



## Mini Review

**Molecular beacons: An optimal multifunctional biological probe**

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## ABSTRACT

Molecular beacon technology is set up based on fluorescence resonance energy transfer (FRET) and the complementary pairing principles. These fluorescent molecular probes, which are very highly specific and sensitive, have now become one important tool in medical and biological researches. This review introduces the molecular beacons structure, principle, the main impact factors, the labeling of the molecular beacons, and research progress on molecular beacons fluorescent-label in the polymerase chain reaction (PCR), DNA sequence analysis, gene dynamic detection in living cells, protein (enzyme)-nucleic acid interactions and applications in clinical medicine.

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In the era of gene and protein, the requirement that high-sensitivity and high-affinity of molecular biological probes used for qualitative and quantitative detection is becoming increasingly urgent. It was providential that Tyagi first established the molecular beacons (MBs) technology in 1996 [1], which produce fluorescence without having to separate probe-target hybrids from excess probes in hybridization assays [1,2]. Because of the characteristics of simple operation, high-sensitivity, and specificity, MBs have been used for real-time quantitative determination of nucleic acid [3], for the construction of self-reporting oligonucleotide arrays [4], and even for analysis *in vivo* [5]. In recent years, the structure of MBs has been made many improvements. MBs technology not only shares a wide range of applications in the study of biology, but also will play an important role in the detection and diagnosis of genetic diseases, basic and clinical biomedical research.

**Principle of MBs operation**

MB is a fluorescent-labeled oligonucleotide chain, typically has 25–35 nucleotides, and constructed by three parts [1]: (1) Loop portion generally constructed by 15–30 nucleotides, specifically bind with target molecules. (2) Stem portion concludes 5–8 base pairs. During the binding process of the MBs and target molecules, this portion is reversibly dissociated. The thermodynamic equilibrium relations between the stem portion and double-stranded structure of loop portion targets hybrid molecular so that the hybridization specificity of MBs was significantly stronger than that of the conventional linear probe. (3) Fluorescence groups connected to 5' end and quenching groups in 3' end.

Fig. 1 presents a classic structure of the MBs and the working principle of MBs. N-terminus 4-((4-(dimethylamino)phenyl)azo)-benzoyl (DABCYL) is used as the quenching group; Texas red, or the luciferase (5-(2-aminethylamino)-1-naphthalenesulfonic acid, EDANS) as the fluorescence group. According to Foerster theory [6], the efficiency of center fluorescence energy transfer is inversely proportional with 6 power of the distance between the groups. Therefore, only to a certain distance between fluorescence and quenching groups, will the fluorescence be produced. In free state, MB is structured as a hairpin, thereby fluorescence and quenching groups are in close distance (about 7–10 nm). At this point fluorescence resonance energy transfer (FRET) occurs that the fluorescence emitted by fluorescence group is almost entirely absorbed by the quenching group and is dissipated in the form of heat, and thus the fluorescent background is very low. When the target molecules combine MBs target complementarily and form hybridized double-strand, the stem complementary portion of the beacon will be melted, and the quenching and fluorescence molecular will be separated. After hybridization, the fluorescence of MBs almost recovers completely. And thus the fluorescence intensity detected is proportional to the quantity of target in the solution.

DABSYL, which most commonly used as the fluorescence quenching agent in MBs technology, has a strong quenching efficiency a variety of fluoresceins [7]. Recently Dubertret used gold nanoparticles cluster instead of DABSYL, they also worked out various quenching agents by altering the shape, size and composition of metal nanoclusters [8]. The nanocluster fluorescence quenching reagent has a higher efficiency, greater sensitivity and specificity. Demidov designed DNA/PNA without double-stranded stem portion structure [9]. As DNA Pentose skeleton or PNA polyamide skeleton has a good flexibility, in the absence of the target, the hydrophobic interaction between quenching

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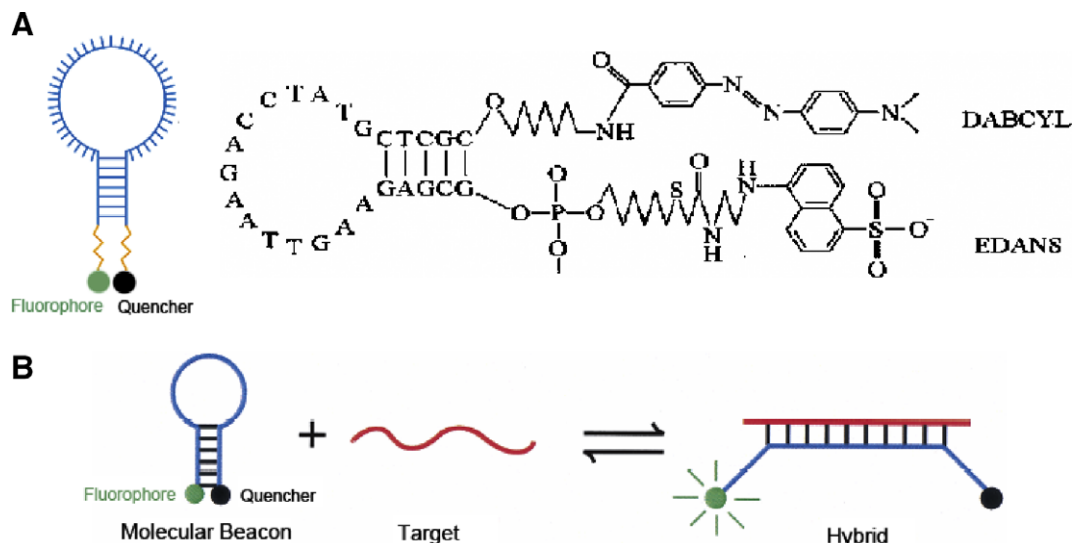


Fig. 1. (A) Classic structure of the molecular beacons and (B) the working principle.

and fluorescence groups makes no stem MBs structure be similar to a closed-loop. After MBs combine with the target, probe and target molecules will form a rigid double-stranded structure of the double-stranded structure and separate the fluorescence and quenching molecules. Compared no stem MBs with classical MBs, it does not contain any sequence irrespective to the target. Hence no stem MBs are simpler in designing and synthesizing. Moreover, no stem MBs also response faster, have higher specificity, and its fluorescent background is higher in quiescent condition.

### Main impact factors on MBs function

There are many factors influence the effect of MBs. These factors often lead to false-positive and false-negative. The major factors are described below:

The distance between fluorescence–quenching groups is the most important factor [1,10]. According to fluorescence energy transfer theory, the efficiency of transferring is proportional to 6 power of the distance. Previous studies reported that the fluorescence intensity generally increased tenfold or even a hundredfold after hybridization. In addition, MBs purity will also have a certain impact [1].

Temperature also plays an important role. MBs can be maintained a stable hairpin structure at low temperature, but this structure will be undermined under high temperature, and the MBs even extend to be a random line, thus the two group of fluorescence and quenching separate and fluorescence is emitted. Its melting temperature depends on the chain length of the stem, GC content, as well as the ionic concentration of the buffer. Bonnet studied the impact of temperature on the MB nature with perfectly matched target sequences [10]. Their research showed that the performance of the fluorescence intensity presented a primarily weakened and then enhanced process. Its principles can be interpreted from Fig. 2. In low temperature, MBs and the matched the target sequences form a stable hybrid combining binary state (S1), which has strong fluorescence signal. With the temperature increasing gradually, MBs and targets separates and MBs form the hairpin structure (state S2), the fluorescence is weakened. With continue increasing temperature, MBs hairpin structure is destroyed and turns into a linear structure (state S3), the fluorescence and quenching groups separate, and the fluorescence recovers.

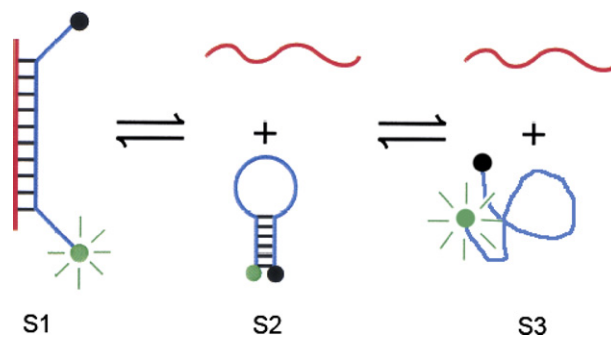


Fig. 2. Three states of molecular beacons.

Environmental pH value also affects the MBs function [1]. Too high pH value will also break down the stem portion, the MBs degenerate and leads to a false-positive result.

The design of loop and stem portion is also a key element in MBs research and application. The design not only determines its conformation, makes the distance between the groups far enough after hybridization, but also determines its appropriate temperature. On the sequence of bases, the stability of stem portion should be appropriate and the loop portion should be avoided to form any possible secondary structure. Too long stems will make hairpin structure too stable and produce false-negative results, whereas too short, the hairpin structure will be unstable and prone to give false-positive results [1]. Probe sequences are usually over double longer than the stem portions so as to ensure the structure changes after hybridization, separating the two groups far enough.

### MBS labeling

MBs are usually modified with a single luminous group. In a homogeneous system, through fluorescence signal changes after hybridization, one MB can detect the only corresponding target sequence. In order to detect a variety of target sequence in a homogeneous detection system at the same time, different MBs can be modified with different fluorescence groups. Tyagi used four types of MBs for allele detection [2]. Only one base of the same location is different and thus the four MBs had four different excitation and emission wavelengths. Four MBs in equal dose were mixed into four tubes and added target sequences, respectively. Only perfectly

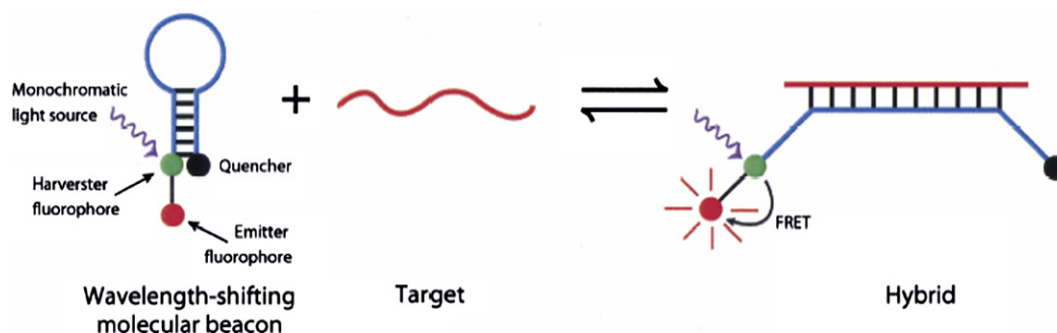


Fig. 3. Wavelength-shifting molecular beacon.

matched with the target sequence can the MBs bind specifically and emit fluorescence at corresponding excitation wavelengths. The stability of perfectly matched sequence was higher than that of single-base mismatch sequences. Clearly, with this method, corresponding fluorescence signal should be acquired in accordance with different groups of fluorescence wavelength of the excitation light sources.

In order to adopt the single excitation source, Tyagi also reported a wavelength-shifting MB, in which two independent groups absorbed excitation energy and emitted fluorescence [11]. Its structure is shown in Fig. 3. Under a single light source excitation, fluorescence group emitted fluorescence of different wavelengths in visible spectrums. They used DABCYL as the quencher moiety linked at the 3' end of the MB probes and luciferase as a harvester fluorophore at an internal location in its 5' arm that is opposite to the quencher in the hairpin conformation, and the distal end of the 5' arm was linked with a fluorescence emission group Texas red. In MBs, fluorescence and quenching groups integrated closely and shared electrons, forming a temporary complex without fluorescence. The fluorescence absorbed was exhaled in the form of heat. In this complex, the speed of absorbing energy from the fluorescence molecular transmitted to the quenching group was much faster than that of the FRET. The wavelength-shifting MBs not only have higher fluorescence efficiency, but also maintain higher specificity and sensitivity, can effectively distinguish single-base mismatch sequences. The benefit of this labeling method is: if the different MB probes modified by fluorescence groups with different wavelengths can be excited in the same light and multi-gene can be analyzed by detecting of these fluorescence intensities with characteristic wavelength in the same system.

## The apply of MBs

### MBs in the application of basic medicine

#### Detection of nucleic acid

In recent years, gene detection method has been developed rapidly. Many scientists used MB technology to detect gene, and achieved some initial results, such as detecting liquid phase hybridization [12], effectively reducing cross-contamination of nucleic acids [13], real-time nuclear acid detection [3,14]. Because of the high specificity and sensitivity [15], it has been increasingly applied in large-scale automated detection [16] and even dynamic detection nucleic acid *in vivo*. [5]

**Quantitative real-time PCR detecting concentration of target.** MB technology was first used to analyze concentration of target by real-time quantitative PCR, which is based on real-time detection fluorescence signal throughout the PCR process, quantitative analysis through the standard curve of the unknown template [17]. On

the basis of conventional PCR, corresponding MB probes were added. In every cycle of the PCR process, MBs combined with amplified products, fluorescence and quenching groups separated and fired fluorescent, but it did not affect the accumulation of PCR products index. Only the DNA template which can combine with MBs can produce fluorescence signal during amplification. Therefore, the fluorescence intensity is proportional to the amount of specific amplification products, and it is the direct markers of template amplified by PCR. Compared with conventional methods, containing of the operation effectively eliminated the cross-contamination of DNA. It is real-time, quantitative, highly sensitive, and highly specific.

**Gene mutation, SNP (single nucleotide polymorphism), allele, multi-determination [18–20,16].** Currently there are many methods used for detection of mutation, SNP, allele, and multiple ingredients, but only those methods based on the real-time PCR are very effective. Compared with the linear oligonucleotide probes, stem-loop structure MBs have higher specificity. Even a single-base mismatch of target sequences or insertion mutations can be detected. Hence the real-time PCR system established with multicolor MB is perfectly suitable for the determination of gene mutation, SNP, allele, multiple ingredients.

**Detection of the double-stranded DNA, biochips and biosensors probe.** Different from RNA, DNA is the double helix structure and using conventional methods is difficult to detect. PNA combines double-stranded DNA complementarily and non-complementary strand can be replaced and dissociated into single-chain, a P-loop structure [21]. Dissociated double-stranded DNA can combine with the MBs, and produce fluorescence.

Because of the advantages of MB for the analysis of nucleic acid, it can be used as the DNA probe as biochips and biosensors. MBs marked with different fluorescent molecules in the form of bioepiderm are fixed to the surface of silicon wafers, the MB chips. Compared to other types of DNA chips, MB chips have low fluorescence background, are obligated to remove redundant probes, and can be used multiple times [22].

**Dynamic nuclear acid detection *in vivo*.** Using MBs technology, the process of biological macromolecules metabolism *in vivo*, for example, mRNA in cells, can be dynamic analyzed to investigate the process of transcription and other changes. Perlette designed and synthesized a MB specific for beta-actin mRNA and microinjected it into the cytoplasm of single living kangaroo rat kidney cells (PtK2 cells). The results showed that MBs can be used for effective and real-time detection of RNA in living cells and research the process of RNA/DNA hybridization [23]. Matsuo studied intracellular bFGFmRNA metabolism with MBs [24]. They designed and synthesized MBs of complementary analyte target mRNA sequence and control MBs, respectively, and intake by the cells with lipo-

somes. Using laser scanning confocal microscope or fluorescence microscopy, the results showed that the fluorescence of experimental group cells was much higher than that of control cells. Extended incubation time, fluorescence of the control group would be gradually enhanced. MBs can be hydrolyzed by nucleases and thus emit fluorescence. PNA MBs can effectively avoid being hydrolyzed by DNA enzyme, so that the fluorescence intensity can accurately reflect the metabolism of mRNA *in vivo*.

#### Protein detection

Following the development of genomics, proteomics emerged. Studies on interactions between two biological macromolecules (nucleic acids and proteins) have become one of the fastest growing areas of modern molecular biology and biotechnology. Considerable previous work has already been done, and a large number of methods and techniques such as DNA blotting, cross-linking technology, filtering technology, block gel analysis, affinity chromatography, circular dichroism spectroscopy (CDS), X-ray crystallography, fluorescence spectroscopy, and so forth also have been established. These methods and techniques can provide a large number of molecular interaction information such as: DNA-binding sites, the length and specificity of combining portion. In these methods, fluorescence analysis is a relatively sensitive method for studying DNA-protein interactions, which can be detected in the concentrations of mmol level or even lower. Particularly, in recent years various types of probes designed with the principle of FRET, were used for the analysis of DNA-protein interactions and had made great progress, but the detection can not be real-time. MBs, which were simple, sensitive, and versatile (single-chain or double-stranded DNA-binding protein), retrieved all the disadvantages. Furthermore, it can be used to dynamic study in living cells, and even can be used for detection *in vivo* [25].

Tan [26] reported their results on protein research with MBs technology. They used MBs to detect single-chain-binding protein and found that the fluorescence intensity was weaker than that generated by the MBs with a combination of nuclear acid, but its strength is far greater than that produced by mismatched MBs and nucleic acid. At the same time they also found that MBs can not be combined with dsRNA-binding protein. McCauley [27] designed Aptamer beacons according to the working principle of MBs. Aptamer can be used to directly detect proteins. This technology, compared with the traditional enzyme-linked immunosorbent assay of protein, is much simpler and more sensitive. However, it can not be used to detect non-specific ssDNA-binding protein, and molecular conformation of the beacons was affected by metal ions, as the existence of metal ions can interfere the fluorescence signal observation.

#### MB technology application in clinical medicine

The use of MBs probes to explore gene and protein identification at a global level has revolutionized the area of probe labeling and it seems to become more powerful way for different applications in human health. Some important applications of these probes in clinical medicine are enumerated below:

One of the challenges in clinical oncology is the early detection of small subsurface tumors embedded inside a large organ. Through hard and careful studies, Chen et al. [28] developed an efficient instrument for detection and localization of tumors labeled by the molecular specific fluorescence contrast agent inside the scattering media. The localization error is within a few millimeters. This instrument can sensitively detect the fluorescence signal from a less than one-nanomole contrast agent in the phantom experiment. This portable device would be complementary to x-ray mammography and provide add-on information on early diagnosis and localization of breast tumor. Development of novel ap-

proaches for quantitative analysis of gene expression in intact tumor cells should also provide new means for cancer detection and for studying the response of cancer cells to biological and therapeutic reagents. Yang [29] and Peng [30] developed procedures for detecting the expression levels of multiple genes in fixed as well as viable cells using MB imaging technology and found that MBs can detect changes of surviving mRNA and other cancer related gene expression in viable cancer cells. Their results proved that MB based expression imaging of tumor marker genes provides a novel approach for the detection of pancreatic cancer cells.

In 2003, SARS virus spread in China, as well as some other countries in the world, and posed a severe challenge to human health. Methods and techniques which can fast and accurately detect SARS virus, such as RT-PCR, ELISA and immunofluorescence assay, had been applied; even the corresponding Test Kit had been introduced. However, at present, these tests still need to judge with the clinical signs, which also have some shortcomings. Take RT-PCR detection for example [31], the sensitivity in detection of the SARS is not satisfactory. There is a tendency of leading false-negative results. Moreover, leaking detection had been reported. The same outcomes also reported by using immunofluorescence assay and ELISA [32]. Immunofluorescence assay can only detect the antibodies in patients 10 days after infection, while ELISA needs to be taken 20 days later. MB technology is based on the principle of complementary base pairing, which guarantees its specificity on detection of SARS virus. In addition, the gene detection sensitivity of this method is higher than those of the above technologies [33–35]. Compared with the above mentioned methods, which can only detect infection after a few days, MBs technology can detect the nuclear acid as long as the SARS virus appears. Thus it made great clinical sense in saving the valuable time for clinical treatment.

Yates promoted a method by using MBs to detect hepatitis B virus [36]. The quantitative detection of HBV DNA was easy to perform and saved time. The risk of carryover contamination is minimized by performing the entire method in unopened microtubes. Furthermore, the test has adequate sensitivity, reproducibility, precision, and a broad dynamic range for monitoring the condition and prognosis of HBV carriers and patients, including those undergoing interferon or nucleoside analog therapy.

#### Prospect

Since its inception, because of the high specificity and sensitivity characteristics, MB technology has been quickly adopted in biology, basic medicine, clinical medicine, and other related researches. People conducted a number of structural improvements and development on the MBs, so that it can be used in a broader context and play a greater role. As a result, a number of nucleic acid probing systems with close resemblance to the MBs are being reported from time to time. Used with nano-technology, laser scanning confocal technology and other advanced technology, it will certainly promote the MB technology amendment. With the development of MBs technology and emerging of new MBs, MBs will definitely be applied in gene diagnosis, gene therapy and new drug researches wider and wider.

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