them to observe hybridized probes.

Two forms of energy transfer may exist in MBs: direct energy transfer and fluorescence resonance energy transfer (FRET) (*28*). Direct energy transfer requires contact between the two moieties. The collision between the fluorophore and the quencher distorts the energy level of the excited fluorophore, which causes quenching. The quenching moiety dissipates the energy that it receives from the fluorophore as heat, rather than emitting it as light. FRET can occur between the two moieties in an MB with a relatively long distance (20–100 Å). It requires a spectral overlap between the donor's emission and the acceptor's absorption spectra. The rate of FRET is inversely proportional to the sixth power of the separation distance of the donor and the acceptor or the quencher (*28*). Because dimethylaminophenylazobenzoic acid (DABCYL) has been found to efficiently quench a large variety of fluorophores, independent of the spectral overlap, direct energy transfer may be dominant in an MB (*2*). Both forms of energy transfer strongly depend on the distance between the dye moieties. Therefore, the spatial separation of the fluorophore and the quencher, resulting from binding to its target, determines the energy transfer efficiency. When a target DNA hybridizes to an MB, fluorescence increases substantially because of the larger separation distance between the two moieties.

Advantages of MB probes

The hybridization of a nucleic acid strand to its complement has been widely used in many areas, and many different types of DNA fluorescent probes have been designed for these applications. For example, nucleic acid blotting techniques have been used to make great strides in our understanding of gene organization and function. In these techniques, a solid support is used to immobilize DNA fragments (*29*). A labeled oligonucleotide probe containing the sequence of interest is then used to hybridize with its counterpart. Nucleic acid techniques are increasingly being harnessed for use in practical applications, such as the molecular diagnosis of disease. But what if real-time synthesis of nucleic acids needs to be monitored? Or what if nucleic acids within living cells need to be labeled? Methods based on immobilizing hybridized nucleic acids, probes requiring intercalation reagents, or other means requiring the isolation of probe–target complexes cannot be used for these applications.

The inherent fluorescent signal transduction mechanism enables an MB to function as a sensitive probe with a high signal-to-background ratio for real-time monitoring. Its fluorescence intensity can increase more than 200-fold when it meets the target under optimal conditions (*2*). Therefore, MBs have a significant advantage over other fluorescent probes in ultrasensitive analyses. With this inherent sensitivity, individual MB DNA molecules have been imaged, and the hybridization process for a single molecule has been monitored (*10*). MBs can be used in situations where it is not possible or desirable to isolate the probe–target hybrids from an excess of the unhybridized probes, such as in the real-time monitoring of polymerase chain reactions (PCRs) in sealed tubes or in the detection of mRNAs within living cells. The usefulness of detection without separation for these applications cannot be overemphasized. This feature enables the synthesis of nucleic acids to be monitored as it is occurring, in sealed tubes or in living specimens, and without additional manipulation.

Another major advantage of MBs is their molecular recognition specificity. They are extraordinarily target-specific, ignoring nucleic acid target sequences that differ by as little as a single nucleotide. Although current techniques for routine detection of single base pair DNA mutations are often labor-intensive and time-consuming, MBs provide a simple and promising tool for the diagnosis of genetic disease and for gene therapy study (*3*). This specificity of an MB comes from its loop-and-stem structure. The stem hybrid acts as a counterweight for the loop hybrid. Experiments have shown that the range of temperatures within which perfectly complementary DNA targets form hybrids but mismatched DNA targets do not is significantly wider for MBs than for the corresponding range of conventional linear probes (*15*). Therefore, MBs can easily

discriminate DNA targets that differ from one another by a single nucleotide (Figure 2). Thermodynamic studies reveal that the enhanced specificity is a general feature of structurally constrained DNA probes.

Synthesizing MBs

The synthesis of MBs is similar to that of dual-labeling a short oligomer with two dyes. The length of the loop sequence (15–40 nucleotides) is chosen so that the probe–target hybrid is stable at the probing temperature. The stem sequences (5–7 nucleotides) should be strong enough to form the hairpin structure for efficient fluorescence quenching, yet still weak enough to be dissociated when a complementary DNA hybridizes with the loop of the MB. Also, the stem sequence must be designed not to interfere with the probe sequences.

Because DABCYL can serve as a universal quench er for many fluorophores (*2*), an MB is generally synthesized using DABCYL-control led pore glass (CPG) as the starting material. Different fluorescent dye molecules can be covalently linked to the 5′ end to report fluorescence at different wavelengths. There are carbon chain linkers between the bases and the dye molecules. The stem and the linker keep the fluorophore and the quencher in close proximity and increase the probability for their direct contact.

There are four important steps in this synthesis. First, a CPG solid support is derivatized with DABCYL and used to start the synthesis at the 3′ end. The rest of the nucleotides are added sequentially, using standard cyanoethylphosphoramidite chemistry. Second, a primary amine group at the 5′ end is linked to the phosphodiester bond by a six-carbon spacer arm. A trityl group at the ultimate 5′ end protects the amine group. Third, the oligonucleotide is hydrolyzed, removed from the CPG, and then purified by reversed-phase LC. Fourth, the purified oligonucleotide is removed from the trityl group and labeled with a fluorophore. After labeling, the excess dye is removed by gel filtration chromatography on Sephadex G-25. The oligonucleotide is then purified again by reversed-phase LC, and the product is collected (*1*).

The synthesized MB is characterized by UV and MS (*12*). The purification of the MB after synthesis is critical to ensure a high S/N to achieve ultrahigh sensitivity. A detailed protocol for MB synthesis can be found at http://www.molecular-beacons.org. Approximately 10 commercial companies specialize in the custom synthesis of MBs at affordable prices.

Surface-immobilizable MBs

Because the typical MB can only be used in a homogeneous solution, surface-immobilizable MBs are critical for the development of highly sensitive biosensors for in vivo detection and biomolecular recognition studies at an interface. A biotinylated ssDNA MB has been designed (*4*).

Biotin–avidin binding is one of the most common ways to immobilize biomolecules onto a solid surface, and it is suitable for DNA hybridization. For biotinylated MB synthesis, there are several important design considerations (*4*), the most important being the position of the biotin, which should be carefully chosen to minimize the effect of the avidin–biotin bridge on the MB hybridization. It is desirable to add the biotin functional group to the quencher side of the MB. A spacer added between the biotin and the sequence should provide an adequate separation to minimize potential interactions between the avidin and the DNA sequence.

A photostable dye, such as tetramethylrhodamine, should be chosen to minimize photobleaching because only a small number of fluorophores are immobilized on a surface. To immobilize biotinylated MBs, a silica surface is first physically or covalently coated with avidin (*4, 8, 9, 12*). The biotinylated ssDNA MB then binds to avidin. The binding process is fast and stable. The hybridization properties of MBs on

the surface are similar to those in solution.

MB biosensors

Chemical etching was used to expose the core surface of an optical fiber to prepare an evanescent wave biosensor. An evanescent wave generated on the core surface was used for fluorescence excitation in the longitudinal surface of the fiber where the MBs were immobilized (Figure 3). The microscopic optical fiber probe was fabricated using either pulling or etching technologies (*30, 31*). MBs were immobilized only at the submicrometer tip of the probe. A highly sensitive optical imaging and detection system with an avalanche photon diode or an intensified charge coupled device (ICCD) was used to detect the fluorescence signal (*10*).

The biosensors were used to detect nonlabeled DNA targets in real time with high sensitivity and one-base mismatch selectivity. There was a linear relationship between the initial hybridization reaction rate and the concentration of complementary DNA. The concentration detection limit of the target complementary DNA was 0.3 nM. The sensors are stable, reproducible, have remote detection capability, and can be easily regenerated by a 1-min rinse with a 90% formamide solution (*8*). They have been applied to the quantitative detection of mRNA sequences and DNA hybridization kinetics (*8, 9*). They hold the potential of direct detection of DNA/RNA targets in living cells without amplification.

Multiple analyte MB sensor

The immobilization method for biotinylated MBs has also enabled the exploration of MB probe arrays for simultaneous multiple analyte detection (*9, 11, 12*). MBs with different loop sequences can be immobilized onto the tips of different microscopic fiber probes (*9*). Those biosensors are individually addressable with a spatially resolved imaging system based on an ICCD camera (*10*). The fluorescence changes from each fiber are monitored to obtain all the hybridization information in one run for a sample containing multiple DNA targets.

Recently, a gene array has been developed on the basis of MBs using particles and an imaging fiber bundle (*11*). Different MB-coated microspheres are randomly distributed in an array of wells etched in a 500-µm diameter optical imaging fiber. To recognize different DNA targets, an optical encoding scheme and an imaging fluorescence microscope system are used for positional registration and fluorescence response monitoring. These multianalyte DNA sensors are expected to provide fast and easy gene analysis and disease diagnosis (*11*).

Protein studies

Although MB probes were originally designed for nucleic acid studies, their hairpin structures can also be disturbed to restore fluorescence upon binding to some proteins. The introduction of MB probes for protein–DNA interaction studies will greatly enhance researchers’ understanding of many important biological processes.

Protein recognition ability was first realized using an *E. coli* single-stranded DNA binding protein (SSB) (*5*). The fluorescence enhancement caused by SSB and by complementary DNA is very comparable (Figure 2). Using MB–SSB binding, it was possible to detect SSB at a concentration as low as 2×10^{-10} M using a conventional spectrometer with a mercury lamp. The interaction between an SSB and an MB was much faster than that between the cDNA and MB. In addition, there are significant differences in MB binding affinity with different proteins, such as albumin and histone (Figure 2), which will lead to selective binding studies of a variety of proteins.

An MB DNA probe has also been used for detailed binding studies of the enzyme lactate dehydrogenase (LDH) (*13*). Different LDH isoenzymes were found to have different ssDNA binding affinities. The stoichiometry of LDH-5/MB binding is 1:1, and the binding constant is 1.9×10^{-7} M^{-1}. Detailed studies of LDH/MB binding,

such as salt effects, temperature effects, pH effects, binding sites, binding specificity for different isoenzymes, and competitive binding with different substrates, were carried out by this simple fluorescence method using the MB probe.

Using MB probes for quantitative, ultrasensitive protein detection shows great potential for increasing understanding of the many important biological processes involving nucleic acids and proteins. Although only nonspecific DNA binding proteins have been investigated so far (*5, 13, 14*), the method opens the possibility for further development of easily obtainable, modified DNA molecules for real-time specific protein detection. On the other hand, the study of the nonspecific DNA binding proteins is also very important, for example, the development of an easy and efficient DNA cleavage enzyme assay using MBs (*14*).

Recent MB applications

Real-time monitoring of PCR. MB probes are suitable tools for the real-time monitoring of DNA/RNA amplification during PCR (*1, 2*). They can simply be added to a sealed PCR tube. The fluorescence signal is monitored at the annealing step of every cycle. At the annealing temperature, the target amplicons' products bind to MB to generate fluorescence, while the unbound MB remains in the closed form without fluorescence. The MB–amplicon hybrids dissociate at elevated temperatures, which keep MBs from interfering with polymerization. The fluorescence signal increases with the increased number of cycles and directly indicates the concentration of the amplicons in the PCR process. The MB assay is fast, sensitive, and nonradioactive. The PCR tubes are sealed during the entire measurement, thus avoiding carryover contamination. Compared with other fluorescent probes used in PCR monitoring, MBs provide more reliable genotyping results, especially in a GC-rich region (*16*). Moreover, MBs allow sensitive and quantitative detection of minority sequence variants over a wider range.

Gene typing and mutation detection. For detecting genetic mutations, a method called "spectral genotyping'' has been developed (*3*). The principle involves two different MB probes with different loop sequences: one specific for a wild-type allele and the other for a mutant allele. These two MBs also have two different fluorophores. The fluorescence measured at the two emission wavelengths during amplification indicates whether the samples are homozygous wild type, homozygous mutants, or heterozygotes. The MB-based PCR mutation detection method has been used for the study of many diseases (*18–25*), especially AIDS. The polymorphisms in the gene for human CC-chemokine receptors CCR5 and CCR2, which are associated with HIV-1, have also been studied (*18–21*) to investigate the HIV disease mechanism and the disease progression process.

MB-based PCR is also a promising tool for rapid and reliable clinical diagnosis. An example is the method developed to assay the pathogenic retroviruses HIV-1, HIV-2, and human T-lymphotrophic virus types I and II (*21*). The retroviral DNA sequences were amplified by simultaneous PCR, which contained four sets of primers and four MBs, each specific for one of the four amplicons and labeled with a differently colored fluorophore. The color of the fluorescence generated during amplification identified which retroviruses were present, and the number of thermal cycles required for the generation of each color were used to measure the number of copies of each retroviral sequence originally present in the sample. Fewer than 10 retroviral genomes can be detected. Moreover, 10 copies of a rare retrovirus can be detected in the presence of 100,000 copies of an abundant retrovirus. There were no false positives for 96 clinical samples. This method will be useful in screening donated blood and transplantable tissue.

Real-time enzymatic cleavage assay. Traditional methods to assay the enzymatic cleavage of ssDNA are discontinuous and time-consuming. On the basis of MB probes, a novel method has been proposed to assay the ssDNA cleavage reaction by single-stranded specific nuclease (*14*). The single-stranded nuclease binds to and

cleaves the single-stranded loop portion of the MB. The cleavage results in the dissociation of the stem, because the 5–7 base pairs in the stem are unstable at the cleavage temperature (37 °C) when the loop is broken. Consequently, the fluorophore and quencher are completely separated from each other, giving rise to an irreversible fluorescence enhancement, which is higher than that caused by the MB's complementary DNA. There is good agreement between traditional gel electrophoresis and the fluorescence assay based on MBs. The fluorescence method permits the real-time monitoring of the enzymatic cleavage reaction process, the easy characterization of the activity of DNA nucleases, and the measurement of steady-state cleavage reaction kinetics.

RNA detection in living cells. In biological studies and recent antisense research, demonstrating hybridization between an antisense oligodeoxynucleotide and its mRNA target in living cells has been a constant challenge. Little progress has been made in living cell studies because of the limited abilities of DNA probes and the problems associated with signal transduction in monitoring the hybridization processes inside living specimens. The MB probes are ideally suited to overcome these difficulties. They have been introduced into living cells by liposome delivery for the visualization of the human basic fibroblast growth factor mRNA in human trabecular cells and by microinjection for the detection of MB/mRNA hybridization in K562 human leukemia cells (*6, 7*).

Recently, we have used microinjections to introduce MBs into living cells for mRNA detection using an ICCD-based fluorescence imaging system. Picoliter amounts of a 35-base MB probe, which is specific for β-actin mRNA, were directly injected into the cytoplasm of kangaroo rat kidney cells. The MB used for this study has the following sequence for β-actin mRNA hybridization: 5′-TMR-GCGAG CGC GGC GAT ATC ATC ATC CAT AAC CTCGC-DABCYL-3′.

Before microinjection, cells were transferred to 3-cm cell dishes and grown in a microincubator at 37 °C for a minimum of 6 days to ensure adhesion to the bottom of the dish. Cells were manually injected with picoliter amounts of the MB directly into the cytoplasm of the cells, while being viewed in the microscope at 60×.

Before injection, optical and fluorescence images of the cells were taken for cell location determination and background measurement. A series of CCD images of the cells were then taken after injection. The fluorescence signals in those single cells injected with MB increase with time (Figure 4). Control experiments showed no fluorescence increase after the injection of other MBs, which are not complementary to b-actin mRNA. These studies suggest that MBs can be used for the real-time detection of mRNA and the visualization of DNA/mRNA interactions.

Outlook

Now that the human genome-sequencing project is completed, the focus will change from collecting and archiving genomic data to analysis and use in prediction and discovery. Although there are many possibilities and great potential benefits, we believe that the following three areas are of foremost interest to the analytical sciences and are feasible in the near future.

First, more research is expected for using MBs to detect mutations in a variety of diagnostic and mechanism studies of disease. Efforts will also be made in finding suitable mRNA sequences and developing real-time studies of RNA processing in living cells and other living specimens without using amplification. This will be expedited with the development of MB biosensors (*8, 9, 11, 12*) and highly sensitive optical detection methods (*10, 32*).

Second, their extraordinarily target-specific capability, along with the availability of different fluorophore–quencher pairs, make MB probes extremely useful for multiplex

applications. The detection of many different targets in the same solution can be achieved by simultaneously using several MBs, each of which emits or can be excited by the light of a different wavelength. These excellent properties also make MBs exquisitely suited for identifying genetic alleles or particular strains of infectious agents. The multiple MB probe approach will also be highly useful for developing high-throughput methods for combinatorial chemistry, drug design, and molecular diagnoses of a variety of genetic diseases, especially with the abundance of genetic information available in the post-genome era.

Third, applications of MBs as novel biomolecular recognition reagents for proteins will be further explored. The MBs are expected to be useful as intracellular protein recognition reagents to probe proteins in different environments and to monitor protein–DNA/RNA interactions. Preliminary results show that there are significant differences in MB binding affinities by different proteins, which will constitute the basis for highly selective bioassays. Approaches include using designer DNA molecules or aptamer-based MBs (*33*) for better specificity and/or using a large array of MB biosensors for pattern recognition. Better understanding of the conformational kinetics of MBs and their fluorescence energy transfer mechanisms will result in optimally designed MB probes for biomolecular recognition with high sensitivity and excellent specificity (*2, 15, 34*). All of these developments will open the possibility of using easily obtainable designer DNA molecules for genomics and proteomics studies, molecular diagnoses of diseases, and new drug development.

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