stronger than DNA–DNA hybridization,[56–58] LNA-MBs are more selective than DNA-MBs. Melting-temperature measurements indicate that the LNA-MBs remain in a hairpin structure even at 95 °C, a temperature at which DNA and RNA would be denatured. The hybridization of LNA-MBs with target DNA at 95 °C has also been observed.[54] Thus, LNA-MBs hold promise for use in special biological applications for which normal DNA-MBs are unsuitable. Besides LNA-MBs, other temperature-stable stem systems have been developed and could be considered in the design of MBs.[143–145]

3. Bioanalytical Applications of Molecular Beacons

The advantages described in the previous sections have made MBs attractive systems for a variety of bioanalytical applications in the biomedical sciences, biotechnology, and chemistry.[8,53,60] The target molecules in these applications have expanded from DNA and RNA molecules to other biomolecules, such as proteins and enzymes. We discuss the major applications briefly in the following sections.

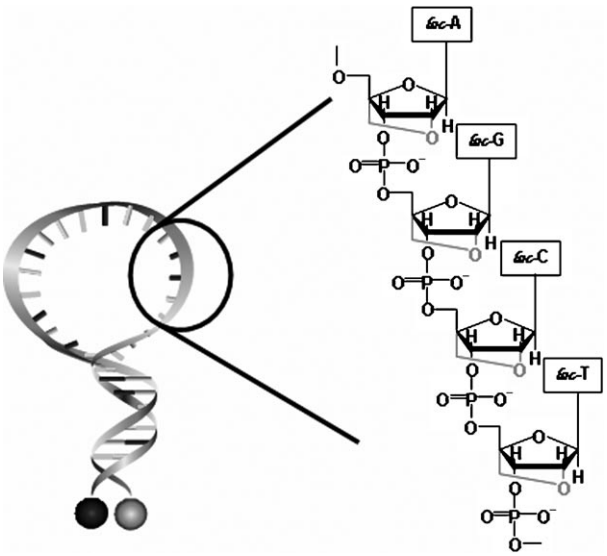

Figure 2. Composition of an LNA-MB. The sugar backbone of the LNA forms a rigid C3′ conformation owing to a 2′-O, 4′-C methylene bridge.

3.1. Real-Time PCR and Other Gene-Detection Assays

MBs are most commonly used for real-time PCR and other gene-detection assays.[53,60] To monitor the DNA amplification of the target sequence during the PCR process, MBs designed to hybridize with the forward/reverse PCR product are introduced into the PCR solution. With an increasing number of PCR cycles, a growing number of amplified target DNA molecules are produced and hybridize with the MB during the annealing stage. This process generates increasing fluorescence from the MB, and this fluorescence provides a measure of PCR progress in real time. MBs offer higher sensitivity and better specificity for target detection than TaqMan probes.[6,10] Moreover, the signaling mechanism of MBs enables multiplexed detection in homogeneous PCR reactions, which improves the throughput and precision of the assay.[9,10]

In other methods for DNA and RNA amplification, such as rolling cycle amplification (RCA) and nucleic acid sequence based amplification (NASBA), MBs are also effective reporters.[61–63] For the detection of RCA products, MBs constructed from 2′-OMe RNA are used to avoid degradation by 3′ exonuclease activity of polymerase. A stem length of four base pairs is sufficient in the 2′-OMe RNA MBs because of binding of 2′-OMe RNA to DNA, compared to DNA–DNA hybridization.[62] NASBA is an efficient, robust, and sensitive RNA-amplification method, but had the disadvantage of labor-intensive and time-consuming postamplification product detection. However, with the introduction of MBs designed to complement the antisense RNA amplicon, the NASBA product can be detected in real time and quantitatively.[62,63]

3.2. Detection of Triplex DNA

MBs are ideal for monitoring DNA and RNA interaction processes[8,48] and have been used to study the kinetics of triplex-DNA formation.[64] The formation of triplex DNA is known to inhibit the promoter site of disease-relevant genes. Real-time analysis of these processes could very well facilitate the development of gene and antigene therapeutic strategies. As an example, the melting profiles of intramolecular and intermolecular DNA duplexes, triplexes, and quadruplexes were investigated by using DNA probes that mimic the signal-transduction mechanism of molecular beacons.[65] Furthermore, MBs have been used to monitor DNA sticky-end pairing[130] (Figure 3). The study of DNA sticky-end pairing

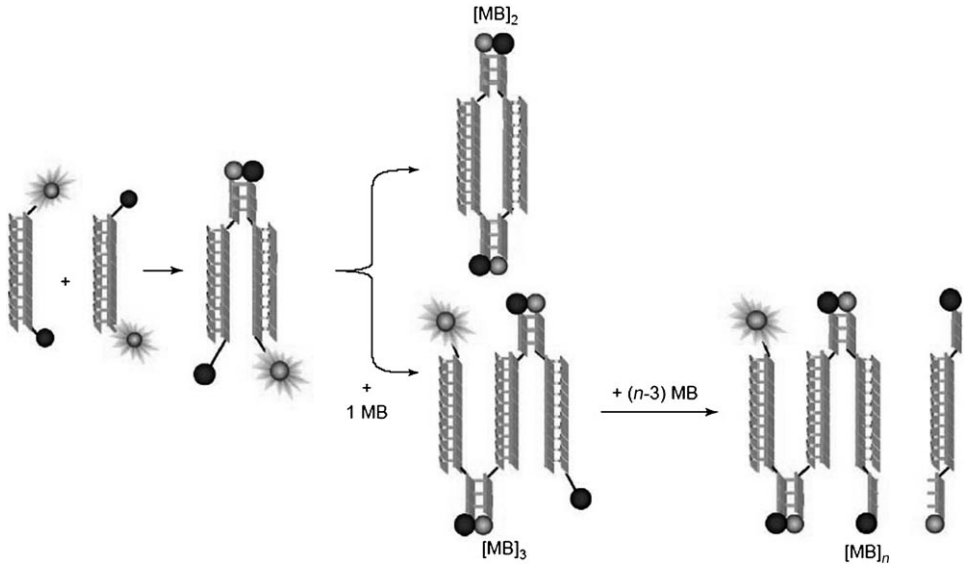

Figure 3. Sticky-end pairing of MBs. Two complementary sticky ends from two MB hybrids can pair to form a short double helix, so that two hybrids are associated at one end. These two MB hybrids can form a closed structure, $[MB]_2$, through pairing of the other two sticky ends, or polymerize into a larger structure, $[MB]_n$ ($n > 3$), by pairing with more hybrids. Sticky-end pairing draws the fluorophore and quencher together again and thus causes fluorescence quenching.

advanced our understanding of DNA characteristics, DNA assembly, and heterogeneous pairing at DNA ends and thus resulted in an overall improvement in MB design.

3.3. SNP and Genetic Screening

Single-nucleotide polymorphisms (SNPs), which make up about 90% of human genetic variation, are regarded as potent molecular genetic markers and valuable indicators for biomedical research, drug development, clinical diagnosis, and disease therapy.[66–70] The inherent signaling mechanism by energy transfer and the high selectivity of MBs enable the simple, rapid, and sensitive detection of SNPs.[10,71–73] MBs can be used to score SNPs in a homogeneous solution during the PCR process by introducing MBs with sequences complementary to those of the wild type and variant alleles, respectively. The loop portion of the MB is designed to have a melting temperature slightly higher than the PCR annealing temperature, and the stem is selected to dissociate at a temperature about 7–10 °C higher than the PCR annealing temperature. The allelic base is typically located in the middle of the loop portion of the MB to enhance its SNP-discrimination ability. To obtain an optimum signal, PCR primers are generally designed to produce a PCR product with a length of fewer than 250 base pairs; the detection zone should be within 20–30 bases from a primer end.[71] In comparison with other FRET-based homogeneous hybridization methods for SNP scoring, such as TaqMan assays, MBs can provide more reliable genotyping results as well as more flexible fluorescence detection for multiplexed analyses. Homogeneous and simultaneous signal amplification and target detection make MB-based SNP assays suitable for high-throughput genotyping studies. The identification of multiple SNPs in this manner is of interest to applications such as the construction of criminal DNA databases.[74]

3.4. Monitoring of Proteins and Small-Molecules

MBs are sensitive probes for monitoring interactions of proteins and enzymes with DNA.[7,8] The protein-recognition ability of MBs was first demonstrated with a single-stranded DNA-binding protein (SSB) from *E. coli*. The binding stoichiometry and binding constant were measured by monitoring the fluorescence enhancement caused by SSB-induced conformational changes of the MB (Figure 4a).[22]

Interactions between enzymes and DNA, for example, the digestion of ssDNA by nucleases, can also be detected with MBs (Figure 4b).[16] The cleavage of the loop sequence destabilizes the stem duplex and restores fluorescence. The cleavage processes induced by three different nucleases (S1 nuclease, DNase I, and mung bean nuclease) were monitored and studied. Similarly, interactions between lactate dehydrogenase (LDH) and ssDNA have also been studied by using MBs (Figure 4c).[21] Variant LDH isoenzymes were found to have different binding affinities for ssDNA; for example, a binding stoichiometry of 1:1 and a binding constant of $1.9 \times 10^{-7}\,\text{M}^{-1}$ were measured for the complex LDH-5/MB.

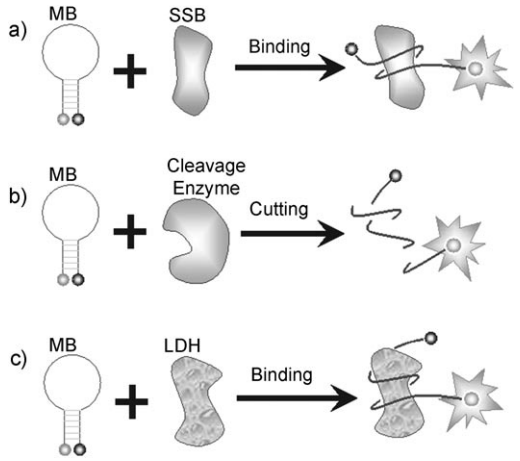

Figure 4. Application of MBs in enzymatic studies. a) Real-time monitoring of SSB–DNA binding: The MB binds to the SSB protein whereby its structure is disrupted and its fluorescence is restored. b) Detection of the enzymatic digestion of DNA: The enzyme cleaves the MB and destroys the hairpin structure to restore fluorescence. c) Detection of LDH–DNA interactions: LDH binds the MB and disturbs its structure, whereby fluorescence is enhanced.

As understanding of the characteristics and functions of MBs has increased, MB-based assays have been designed to study more-specific and more-sophisticated processes involving DNA and proteins, such as DNA ligation and phosphorylation,[17,18,75,76] which are involved in DNA duplication, DNA recombination, and the repair of DNA damage. For the monitoring of DNA ligation, MBs are designed to bridge two ssDNA sequences to form a nick structure (Figure 5a). The two ssDNA sequences are complementary to two adjacent zones of the loop portion of the MB. The melting temperature of these two short sequences is much lower than that of the MB; therefore the loop–stem structure of the MB will remain intact. When the DNA ligase is introduced, it catalyzes the junction of the nick structure by using the loop of the MB as the template to form a longer DNA sequence that is complementary to the entire loop sequence. This process leads to the separation of the stem and the restoration of fluorescence. The DNA-ligation process can thus be monitored in real time.[18,75,76]

A number of DNA ligations catalyzed by different DNA ligases, including T4 DNA ligase and the DNA ligase from *E. coli*, have been explored by using this principle. The DNA ligase from *E. coli* operated much more precisely than T4 DNA ligase; however, a T-G mismatch at the 5′ end of the nick led to the highest error rate for the ligation with both ligases. The effects of metal ions, small biomolecules, and drugs on the activity of DNA ligases have also been examined. This assay might be useful for the identification and development of ligase-targeted drugs.[76] However, as we still do not know exactly how the ligation process takes place, the results of the studies with molecular beacons should be interpreted with caution.

A similar strategy with MBs was used to study DNA phosphorylation, an important process for the repair of DNA damage (Figure 5b).[17] In this case, the DNA fragment

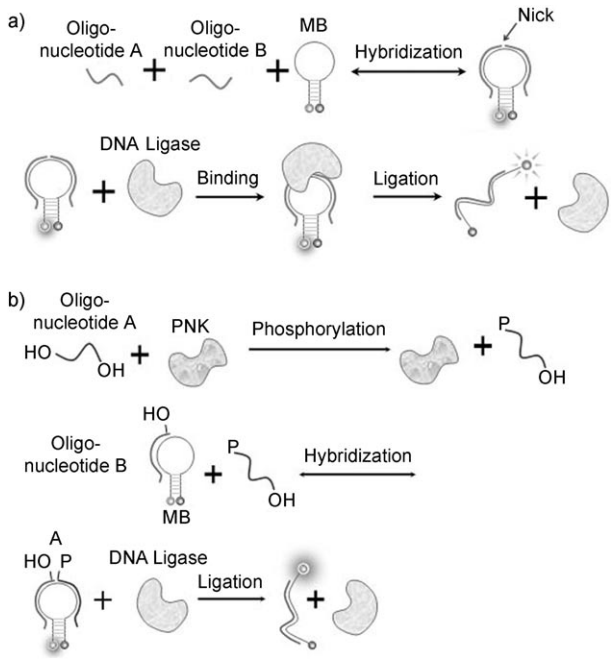

Figure 5. Application of MBs in phosphorylation and ligation studies. a) Real-time monitoring of nucleic acid ligation: Two oligonucleotides that are complementary to opposite halves of the MB loop hybridize with the MB, whereby a nick is formed, and the stem may be opened slightly. The DNA ligase binds to the nick and catalyzes the ligation of the two short oligonucleotides to form a longer oligonucleotide. The ligation product hybridizes with the MB to restore fluorescence. b) Monitoring of nucleic acid phosphorylation: Oligonucleotide A is first phosphorylated at the 5′-hydroxy group by the polynucleotide kinase. The nick formed upon the hybridization of oligonucleotide B and phosphorylated oligonucleotide A with MB can be sealed by the DNA ligase, whereupon the stem helix of the MB is opened, and fluorescence is restored.

located at the 5′ side of the nick was modified to mimic dephosphorylation damage at the 5′ end of DNA (that is, one hydroxy group at the 5′ end of the DNA is not phosphorylated). Consequently, the nick cannot be sealed by a DNA ligase. Phosphorylation of the damaged DNA under the catalysis of a polynucleotide kinase (PNK) is required before the two DNA fragments can be ligated to form the longer oligonucleotide sequence complementary to the MB loop.

The use of MBs to investigate interactions of small molecules has also been attempted recently.[76,133] A new molecular scheme was developed for the detection of small molecules, such as nicotinamide adenine dinucleotide (NAD) and ATP. For the NAD assay, MBs were used with the DNA ligase from *E. coli*. The MB was designed to bridge two short oligonucleotides with its loop sequence. Under these conditions, the DNA ligase from *E. coli* will only catalyze the ligation of two short oligonucleotides in the presence of NAD. The ligation results in the opening of the MB and the recovery of the fluorescence signal. This MB assay is faster, more selective, and about ten times more sensitive than currently used NAD assays and can be used conveniently to analyze for NAD in biological samples, such as cell lysates.

3.5. Biosensors and Biochips

The use of MBs on solid surfaces as biosensors is expanding rapidly.[19,20,24,25,51,77–82] Two important issues must be addressed in the development of such systems: 1) The immobilization of the MBs on the surface must proceed with high efficiency and lead to stable sensor architectures; 2) the MBs must be optimized for use at the liquid–solid interface. In analogy with the immobilization strategies reported previously for DNA and RNA probes, three types of interaction are employed for most applications for the immobilization of MBs: the biotin–avidin interaction, the thiol–gold linkage, and amide bonds. Corresponding to the various immobilization strategies, different support substrates, such as glass,[19,24] gold,[51,84] polyacrylamide[85] and agarose,[54] are used. All of these strategies provide the highly efficient and stable immobilization that is essential for the reproducibility, sensitivity, and response speed of MB-based biochips and biosensors.

The immobilization of MBs on a surface often results in increased background fluorescence. Several approaches have been developed to minimize this effect. One possibility is to add a spacer between the MB and the surface to minimize potential MB–surface interactions.[20,86] In one case, a polyT linker at one end of the MB was used as a spacer.[24] However, this approach provides only a limited improvement in sensitivity. In another approach aimed at providing a homogeneous local environment for immobilized MBs, MBs were attached to the surface of a functionalized hydrophilic gel film, such as agarose or polyacrylamide.[25,85] The MB–agarose system showed lower background fluorescence, higher sensitivity, a faster response, and better selectivity than MBs fixed on glass. The immobilization of MBs on a gold surface is yet another way to decrease background fluorescence, owing to the highly efficient quenching effect of the gold surface on fluorophores in close proximity.[51,84] MB fluorescence was enhanced more than 100-fold after hybridization with target DNA.

Novel signal-transduction strategies, such as enzymatic signal detection and electrochemical electron transfer, have been developed as substitutes for fluorescence analysis.[87,88,131,138] In these detection methods, the reporter is an enzyme or electrochemical agent linked to the free end of an MB. The conformational change of the MB upon hybridization with the target nucleic acid triggers the release of a signal by the reporter.

MB-functionalized beads have been used for multiplexed analyte detection.[80,86] For this application, various MB-coated microspheres are entrapped randomly within an array of wells etched on an optical fiber of 500 μM in diameter.[80] The high-throughput capacity and fast response time of this array enables the accurate analysis of multiple genetic mutations. In combination with analysis by flow cytometry, MB-functionalized microspheres have been used to detect multiple unlabeled nucleic acid targets.[86] The signals from various MB-coated beads were differentiated by using a classification strategy based on size and color coding. Such assays based on MB beads are expected to enable simple, fast, and accurate genetic analysis and genotyping for disease diagnosis and therapy.

Another class of powerful biosensors are ultrasmall optical-fiber probes, which are characterized by their extreme sensitivity and enable spatial resolution.[89,90] By taking advantage of immobilized MBs and evanescent wave excitation, biosensors were fabricated from submicrometer fibers and used to detect DNA and RNA in small-volume samples with exceptional sensitivity and selectivity. These renewable fiber probes could be applied to the detection of specific DNA or RNA targets in living cells.

4. Real-Time Intracellular Monitoring with Molecular Beacons

One distinct advantage of methods based on MBs is that analytes can be detected without prior separation of unbound probes. These methods can therefore be used for intracellular measurements. For example, the synthesis, transport, and distribution of mRNA in living cells can be monitored with good spatial and temporal resolution to provide important information for functional genomics.[12,14,55] A number of probes have been developed for intracellular mRNA detection in recent years, including linear binary FRET probes,[91] the nucleic acid stain SYTO 14,[92] GFP-fused RNA-binding proteins,[93–96] quenched autoligation probes,[97,98] and MBs.[12,14,99] Despite these advances, the field is in its infancy, and there is still no single effective way to monitor intracellular events with all required parameters.

4.1. Molecular Beacons for mRNA Detection in Living Cells

As a result of their unique signaling mechanism, MBs are ideal for studying gene expression in living cells: 1) Target RNA is detected in real time without the need to separate the bound and unbound probes. This feature is crucial for studies on living cells, as it is impossible to separate the MB–mRNA hybrids from an excess of the unbound MB without destroying the cell. 2) The high sensitivity of MBs (in the nanomolar to subnanomolar range) is important for sensing mRNA molecules with a low copy number in a single cell. 3) MBs have exceptional selectivity and can differentiate between sequences with single-base mismatches.

In the design of MBs for intracellular mRNA monitoring, there are two important issues to consider: The MBs must be designed to find the target mRNA inside the cells, and an effective method must be selected for efficient delivery into the cells. The selection of an appropriate target region of the mRNA is perhaps the primary obstacle to sound MB design. This complication arises from the complex secondary structure of the long mRNA sequences and the fact that a given MB needs to have free access to its complementary sequence. Therefore, the selection of a suitable target site usually begins with the prediction of possible mRNA secondary structures. The target site is chosen in regions that have a high probability of remaining single-stranded to ensure that the native mRNA structure experiences only minimal interference from hybridization with the MB. High-affinity oligonucleotides of different lengths that are complementary to the target sequences in the chosen regions are then used as the loop sequences of MBs. To minimize false hybridization with nontarget mRNA molecules, BLAST (basic local alignment search tool) analysis for selected sequences is a necessary step.[100]

The most common methods for DNA delivery into living cells are microinjection,[14,101] electroporation,[102] reversible permeabilization,[15] and peptide-assisted delivery.[103] Microinjection has several advantages: It enables delivery into a single cell of interest, the probe can be delivered in relatively reproducible amounts, and the response of the probe can be observed immediately. Disadvantages of the microinjection technique are the relatively complex instrumentation and low throughput. Electroporation and reversible permeabilization offer a much higher throughput. Both methods produce pores in the cell membrane through which probes enter the cell by passive diffusion.[15,102] However, intracellular molecules may also diffuse out of the cell, and there are variations in the amount of the probe delivered into each cell. Another approach, peptide-assisted delivery, enables probes to pass through the cell membrane without disturbing the integrity of the cell.[103] However, this technique requires the peptide to be conjugated to the probe, which may increase the cost and complexity of probe synthesis. Furthermore, the delivery process requires an incubation period, which may compromise the monitoring of hybridization dynamics. Also, the binding of the peptide-linked MB on the cell membrane may trigger endocytosis-like processes, so that strategies to facilitate endosomal escape may be required.

4.2. Gene-Expression Studies in Living Cells

The increasing use of MBs for intracellular analysis has led to the development of MB methods for the visualization of the localization, distribution, and transport of mRNAs inside living cells. Early studies with MBs in vivo focused on the detection of hybridization to the target RNA sequence, rather than on localization and distribution.[13,104] However, in 2003, Tyagi and co-workers demonstrated that MBs could be used to visualize the distribution and transport of mRNA.[12] In this study, an MB for Oskar mRNA in oocytes of *Drosophila melanogaster* was investigated. To eliminate the background fluorescence of the MB, an approach was developed with two MBs that targeted adjacent regions on the mRNA. When both MBs were hybridized to the mRNA sequence at adjacent positions, the FRET signal would occur and indicate the presence of the target. This method could be used not only to visualize mRNA distribution, but also to track the migration of the mRNA through the cell and even into adjacent cells in the oocyte. To study the behavior of a virus, viral mRNA was imaged by using MBs inside host cells. The localization of the mRNA was examined, and diffusion of the mRNA was monitored by fluorescence recovery after photobleaching (FRAP). Bao and co-workers expanded on mRNA visualization by showing the colocalization of mRNA and intracellular organelles in human dermal fibroblasts.[15] In this study, MBs were used in conjunction with a fluorescent mitochondrial stain. Since the fluorescence of the MBs and

that of the stain could be resolved spectrally, Bao and co-workers were able to demonstrate that the mRNA of both glyceraldehyde 3-phosphate dehydrogenase and K-ras were localized specifically within the mitochondria.

It is commonly observed that MBs introduced into living cells are quickly sequestered into the cell nucleus and then opened. The high fluorescence background that results in the intracellular compartment and particularly in the nucleus area seriously affects the study of mRNA in the cytoplasm. To solve this problem, tRNA translated in vitro was attached to one end of an MB by hybridization with an extended arm of the MB.[99] The results of this study suggested that the tRNA-coupled MBs had a longer residence time in the cytoplasm and were not concentrated into the nucleus. In this form, they were suitable for intracellular mRNA monitoring. A similar strategy, whereby molecular beacons are attached to macromolecules or nanoparticles, can also help to keep the MBs out of the nucleus.[139,140]

The expression level of mRNA is also an important area of investigation in MB-based studies of living cells. Two MB approaches have been used to determine the relative expression levels of K-ras and surviving mRNA in human dermal fibroblasts.[15] We used MBs and a reference probe as an internal standard for ratiometric analysis to study the stochasticity of mRNA expression of manganese superoxide dismutase (MnSOD) in human breast carcinoma cells.[105] We analyzed the MnSOD expression of three different cell groups and compared the results with the expression of β-actin mRNA. The results for the different cells were normalized by ratiometric analysis to compensate for many experimental and instrumental variations and could then be in order for direct cell-to-cell comparisons. This method offers a new strategy for the quantitative detection of mRNA in living cells.

Whereas several examples of the study of single mRNA targets inside cells have been described, the monitoring of multiple mRNA molecules simultaneously in a single living cell remains a challenge. Such an experiment would enable direct comparison of the expression levels of related mRNA molecules in a single cell and would be extremely useful for the study of certain diseases. It has been shown that MBs can be labeled with different fluorophores to report different mRNA targets. In one study, MBs for MnSOD and β-actin mRNA, along with an MB as a negative control, were microinjected into cells, and a confocal microscope with multiple fluorescence-detection channels was used to monitor the relative expression levels of MnSOD and β-actin simultaneously by ratiometric analysis (Figure 6).[14,105,146] Ratiometric analysis enables more accurate and more reproducible measurements than direct intensity measurements. The fluorescence of the MnSOD-specific MB in the cells increased considerably during incubation for 4 h with lipopolysaccharide (LPS), a prototypical endotoxin known to stimulate MnSOD expression. In control cells without LPS, the fluorescence of the MnSOD MB remained at a low level. This study demonstrates that MBs are suitable for effective intracellular monitoring and could potentially be used to study diseases where the comparison of expression level of different mRNA species is important.

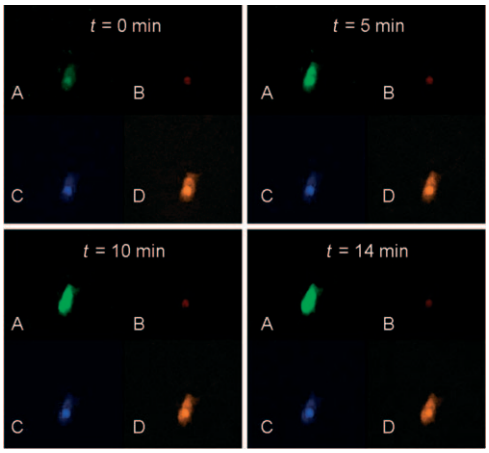

Figure 6. Simultaneous detection of multiple mRNAs inside a living cell. Time-lapsed fluorescence images of MBs inside a single MDA-MB-231 cell are shown. A: MB for β-actin (green); B: control MB (red); C: MB for MnSOD (blue); D: tris(2,2′-bipyridyl)ruthenium(II) (RuBpy) reference probe (orange).

4.3. Neuron-Cell Genomics

Because of the extreme complexity and diversity of the nervous system, the mechanisms underlying brain function and consciousness remain enigmatic, even after many years of extensive research.[106] For example, the family of olfactory receptors (ORs) is one of the largest known mammalian gene families and contains about 900 genes for humans and 1500 genes for mice.[107] Among the approximately 1000 (or even more) possibilities encoded in the genome, every olfactory sensory neuron chooses just one OR and transcribes it from just one allele in order to establish the correct olfactory circuitry between the nose and the brain. Although our knowledge of the brain has been limited by an inability to identify and classify its various cell types,[108] progress in genomics and proteomics now enables us to study the development and operation of neurons and circuits at the molecular level. New methods in functional genomics and proteomics, such as comparative genomic hybridization (CGH) profiling, are being applied increasingly to gain insight into neural networks.[109]

Investigations into the function of single neurons would be enhanced by real-time monitoring of the expression level of relevant genes in living neuron cells. We attempted to use MBs to detect the mRNA of living neuron cells by the same approach that we used to study breast carcinoma cells (Figure 7).[14,105] Unfortunately, the MBs produced a high fluorescence signal soon after microinjection into the neuron cells. This result indicates that the cytoplasmic contents of the neuron cells were very different to those of the cancer cells tested previously. This high background fluorescence might also be caused by nuclease digestion or protein binding, which are two major concerns regarding the use of MBs in living systems. To increase the stability of MBs in neurons, we designed and synthesized new MBs from LNA (locked nucleic acid) bases.[54,55] The preliminary results suggest that LNA-MBs are much more stable in the cell cytoplasm than

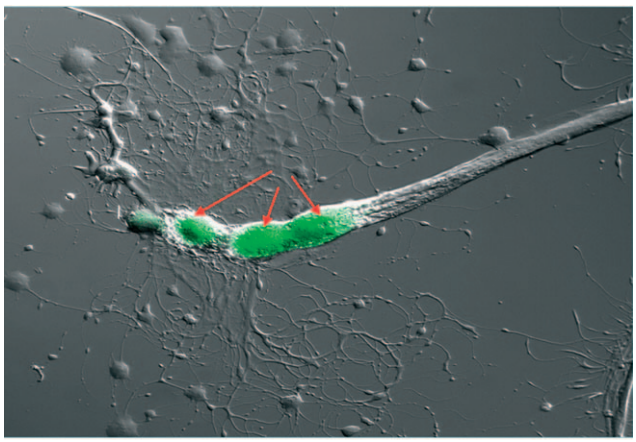

Figure 7. Living neuron into which MBs with polyU sequences were injected. The green fluorescence is caused by opening of the MBs during neuronal processes.

conventional MBs because of the rigid structure of the LNA bases (see Table 1 in Section 2.1).

4.4. Optimization of DNA Probes by Molecular Engineering

Much optimization is necessary before MBs can be used to monitor mRNA expression in living cells, in particular in terms of the sensitivity of the probes and the prevention of nonspecific protein binding and nuclease digestion.

4.4.1. Increasing the Sensitivity of Molecular Beacons

The generation of MB probes with high sensitivity is a complex problem. For target sequences with a very low copy number in single living cells, the sensitivity of conventional MBs is often inadequate. Therefore, most applications are limited to the reporting of abundant or stimulated gene products.[12,14,99] As a basic principle, the sensitivity of MBs can be improved by either increasing the fluorescence intensity of the fluorophore or enhancing the quenching efficiency of the quencher. However, the latter solution is confounded by the fact that there is always a certain background signal from the cellular compartment and the environment, regardless of the extent to which the fluorescence of the donor can be quenched. Accordingly, most strategies for improving the sensitivity of MBs have focused on increasing fluorescence intensity.

As mentioned in Section 2.1, the typical efficiency of static or contact quenching observed is 85–97%.[26,42] Theoretically, higher quenching efficiencies would lead to a higher signal-to-background ratio and thus better sensitivity and a greater dynamic range for target detection.

A novel strategy for highly enhanced quenching, termed superquenching, involves a molecular assembly of multiple quenchers.[43] For example, with a FAM as the fluorophore, quenching efficiency was improved from 92.9 to 99.7% when an assembly of three dabcyl molecules was used instead of just one dabcyl molecule. Furthermore, the signal-to-background ratio increased significantly from 14 to 320; thus, the potential for detecting trace amounts of a target was improved considerably (Figure 8). The increased hydrophobic interaction between the fluorophore and multiple quenchers resulted in an increase of 5 °C in the melting temperature of the MB. The higher melting temperature could possibly improve the capacity of the MB to distinguish single-base mismatches. However, the method of reducing probe background by superquenching cannot reach its full potential unless the cellular background is greatly minimized by other strategies such as time-resolved measurement or wavelength shifting.[124]

4.4.2. Increasing the Sensitivity of Molecular Beacons with Conjugated Polymers

Another strategy for the improvement of MB sensitivity is the use of fluorescent dyes. Poly(phenylene ethynylene) (PPE)[44] is a water-soluble polyelectrolyte with a high quantum yield.[45] A single PPE chain can generate a fluorescence signal of much higher intensity than that of a single fluorophore in a conventional MB. In a novel synthetic method for coupling PPE to oligonucleotides, the polymerization reaction is carried out directly on the oligonucleotides, which are linked to a glass support with pores of controlled size. It was found that the PPE interfered with neither the hairpin structure of the MB nor its hybridization rate. We compared the brightness generated by PPE with the fluorescence intensity of different common fluorophores at their optimal excitation and emission wavelengths. The concentration of these fluorophores was kept constant at 10 nM. The data obtained show clearly that PPE is much brighter than regular organic dyes, such as Cy3, tetramethylrhodamine (TMR), FAM, and Alexa Fluor 488 (ALX488; Figure 9). Under our experimental conditions, PPE is about 20 times brighter than Cy3 and more than six times brighter than Alexa Fluor 488 (Figure 9). Moreover, the fluorescence intensity of a single PPE chain is about 75% that of a quantum dot (QD); in other words, its brightness is comparable to that of a nanoparticle.

The quencher dabcyl showed high quenching efficiency in combination with PPE. An experiment was performed to determine whether the superquenching of PPE by dabcyl was possible. PPE (2 μM: concentration of the repeat unit) was quenched very efficiently by dabcyl at increasing concentrations (Figure 10). The Stern–Volmer quenching constant at lower quencher concentrations (< 0.4 μM) was approximately $4 \times 10^6\,\text{M}^{-1}$. At higher concentrations of the quencher, an upward curve was observed in the Stern–Volmer plot, and a much higher quenching constant of approximately $1.4 \times 10^7\,\text{M}^{-1}$ was found. Upward curvature in the Stern–Volmer plot is typical for conjugated polyelectrolytes with oppositely charged quenchers. The mechanism for this behavior has been discussed previously. In our experiment, the MB conjugated with PPE generated a strong fluorescence signal upon binding to the target. This result is promising for bioanalytical applications.

New strategies of this type, which improve quenching capability and strengthen overall signal intensity, offer a

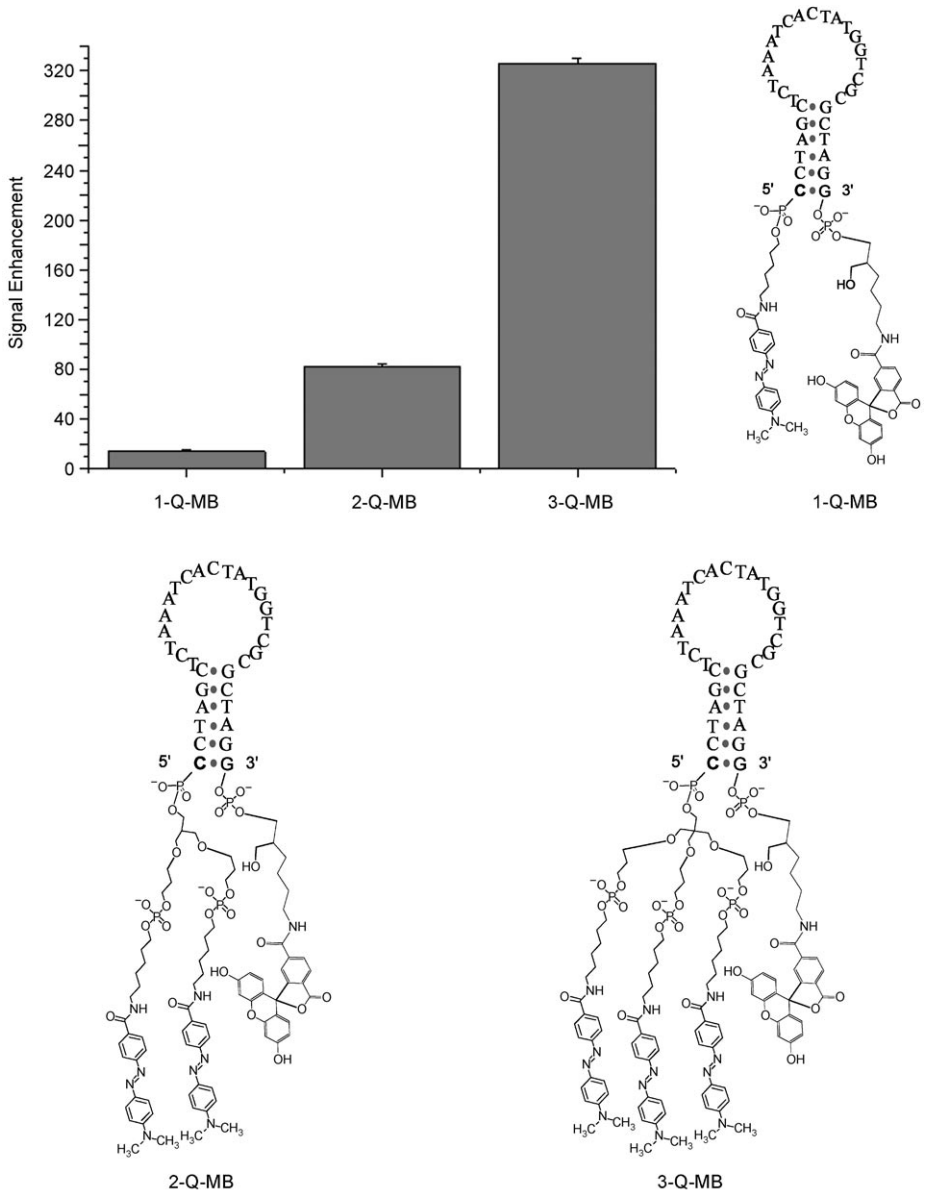

Figure 8. Superquencher MBs. Signal-to-background ratio of the MBs with one (1-Q-MB), two (2-Q-MB), or three (3-Q-MB) quenchers. The MBs have the same sequence and are labeled at the 3′ end with fluorescein as the fluorophore. One, two, or three dabcyl molecules were attached to the 5′ end of the oligonucleotide. The MB containing three quenchers produced a more than 320-fold signal enhancement upon hybridization; a 14-fold signal enhancement was observed with the single-quencher MB.

potential solution to the difficulty of detecting targets of low abundance in living cells. These strategies can also be transferred readily to the design of other DNA and RNA probes for bioassays that require high sensitivity.

4.4.3. Increasing the Stability of Molecular Beacons with Modified DNA Bases

A general problem associated with the use of MBs is their cleavage by nuclease digestion, a mechanism by which cells protect themselves from invasion by heterogeneous genes. MBs have been shown to degrade inside cells within about 45 min, after which time MB fluorescence increases significantly.[14,55] To improve the cytoplasmic stability of MBs, modified DNA bases, including 2′-OMe derivatives,[110–112] phosphorthioate derivatives,[113,114] and peptide nucleic acid (PNA) bases,[115,116,141] have been incorporated into their structure. The 2′-OMe-modified MBs offer good nuclease resistance against intracellular digestion and are not subject to cleavage by RNase H.[12] However, they exhibit high background fluorescence in living cells, possibly as a result of nonspecific protein binding.[21,22,138] PNA-MBs resist nuclease degradation and have a good affinity for DNA and RNA targets; however, their low solubility and occasional aggregation have limited the scope of their application.

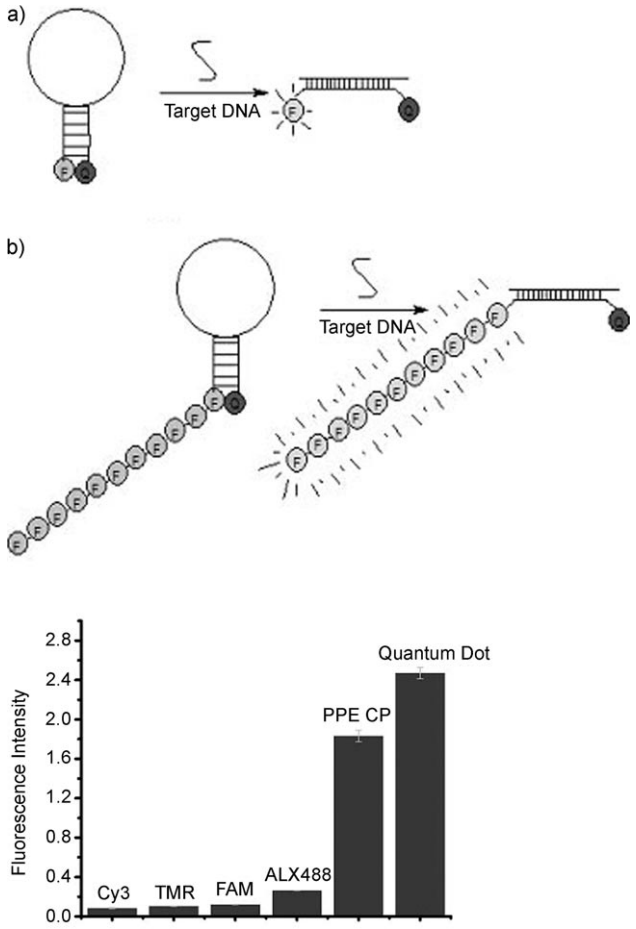

Figure 9. PPE-MBs. Top: Working principle behind a) a conventional MB and b) a conjugated-polymer-labeled MB. In the conventional MB, only one fluorophore is used to report a target-binding event, whereas in the conjugated-polymer-labeled MB, a fluorescent polymer chain is used. Bottom: Comparison of the fluorescence intensity of different fluorophores. The excitation/emission wavelengths for Cy3, TMR, FAM, ALX488, PPE, and quantum dots are 543/560, 557/581, 488/514, 488/515, 440/520, and 400/520 nm, respectively. The concentration of all dyes is 10 nM.

To take full advantage of LNA-MBs (mentioned in Sections 2.3 and 4.3) for a variety of applications, including the analysis of complex samples and intracellular monitoring, Yang et al. synthesized a series of chimeric DNA/LNA MBs systematically and studied the effect of the DNA/LNA ratio in MBs on their thermodynamics, hybridization kinetics, protein-binding affinity, and enzymatic resistance.[132] The LNA bases in the stem sequence of the MB had a significant effect on the stability of the hairpin structure. The hybridization rates of LNA-MBs were improved significantly by lowering the DNA/LNA ratio in the probe. Furthermore, a shared-stem design was important; that is, the stem region of the LNA-MB should also hybridize to the target sequence to prevent sticky-end. Only MB sequences with alternating DNA and LNA bases or entirely composed of LNA bases were found to resist nonspecific protein binding and digestion by DNase I. Additional results showed that a sequence consisting of a DNA stretch of less than three bases between LNA bases was able to block RNase H function.

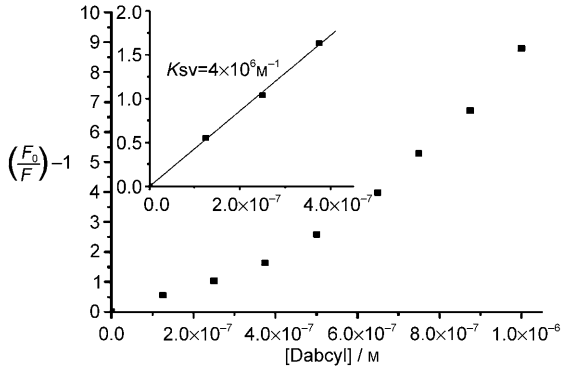

Figure 10. Stern–Volmer plot of the fluorescence quenching of PPE by dabcyl in glycine–HCl buffer (40 mM, pH 2.3). The buffer was used to protonate dabcyl as a counterion to the polymer PPE.

Overall, the study suggested that a shared-stem MB with a 4 base-pair stem and alternating DNA/LNA bases is desirable for intracellular applications as this configuration ensures reasonable hybridization rates, reduces protein binding, and resists nuclease degradation for both target and probes. These findings have enormous implications for the design of molecular LNA probes for intracellular monitoring, disease diagnosis, and fundamental biological studies. Further modifications provided LNA-MBs that were suitable for long-term (from a few hours to days) intracellular monitoring in real time with high sensitivity and selectivity.[142] The LNA-MBs composed of a mixture of LNA and DNA bases showed extremely high biostability. They were tested with MDA-MB-231 cancer cells to monitor mRNA expression levels in real time for 5–24 h. The LNA-MBs were still functional after 24 h inside living cells and thus enable the measurement of intracellular gene expression over an extended period of time.

To address the problem of stem invasion in hairpin probes, we recently created a modified MB in which non-natural enantiomeric DNA (L-DNA) is incorporated in the stem and natural D-DNA in the loop.[134] Since L-DNA is the mirror-image form of naturally occurring D-DNA, it forms duplexes with the same physical characteristics in terms of solubility and stability as those of D-DNA hybrids. With the exception of forming a left-handed double helix, however, L-DNA cannot form stable duplexes with D-DNA. By incorporating L-DNA into the stem region of an MB, intra- and intermolecular stem invasions are reduced, melting temperature is increased, and selectivity for the target is improved; all these factors lead to enhanced biostability. Our results suggest that the incorporation of L-DNA is a useful strategy for the design of functional nucleic acid probes.

5. Outlook

Since the introduction of molecular beacons in 1996,[6] many efforts have been made to improve their design and extend their scope of application, which has led to the creation of a large family of efficient MB probes. The advantages of MBs include their highly efficient signal-transduction mechanism through energy transfer, the flexi-

bility of their chemical modification, and their cost-effectiveness and have led to the development of a broad spectrum of applications in biology, chemistry, biomedicine, and biotechnology. Efforts to improve MBs will continue to focus on the optimization of signaling transduction, the use of modified bases, and the development of new biochemical and biomedical applications.

An important approach to improve the performance of MBs is the development of a more efficient signal-transduction strategy. A wealth of new signaling strategies, such as FRET,[11] wavelength shifting,[9] quenching at metal surfaces,[51,84,118] enzymatic amplification[87,119,120] and electrochemical signal transduction,[88] have been developed and applied to MB design. Novel fluorophores and quenchers, such as gold nanoparticles,[118] superquenchers,[43] pyrene derivatives,[121,135] conjugated polymers,[44] gold surfaces,[51,84] and ferrocene derivatives,[88] are also becoming increasingly attractive for various applications.

A further advantage of MBs is that chemical modification of the structures is relatively straightforward, which enables the use of highly flexible synthetic approaches. Several base modifications have been developed for MBs, such as the incorporation of 2′-OMe[110–112] and phosphorthioate derivatives,[113,114] as well as PNA,[115,116] LNA,[54,55] and L-DNA bases[134] (see Table 1). MBs with these modifications have additional useful properties, including resistance to nuclease digestion, a higher hybridization affinity, and minimized nonspecific protein binding. MBs can also be linked to carbon nanotubes to generate molecular sensors for studying molecular interactions.[147]

The scope of application of MBs, which were originally used in PCR monitoring and allele analysis, has been extended enormously in the past few years. In combination with other platforms and technologies, MBs are now used for protein assays,[21,22] enzyme monitoring,[16–18] mRNA tracing in living cells,[12,14,99] biosensors,[19,20,80,82] and molecular computing.[119,122] MB aptamers,[123–127] for example, are protein probes that combine the advantages of the signal-transduction mechanism of MBs and the specificity of molecular recognition by aptamers.[128,129,136,137] They can be used for the highly sensitive and selective detection of proteins and cells.

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