Molecular beacons for bioanalytical applications

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Molecular beacons (MBs) are hairpin-shaped oligonucleotides that contain both fluorophore and quencher moieties. They act like switches and are normally in a closed state, when the fluorophore and the quencher are brought together to turn ''off'' the fluorescence. When prompted to undergo conformational changes that open the hairpin structure, the fluorophore and the quencher are separated, and fluorescence is turned ''on.'' This Education will outline the principles of MBs and discuss recent bioanalytical applications of these probes for *in vitro* RNA and DNA monitoring, biosensors and biochips, real-time monitoring of genes and gene expression in living systems, as well as the next generation of MBs for studies on proteins, the MB aptamers. These important applications have shown that MBs hold great potential in genomics and proteomics where real-time molecular recognition with high sensitivity and excellent specificity is critical.

Introduction

In the post-genomic and proteomic era, a continued demand exists for biomolecular recognition probes with high sensitivity and selectivity for use in quantitative studies of genomic and proteomic information. These are particularly important for fundamental biomedical studies, disease diagnosis as well as drug discovery. Since they were first reported in 1996,[1] molecular beacons (MBs) have become a class of DNA probes widely used in chemistry, biology, biotechnology, and medical sciences for biomolecular recognition,[2–5] due in part to their ease of synthesis, unique functionality, molecular specificity and structural tolerance to various modifications. While there have been many interesting applications and fundamental studies on MBs, this Education focuses on the recent bioanalytical applications of MBs.

Principles of molecular beacons

MBs are a class of DNA probes which are single-stranded oligonucleotides that possess a stem-and-loop structure (Fig. 1).[6] The loop portion of the MBs can report the presence of a specific complementary nucleic acid. The stem has five to seven base pairs which are complementary and contain a fluorophore and quencher linked to the two ends of the stem. The stem keeps these two moieties in close proximity, causing the fluorescence of the fluorophore to be

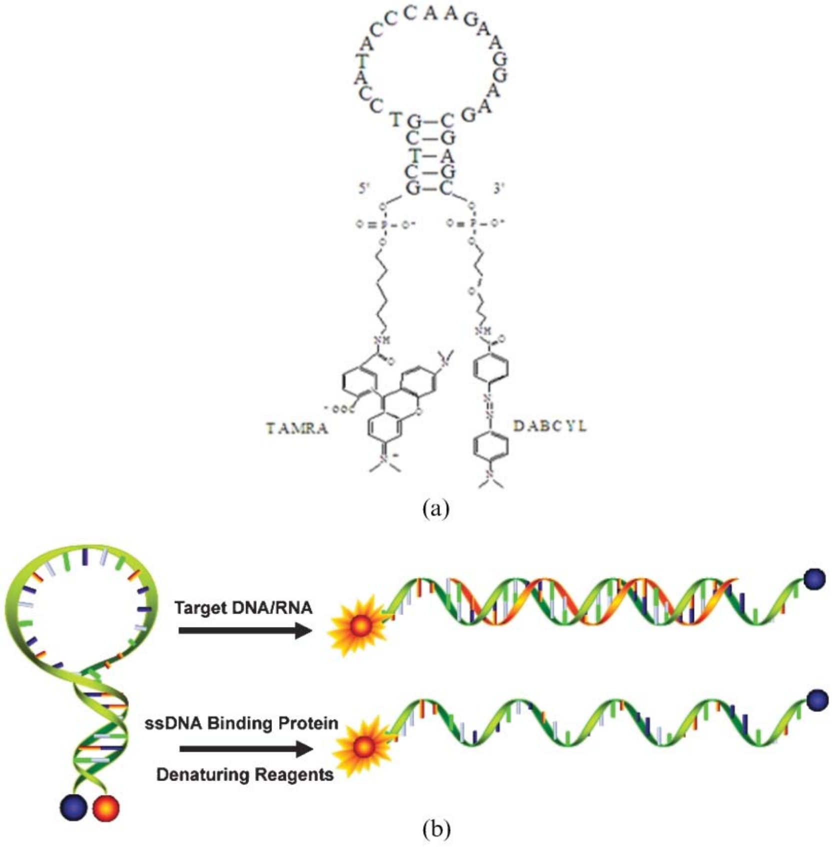

Fig. 1 Structural characteristics of MB probes. (a) A typical MB DNA probe. (b) The MB working principle.

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quenched by resonance energy transfer. When the probe encounters a target DNA molecule, the MB undergoes a spontaneous conformational reorganization that forces the stem apart, leading to the restoration of fluorescence. The conformational state of a MB is thus directly reported by its fluorescence: in the closed state, the MB has minimal fluorescence; in the open state, when the fluorophore and the quencher molecules are apart, it emits a strong fluorescence signal. Different MBs can be designed by choosing loop sequence and size. Different sizes and loop sequences can be chosen to design MBs.[1] Also, the quencher and the fluorophores can be changed according to the application.[7]

Advantages of molecular beacon probes

One of the primary advantages of the MB is that its inherent fluorescence signal transduction mechanism allows it to function as a highly sensitive probe for real-time monitoring. Because of its usually low background signal, a MB can display a fluorescence enhancement of more than 200 times upon hybridization of its target, under optimal conditions.[7] This provides the MBs with a significant advantage over other fluorescent probes in ultrasensitive analysis. MBs can be used in situations where it is either impossible or undesirable to isolate the probe–target hybrids from an excess of the unhybridized probes: "detection without separation". With this inherent sensitivity, individual MB–DNA complex molecules have been imaged, and their hybridization process has been monitored on a single-molecule basis.[8] 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. The selectivity of MBs is a direct result of its loop and stem structure, as the stem hybrid acts as a counterweight to the loop–target hybrid. The specificity provided by the MB loop–stem structure has been demonstrated to be applicable in a variety of biological environments, further extending the applicability of MBs for these types of analyses.

Bioanalytical applications of molecular beacons

MBs can be used in a variety of applications where studies were initially not possible. These have included solution-based RNA–DNA interaction investigations, living systems' measurements, biosensor design, as well as protein–DNA interaction studies.

In vitro RNA/DNA monitoring

MB probes are suitable tools for real-time monitoring of DNA/RNA amplification during PCR.[1,7] Since the non-hybridized MB has minimal fluorescence, the increasing fluorescence signal after each cycle is representative of the increasing concentration of the amplified sequence. Since MBs have excellent characteristics for mutation detection, MB-based PCR is also a promising tool for rapid and reliable clinical diagnosis. For the detection of genetic mutations, a method called spectral genotyping has been developed.[9] MBs have also provided an important tool for DNA sticky-end pairing (SEP) analysis. In terms of MBs, SEP is defined by the intermolecular interaction between the stems of two MBs once target DNA is present (Fig. 2A).[6,10–12] The result of intermolecular sticky-end pairing causes a severe loss in the fluorescent signal in MB–DNA complex molecules during hybridization. Such assays allow for the observation and quantitation of DNA sticky-end pairing as well as the study of various biophysical processes that involve DNA sticky-end pairing, from nonhomologous end-joining to DNA self-assembly. Another important finding was that higher analytical sensitivity can be achieved by designing the MB in such a way that one side of its stem is involved in target recognition. In addition, the cutting of DNA into shorter pieces, or DNA cleavage,[4,13] has been studied using MB probes as "molecular break lights" for monitoring and studying enediynes.[13] In this case, the fluorescence intensity of the MB increases upon

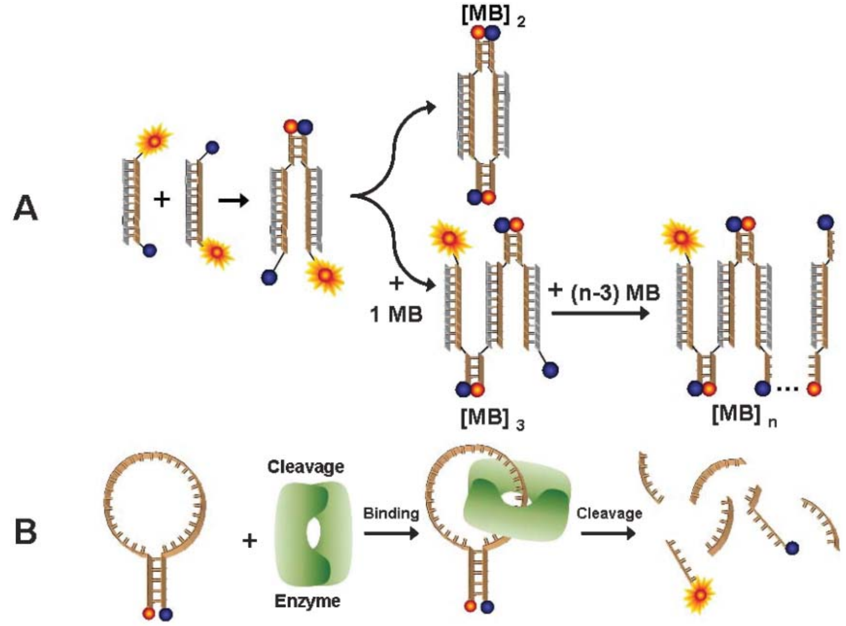

Fig. 2 A wide variety of sensitive assays can be developed based on the MB structural confirmation changes in the presence of both proteins and nucleic acids. (A) MBs can exhibit a substantial amount of intermolecular interactions as a result of sticky-end pairing of the MB stems in the presence of target nucleic acids. Two complementary sticky ends from two MB hybrids can pair to form a short double helix, leading to association of the two hybrids at one end. These two MB hybrids can form a closed structure, ($[\mathrm{MB}]_2$), by pairing the other two sticky ends or polymerize into a multimolecular structure, $[\mathrm{MB}]_n$ ($n > 3$), by pairing with more hybrids. With sticky-end pairing, F and Q are drawn together again, causing fluorescence quenching. (B) DNA cleaving enzymes, or nucleases, can also be monitored by using the ability of the enzymes to cut the MB into short sequences. Eventually, the enzyme cleaves the stem sequence and destabilizes the hybrid in such a manner that the fluorescence of the fluorophore is restored.

cleavage by enediyne when an appropriate cleavage site is integrated into the stem or loop of the probe. Similarly, nuclease activity can be sensitively monitored and detected (Fig. 2B).[4,6] Nucleic acids' ligation is a vital process in the repair, replication and recombination of nucleic acids. The MB, designed in such a way that its sequence is complementary with the product of the ligation process, is used to monitor the nucleic acid ligation in homogeneous solution and in real time.[14,15]

Living systems studies

Gaining knowledge about subcellular localization and cellular transport pathway of RNAs is of crucial importance for our understanding of basic cell biological and developmental processes, and of great interest for the study of functional genomics in the post-genome era. MBs have been reported to help visualize the bFGF (basic fibroblast growth factor) mRNA in human trabecular cells,[16] β-actin mRNA in K562 human leukemia cells,[17] and PTK2 kangaroo rat kidney cells.[18] Some of our recent efforts in understanding mRNA in living neurons are shown in Fig. 3. Successful application of MB in mRNA detection and localization depends mainly on the following three factors: rational design of a MB probe for the specific mRNA, efficient introduction of the MB probe into the cell, and optimization of the experimental conditions for fluorescence imaging and detection. Choosing an appropriate sequence on the target mRNA that will be most accessible to the MB probe hybridization and choosing a corresponding MB loop and stem that will avoid the secondary structure of the loop sequence are the major concerns in the MB probe design. Common delivery methods include DNA transfection,[19] microinjection[20] and electroporation.[21] However, due to the disadvantages that these techniques present (DNA transfection is a long and tedious process, and microinjection and electroporation are both invasive and may cause cell damage), there is a need for another method that can provide a fast, highly efficient and non-invasive delivery of probes inside the cells. Recently, it has been shown that MBs can be linked to certain small protein regions that are known for their efficient penetration through the cell membrane. These regions are called cell penetrating peptides (CPPs). The MB–peptide constructs formed were capable of being internalized into the living cells within 30 min with ~100% efficiency.[22] Intracellular measurements using fluorescent intensity often do not yield reproducible results. To solve this problem, fluorescent ratiometric measurements are usually employed, by microinjecting two probes, one MB probe for the target sequence and one reference probe for optical signal calibration.[23] This approach proved to give more reproducible results, and to be more reliable for the quantitation of target nucleic acids in the cytoplasm of single living cells. Recently dual MB has been designed to reduce false positives, leading to sensitive imaging of K-ras and surviving mRNAs in live HDF and MIAPaCa-2 cells.[24]

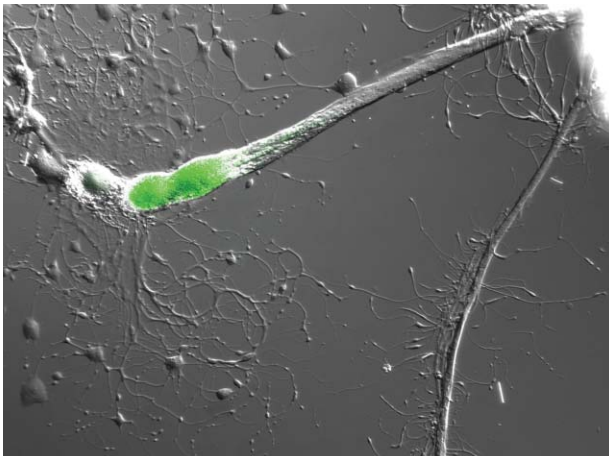

Fig. 3 MB PolyU-probe injections in neuronal processes. The green color is due to opening of MBs inside the processes.

This dual MB uses a pair of MBs, one with a donor and the other with an acceptor fluorophore that hybridize to adjacent regions on the same mRNA target, resulting in fluorescence resonance energy transfer (FRET). It provides a novel technique for sensitive RNA detection and quantification in living cells.[24]

Biosensing applications

As the common MB can only be used in homogeneous solution, surface-immobilizable MBs are critical for the development of highly sensitive biosensors for *in vivo* detection and for the study of biomolecular recognition processes at an interface.[25] Immobilized MBs have been used for the preparation of optical-fiber DNA biosensors such as submicrometer biosensors and fiber-optic evanescent-wave biosensors.[26,27] Multianalyte DNA sensors are expected to provide an easy and fast way for high-throughput gene analysis and disease diagnosis.[28] Overall, a variety of biosensor formats based on MBs have been developed, and their applications are just on the horizon.

Protein detection

Although MBs were originally designed to bind and recognize specific nucleic acids, these probes can also lead to increased fluorescence upon binding to certain proteins. The protein recognition ability of MBs was first recognized with an *E. coli* single-stranded DNA-binding protein.[29] These results demonstrated that while MBs are sensitive and somewhat selective to DNA-binding proteins, they are not specific enough to be capable of distinguishing a particular protein, and a more selective protein recognition mechanism is required. The combined sensitive signal transduction mechanism of MBs with the specific protein-binding capability of aptamers results in a novel class of analytical probes, termed MB aptamers (MBAs).[30] Aptamers are single-stranded DNA or RNA molecules, generally 25–60 nucleotides in length, that have been selected in a process termed SELEX from a combinatorial library by their ability to bind a specific target.[31–33] With further development, MBAs are expected to be useful as intracellular protein recognition agents to probe proteins in different environments and to monitor protein–DNA/RNA interactions.

Conclusion

As the human-genome-sequencing project is coming to fruition, there will be a resulting change in the focus of quantitative studies of genomic information from the collecting and archiving of genomic data to their analysis and use in prediction and discovery. The key to this new era is research across many disciplinary interfaces and the development and use of new quantitative tools. MBs are ideally suited for and hold great promise in genomics and proteomics.

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