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REVIEW

Molecular Beacons: A Novel Optical Diagnostic Tool

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Abstract As a result of the efforts of the Human Genome Project and the rise in demand for molecular diagnostic assays, the development and optimization of novel hybridization probes have focused on speed, reliability, and accuracy in the identification of nucleic acids. Molecular beacons (MBs) are single-stranded, fluorophore-labeled nucleic acid probes that are capable of generating a fluorescent signal in the presence of target, but are dark in the absence of target. Because of the high specificity and sensitivity characteristics, MBs have been used in variety of fields. In this review, MBs are introduced and discussed as diagnostic tools in four sections: several technologies of MBs will be illustrated primarily; the limitation of MBs next; the third part is new fashions of MBs; and the last one is to present the application of MBs in disease diagnosis.

Keywords Molecular beacons (MBs) · Nucleic acid probe · Fluorescence resonance energy transfer (FRET) · Diagnosis

Introduction

Molecular beacons (MBs) are single-stranded, fluorophore-labeled nucleic acid probes that are capable of generating a fluorescent signal in the presence of target, but dark in the absence of target. Since the first report (Tyagi and Kramer 1996), they have been widely used in chemistry, biology, biotechnology, and medical science (Antony and Subramaniam 2001; Tan et al. 2000). The unique hairpin structure and signaling mechanism make MB with several advantages. First of all, the light-up signaling mechanism allows it to function as a highly sensitive probe (Deiman et al. 2002; Tan et al. 2005; Yang et al. 2005). The unbound MB does not emit fluorescent signal, thus the signal from target bound MB can be clearly observed in the presence of unhybridized probe. Such detection without separation property is useful for the MB in situations where it is either impossible or undesirable to isolate the probe-target hybrids from an excess of the unbound MB, for instance, monitoring of mRNA inside of living cells. Another advantage of MBs is their relatively high signal-to-background ratio, providing higher sensitivity (Deiman et al. 2002; Santangelo et al. 2006; Tan et al. 2005; Yang et al. 2005). Upon hybridization of its target, a well-designed MB can generate as high as 200-fold of fluorescence enhancement under optimal conditions. This provides the MBs with a significant advantage to other fluorescent probes. In addition to its sensitivity, MBs offer excellent selectivity (Deiman et al. 2002; Santangelo et al. 2006; Tan et al. 2005; Yang et al. 2005). They are extraordinarily target-specific, and able to differentiate nucleic acid targets with single base mismatches. 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. Because of the advantages above, MBs technology not only share wide range of applications in the study of biology, but also play an important role in the diagnosis of genetic diseases. In recent years, the structure of MBs has been made many improvements (Li et al. 2008). The specificity and

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sensitivity of new fashions MBs are higher than the traditional MBs (Nitin et al. 2004; Santangelo et al. 2004, 2006). Herein, MBs technology has been increasingly applied in diseases diagnosis.

In the next section, after a statement of the basic technologies of MBs, various situations involving possibility knowledge are investigated: firstly, the limitation of MBs; then the new fashions of MBs; lastly, present the application of MBs in disease diagnosis.

Some Technologies of MBs

Molecular beacon is a class of DNA probes (Tyagi and Kramer 1996) that is widely used in chemistry, biology, biotechnology and medical science for biomolecular recognition (Tan et al. 2005). They are single-stranded hairpin shaped oligonucleotide probe and dual-labeled oligonucleotide probes having a fluorescent reporter group at 5′ and fluorescent quencher group at 3′ end of the arm.

Structure of MBs

As show in Fig. 1, MB is constructed by the following four parts (Santangelo et al. 2006; Tan et al. 2005; Tyagi and Kramer 1996):

1. *Loop* It is complementary to the target sequence which is generally the 15–30 bases of the MB. It should not form any secondary structure for the frame of the probe can be moved along the target to obtain the non-self-complementary sequence. Increase in the probe length results in improved affinity but reduces specificity.
2. *Stem* The beacon stem sequence lies on both the ends of the loop which is typically 5–7 base pairs long. They are complementary to each other. The shorter stem length has faster hybridization kinetics but suffers from lower signal-to-background ratio.
3. *5′ Fluorophore* The 5′ end of the MB is attached a dye that fluoresces in presence of a complementary target. The most commonly used dyes are 5-(2′-aminoethyl) aminonaphthalene-1-sulfonic acid (EDNAS), fluorescein (Fam), tetrachloro-6-carboxyfluorescein (Tet), hexachloro-6-carboxy fluorescein (Hex) and tetramethylrhodamine (Tamra) and 5-carboxyrhodamine-X (Rox). Proper selection of fluorophore is critical for improved signal-to-background ratio.
4. *3′ Quencher* (*non-fluorescent*) Capture and transfer of light energy from an excited fluorophore is referred as quenching and the substances involved are termed as quenchers. The quencher dye is covalently attached to the 3′ end of the MB. When the beacon is in closed loop shape, the quencher prevents the fluorophore from emitting light. The commonly used quencher, 4-(4′-dimethylaminophenylazo)benzoic acid DABCYL, is a non-fluorescent chromophore, has a strong quenching efficiency variety of fluoresceins. Dubertret used gold nanoparticles cluster instead of DABCYL, they also worked out various quenching agents by altering the shape, size and composition of metal nanoclusters (Tyagi and Kramer 1996).

Fundamental Principles of MBs

Resonance energy transfer (RET) is one of the fundamental principles of MBs (Ortiz et al. 1998; Tyagi and Kramer 1996). It is used for signal transduction. Energy transfer is the transfer of the excited state energy from the initially excited donor to an acceptor. Donor molecules typically emit at wavelengths which overlap with the absorption spectrum of the acceptor (Yang et al. 2005). RET occurs without the appearance of a photon and is the result of long range dipole–dipole interactions between the donor and acceptor. Energy transfer rates depend on the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the quantum yield of the donor, the relative orientation of the donor and acceptor molecules and the distance between the donor and the acceptor. The distance at which RET is 50 % efficient is characterized as the Forster distance and is typically in the range of 20–60 Å (Tan et al. 2005). Molecular beacons are designed to form a stem-loop (hairpin) structure which can hold the fluorophore at 5′ end and the quencher at 3′ end in close proximity to one another, preventing the fluorophore from emitting a signal as a result of RET. Hybridization with target nucleic acids opens the hairpin and physically separates the reporter from quencher, allowing a fluorescence signal to be emitted upon excitation (Fig. 1) (McDonald et al. 2002; Tan et al. 2005).

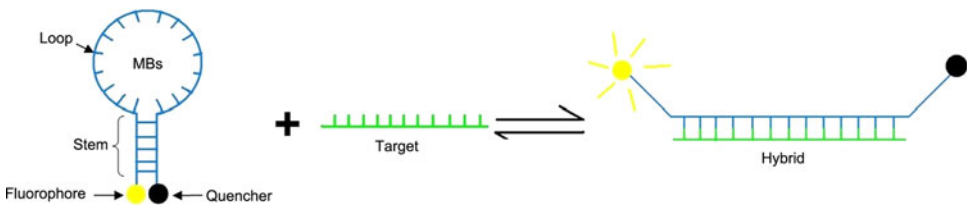

Fig. 1 Classic structure of the MBs and the working principle

Designing of MBs

The length and sequence of probe loop and stem, hairpin structure, and fluorophore/quencher selection are critical design issues for MBs (Santangelo et al. 2006). The design parameters are described below.

The process of MB design begins with the selection of the probe sequence. It is really a crucial step for the functions of MBs, because the probe sequence determines the specificity and the target accessibility. In practice, the choices of the probe sequence are limited by target-specific considerations, such as the sequence context surrounding a single nucleotide polymorphism (SNP) of interest, and using the NCBI BLAST or similar software to select multiple target sequences that are unique for the target nucleic acids. The probe length can be adjusted to a specific application of a MB. Typically, the length of the probe sequence is in the range between 15 and 30 nucleotides in the past.

After selecting the probe sequence, two complementary arm sequences are added on either side of the probe sequence. In general, it has been found that longer stems can make hairpin structure more stable and produce false-negative results, whereas too short, the hairpin structure is astable and prone to give false-positive results (Tyagi and Kramer 1996). Usually, the length of stem contains 5–7 base pairs long and has a very high guanine and cytosine (GC) content (75–100 %), and the stem sequence is designed in such a way that the molecule beacons remain closed and non-fluorescent in the absence of the target.

The hairpin structure rather than other structures is able to place the fluorophore in the immediate vicinity of the quencher. A folding of the selected sequence by the Zuker DNA folding program will reveal such problems. In addition, on the sequence of bases, loop portion and stem portion should be avoided to form any possible secondary structures.

At last, selecting proper fluorophore/quencher for a MB could yield satisfactory additional benefits such as an improved signal-to-background ratio. Fluorescein isothiocyanate (FITC), Texas red and the luciferase (5-(2-aminethylamino)-1-naphthalenesulfonic acid, EDANS) are commonly used as fluorophore. Since each MB possesses only one fluorophore, it is possible to use multiple MBs in the same assay, assuming the fluorophores are chosen with minimal emission overlap. MBs can even be labeled simultaneously with two fluorophores. They will be described in below content (new fashions of MBs). Terminus 4-((4-(dimethylamino)phenyl)azo)-benzoyl (DABCYL) is most commonly used as the quencher in MBs technology. There are many other quenchers besides DABCYL, such as blackhole quencher (BHQ) and Iowa black. They can all effectively quench a wide range of fluorophores. In addition to quenchers mentioned above, gold nanoparticles can also be used as a quencher (Dubertret et al. 2001; Li and Rothberg 2004; Wu et al. 2006). The nanocluster fluorescence quenching reagent is more efficient, sensitive and specific. Recently quencher-free MBs (QF-MBs) are designed for identification of the fully matched target sequences in the presence of even large excess of mismatched DNAs (Venkatesan et al. 2008). And the QF-MBs have several advantages over the quencher-fluorophore MB systems. For example, mono-labeled QF-MBs can be immobilized over solid surface easily through the free end.

Main Environmental Impact Factors on MBs Function

There are many environmental factors influencing the effect of MBs. Temperature and environmental pH values are two most important factors amongst.

To obtain MBs that function optimally under a given set of assay conditions, it is important to know how their fluorescence changes at the temperature in the presence or absence of their targets. It has been proved that at low temperature MBs exist in a closed state, the fluorophore and the quencher are held in close proximity to each other by the hairpin stem, and there is no fluorescence. However, at high temperature, the helical order of the stem gives way to a random-coil configuration, separating the fluorophore from the quencher and restoring fluorescence. If a target is added to a solution containing a MB when the temperature is below the melting temperature of its stem, the MB spontaneously binds to its target, dissociating the stem, and turning on its fluorescence. How the fluorescence of the probe-target hybrid varies with the temperature is indicated by the blue fluorescence versus temperature trace. While at low temperature, the probe-target hybrid remains brightly fluorescent, but as the temperature rising, the probe dissociates from the target and return to its hairpin state, diminishing the fluorescence obviously (Vet et al. 2004). Gregoire Bonnet found that the transition from probe-target duplex to a closed conformation occurred at 42 °C. As the temperature is raised further, the closed MBs melt into fluorescent random coils (Bonnet et al. 1999).

Environmental pH value also affects the MBs function (Tyagi and Kramer 1996). Too high pH value also breaks down the stem portion, the MBs degenerate and lead to a false-positive result.

Limitations of MBs

In some studies, the applications of MBs still presented some limitations, especially in vivo.

One of them is that once the MB is degraded by an endogenous nuclease, the MB stem opens, creating a false-positive signal. In addition, interaction of MB with intracellular proteins will also disrupt the hairpin structure,

resulting in non-specific signals and creating a false-positive signal. The low sensitivity is one of the limitations in applications. The reasons of low sensitivity are low brightness of the fluorophore-labeled MB, high background given by the closed MB, and the autofluorescence from the cell. In general, we know that MBs enable a homogenous assay format where background is low without the need to wash away free probes. However, to detect mRNA in vivo, we need something to deliver MBs into living cells with high efficiencies and fast kinetics (Barton and Medzhitov 2002; Mitchell 2001). So at last, probe delivery is a critical problem in the application of MBs in vivo.

In the following sections, we will introduce three important fashions of MBs. It might be a prospective way to overcome the obstacles above.

Important Fashions of MBs

Wavelength-Shifting MBs

Low signal intensity of single fluorophore limits the sensitivity of the MBs. One way to improve the sensitivity of the MBs is to improve the signal intensity of the fluorophore. Tyagi designed a novel hairpin probe to overcome this problem. As shown in Fig. 2a, in this design, a MB contains two fluorophores: the first fluorophore that absorbs strongly in the wavelength range of the monochromatic light source, and the second fluorophore that emits at the desired emission wavelength due to fluorescence RET from the first fluorophore to the second fluorophore. It has been demonstrated that wavelength-shifting MBs are substantially brighter than conventional MBs which contain a fluorophore that cannot efficiently absorb energy from the available monochromatic light source. In addition, the second fluorophore of wavelength-shifting MBs can possess differently colored fluorophores, enabling assays to be carried out that simultaneously detect different targets in the same reaction (Santangelo et al. 2006; Takacs et al. 2008; Tyagi et al. 2000).

Dual FRET MBs

When it comes to detecting target molecules in living cells, conventional MBs could be degraded by nucleases or opened by non-specific hairpin structure binding proteins, resulting in false-positive signals. The dual FRET MBs (Fig. 2b), which had a pair of MBs, were designed for resolving the problem (Santangelo et al. 2004, 2006). Each beacon contains a fluorescence quencher and a fluorescence reporter, one with a donor fluorophore and the other with an acceptor fluorophore. They had sequences complementary to adjacent regions on the same target so that the two fluorophores would link the FRET range (~6 nm), while both beacons were hybridized to the target. To get the FRET by activating the donor fluorophore and detecting the fluorescence emission at a wavelength characteristic of the acceptor fluorophore, the positive result

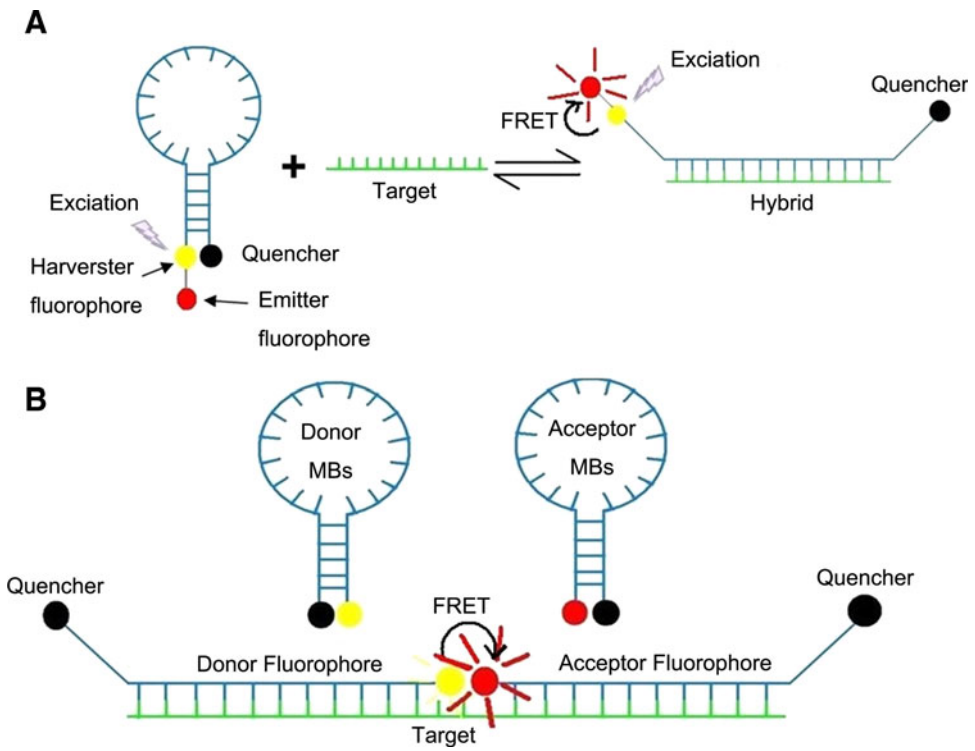

Fig. 2 New fashions of MBs. Wavelength-shifting MBs (**a**), dual FRET MBs (**b**)

should be ensured because of binding both two beacons with the target molecule. And the rate of the false-positive results, because of the probe degradation and non-specific probe opening, was declined. This design makes the MBs with more advantages than before, such as low background signal and high specificity.

Permeation Peptide-Conjugated MBs

Rapid and efficient delivery of imaging probes to the cell interior using permeation peptides has enabled novel applications in molecular imaging. Recently, a novel peptide-linked MB is designed that can quickly and efficiently enter living cells without the need of any other delivery reagent. Membrane permeant peptides based on the HIV-1 Tat basic domain sequence, GRKKRRQRRR, were conjugated to MBs; the resulting peptide-linked MBs were delivered into living cells to target GAPDH and survivin mRNAs (Nitin et al. 2004; Santangelo et al. 2006). The result demonstrated that, cellular delivery of MBs using the peptide-based approach has far better performance as compared with conventional transfection methods. The advantages of permeation peptide-conjugated MBs are blow: firstly, peptide-linked MBs were internalized into living cells within 30 min with nearly 100 % efficiency. Secondly, peptide-based delivery did not interfere with either specificity or function of MBs. In contrast, liposome-(Oligofectamine) or dendrimer-based (Superfect) delivery of MBs required 3–4 h and resulted in a punctate fluorescence signal in the cytoplasmic vesicles and a high background in both cytoplasm and nucleus of cells (Nitin et al. 2004; Santangelo et al. 2006).

Basic Application of MBs

Tyagi and Kramer (1996) developed a novel probe to detect specific nucleic acid and discussed the possibility to use it in real-time polymerase chain reaction (RT-PCR). MBs formed a fluorescent hybrids with the product of PCR. The intensity of the fluorescence had a strong correlation with the amount of DNA synthesized. Since then MBs have been commonly used for RT-PCR. The numerous MB-based PCR experiments laid the foundation for pathogen detection with MBs, as well as the other applications, such as the SNPs detection.

In the last decade, MBs have emerged to become a widely used tool in the multiplex typing of SNPs. Liao et al. (2011) tried to establish the method based on MB RT-PCR for detecting SNP in codon 72 of scar-related p53 gene. The result showed the goodness of fit of this method was 100 % in comparison with direct DNA sequencing, higher than that of reverse dot hybridization (Liao et al. 2011). Many improvements have been made in detection technologies in instrumentation and chemistries (Liu et al. 2010b; Nguyen et al. 2011). So that we could see a better future of MBs application in SNPs.

Another important application of MBs is the nucleic acid sequence-based amplification (NASBA) assay. By combining NASBA amplification with MB probes, this assay becomes a real-time analysis tool that offers faster results than conventional RT-PCR technique. The advantage of this method is the low possibility of pollution and the isothermy of the reaction process. NASBA is also wildly used in pathogen detection (Chen et al. 2012; Sidoti et al. 2012).

MBs Used in Diseases Diagnosis

As a result of the efforts of the Human Genome Project and the rise in demand for molecular diagnostic assays, the development and optimization of novel hybridization probes has focused on speed, reliability, and accuracy in the identification of nucleic acids. High sensitivity is required to provide early diagnosis of pathogenic infections in clinical samples. This can be achieved by either direct detection of nucleic acids, in living cells or by in situ hybridization, or by detection of nucleic acids during amplification of target DNA and RNA sequences.

Although invented not long ago, MBs have already demonstrated their usefulness in assays requiring the rapid and sensitive detection of genomic sequences. Thus, the use of MBs in diagnostic assays has been ever increasing.

MBs Used in Microarray Assays

Molecular beacon methods were adapted to microarray analysis for label-less detection of gene sequences relevant to specific disease states. Because this design is suitable for simultaneous reliable detection of hundreds of DNA target sequences in one test run using a series of beacons immobilized on a single substrate in a spatial format (Epstein et al. 2003).

Recently, Kim et al. (2007) revealed that the false-negative signal of utilizing a MB DNA microarray system to detect of the target sequence is virtually zero probability. This eliminates false-negative signal detection characteristic for the fluorophore-quencher beacon, where bleaching of the fluorophore or washout of a beacon is indistinguishable from the absence of the target DNA sequence. In perspective, the two-color design allows also to quantify the concentration of the target DNA in a sample down to $\leq$ 1 ng/μl (Kim et al. 2007). In another study, MBs were immobilized with polyacrylamide gel for single nucleotide mismatch detection. The result indicates that a polyacrylamide film-coated glass slide provides an ideal solution-like

environment for MB probes. The potential applications of this kind of MB array are mutation detection, disease mechanisms, disease diagnosis, etc. in a parallel, cost saving, and label-free detection way (Wang et al. 2005).

MBs Based Assay for Detecting Pathogen

Molecular beacons can be successfully used to ascertain not only in the presence or absence of a particular causative agent, but also in screening which antibodies will be effective against a particular strain of the causative organism.

In recent years, causative organism detection method has been developed rapidly. Many scientists used MB technology to detect causative organism, and achieved some initial results, such as detection of *Mycobacterium tuberculosis* in sputum (Haldar et al. 2007) and multidrug-resistant tuberculosis in clinic (Banerjee et al. 2010), identifying community-acquired pneumonia causative agents rapidly (Morozumi et al. 2006), and even identification of *Candida dubliniensis* (Park et al. 2000). Nowadays, high-efficiency fluorescent inorganic core–shell quantum dots-CdTe/ZnS (as fluorescent energy donor) and BHQ-2 (energy acceptor) are combined to the single-strand DNA of *Toxoplasma gondii*. And it turns out to be a successful tool of targeting *T. gondii* DNA (Zhou et al. 2009).

Because of the advantages of MBs for the analysis of nucleic acids, it is not hard to imagine a sensitive assay for the rapid and simple detection of morbigenous virus in clinic. Molecular beacon assay is one type of RT-PCR technology capable of fast, specific, sensitive, and reliable viral detection. It has been used wildly in diseases diagnosis.

Hepatitis B is recognized as an endemic in China by the World Health Organization (Liang et al. 2005). Roughly 400 million people are infected with hepatitis B virus (HBV) worldwide (Lai et al. 2003). Over one-third of the world's population has been or is actively infected by HBV. An estimated 130 million Chinese are infected with the disease, about 10 % of China's total population and about one-third of the world's cases. Almost one million new cases were reported in China in 2005. HBV is seriously harmful to human health. Methods and techniques such as RT-PCR, ELISA and immunofluorescence assay, which could detect HBV virus fast and accurately, had been adopted; even the corresponding Test Kit had been introduced. However, nowadays these techniques are still used together with the clinical signs to get an accurate diagnosis. MBs technology can detect the nuclear acid as long as the HBV appears. Thus, it made great clinical sense in saving the valuable time for clinical treatment.

The assay of based amplification (NASBA) technology and real-time detection with MB technology have a detection range of 10^3–10^9 HBV DNA copies/ml of plasma or serum (6 logs), with good reproducibility and precision (Yates et al. 2001). The 1896 precore mutation is the most frequent cause of HBV e-antigen (HBeAg)-negative chronic HBV infection. It was detected using RT-PCR and molecular-beacon technology of the assay in 55.5 % of the children with HBeAg-negative infection (Sum et al. 2004). The HBV genomic DNA level measured using RT-PCR with a MB (HBV beacon assay) showed good correlation with that measured by the commercially available COBAS AMPLICOR HBV Monitor test ($r = 0.901; p < 0.001$) (Waltz et al. 2005). A sensitive and reproducible RT-PCR assay based on the universal MB (U-MB) technique was developed for the detection of HBV DNA in serum (Li et al. 2007).

These studies prove that the technique of MBs for quantitation of HBV is superior to the other HBV DNA assays in higher sensitivity and broader dynamic range. MBs technology may be offered as an ideal tool for monitoring disease progression and treatment efficacy in HBV-infected patients, in particular, for those with low levels of HBV viremia.

Meanwhile, MB assays are also used to detect other kinds of virus, such as hepatitis A virus (Deiman et al. 2007; Yeh et al. 2008); hepatitis C virus (Morandi et al. 2007); hepatitis E virus (Rehman et al. 2008); HIV-1, HIV-2, human T-lymphotrophic virus types I and II (Vet et al. 2004); human herpesvirus-8 (Polstra et al. 2002); influenza A virus (Wang et al. 2008); adenovirus (Poddar 1999); enteroviruses (Landry et al. 2005); cytomegalovirus (Yeo et al. 2005); respiratory syncytial virus A and B (Deiman et al. 2007); human papilloma virus (Molden et al. 2007); bluetongue virus (Deiman et al. 2007), the emerging human coronavirus (Hadjinicolaou et al. 2011), even the prion (Wang et al. 2011) and so on.

Applications of Modified MBs In Vivo

Using MBs technology, the process of biological macromolecules metabolism in vivo, for example, mRNA in cells (Fig. 3), can be dynamically analyzed to investigate the process of transcription and other changes (Santangelo et al. 2006). This ability can provide important information concerning the temporal and spatial processing, localization and transport of specific mRNAs under various conditions. To date, people conducted a number of structural improvements and development on MBs, such as wavelength-shifting MBs, dual FRET MBs and permeation peptide-conjugated MBs, so that MB technology can be used in monitoring the level of gene expression in vivo with high sensitivity and low background signal. Molecular beacons proved its potential in decoding the messages coded on mRNA. The use of MB for in vivo studies depends on the combination of rational sequence design, efficient probe insertion in the cell and choice of target

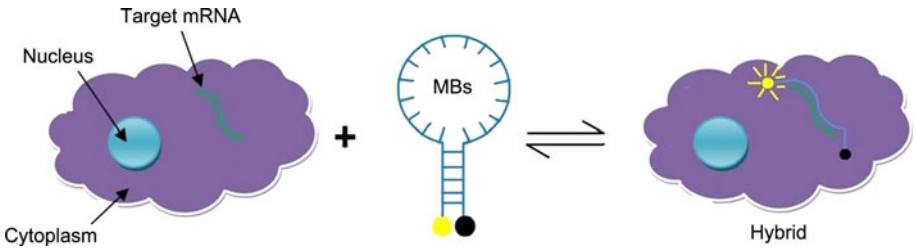

Fig. 3 Illustrations of MBs used to detect mRNA in living cells

mRNA sequence. As a result, a number of rapid diagnosis and prognosis systems of diseases using MBs are being reported from time to time.

Our early study has shown the promise of MBs's applicability in monitoring genes in living systems. And now, we design and synthesis two different labeled MBs. Their sequences are antisense oligonucleotides complementary to survivin mRNA. One of them is survivin mRNA-MB-Cy3 (red), another is survivin mRNA-MB-FITC (green). These two kinds of survivn mRNA MBs, with different color fluorescence, had strong fluorescent signals in cervical cancer cell lines. Though the result was mostly preliminary, the technology of MBs imaging may provide a new approach to diagnosis of early stage cervical cancer and the following-up in the clinic.

Molecular beacons are modified by many ways to apply in the detection of tumors for years. It is the important application for MBs in medicine.

p21 (WAF1/CIP1) is the gene whose transcription is directly activated by the p53 tumor suppressor protein. Strong nuclear signal was observed following treatment of wild-type p53-expressing human H-460 lung cancer cells for 8 h with the chemotherapeutic agent doxorubicin (adriamycin) by a MB. Similar induction was observed in wild-type p53-expressing HCT116 cells and not in p53-null HCT116, or mutant p53-expressing DLD1 cells that are either wild-type or p21-null (Shah and El-Deiry 2004). Molecular beacon was applied for quantitative detection of p21 mRNA in cellular total RNA. The results showed that p21 mRNA is inhibited by silencing p53 in CNE2 cells, while it is upregulated in MCF-7 cells by ING1 transfection, the influence of 5-fluorouracil (5-FU) treatment on p21 mRNA of MCF-7 cells is time and concentration-dependent manner (Liu et al. 2010a). After injecting the p21 MB into nasopharyngeal carcinoma cell and p33-transfected nasopharyngeal carcinoma cell, the consistent increase of fluorescent signal intensity was detected in both cell lines, and the fluorescence increasing rate was significantly different between these two cell lines, which indicate the different p21 mRNA expression levels (Tang et al. 2008). These results demonstrated that the new method with the advantages of high specificity, fast speed and simplicity can be widely applied for p21 mRNA detection in different samples.

By binding K-ras MBs differentially to mutant K-ras mRNAs, it is resulted in strong fluorescent signals in pancreatic cancer cells with specific mutant K-ras genes but not in normal cells or cancer cells expressing either wild-type or a different mutation of the K-ras gene. MBs targeting survivin mRNA produced a bright fluorescent signal specifically in pancreatic cancer cells. Survivin and K-ras MBs have a high specificity in identifying cancer cells on frozen sections of pancreatic cancer tissues (Yang et al. 2005). Using modified MB assay in breast cancer tissues, it can assessed expression of hCG beta-3, -5, -8, which differ by only one nucleotide from other hCG beta genes (Span et al. 2003). There is a simultaneous delivery of MBs targeting survivin and cyclin D1 mRNAs produced strong fluorescence in breast cancer but not in normal breast cells (Peng et al. 2005). The green fluorescent signal was present in the exfoliated cells of patients but not in the healthy adult using survivin MBs in bladder cancer (Zhao et al. 2007).

In addition, MBs have been reported for the detection of human c-fos mRNA in transfected Cos7 cells (Tsuji et al. 2000), bactin mRNA in K562 human leukemia cells (Sokol et al. 1998) and in PTK2 cells (Perlette and Tan 2001), 11 steroidogenic genes' mRNA in H295R human adrenocortical carcinoma cells (Xu et al. 2006). Their results prove that MBs-based expression imaging provides a novel approach for the detection of the corresponding cells. The applications of MBs in tumors are very helpful for the patients to gain the following treatment. It will be wildly used in the future in clinical diagnosis.

Recently, there are more and more new fashion MBs being used in molecular diagnosis in vivo. Some important applications of these MBs are described below:

Through hard and careful studies, Lockett et al. (2007) developed an efficient instrument for the scoring of SNPs in surface invasive cleavage reactions. They found that quenching efficiency in the "dual FRET molecular beacon" format is increased to 88 %, much closer to the 91 % level that has been reported for MB assays. A second benefit of the approach described here is that the portion of probe oligonucleotide that is removed by the enzyme is shorter, thus increasing the rate of probe cleavage. Tsourkas et al. (2003) and Santangelo et al. (2004) used dual FRET MBs for detecting the expression levels and localization of specific endogenous RNA, found that the detection of a FRET

signal leads to a substantially increased signal-to-background ratio as compared with that seen in single MB assays and enables discrimination between fluorescence due to the specific probe/target hybridization and a variety of possible false-positive events. Tang et al. (2008) used a phosphorothioate-modified MB detecting green fluorescent protein (GFP) mRNA in COS-7 cell and GFP-transfected COS-7 cell in real time based on living cell imaging method. The detection shows the advantages of MB, such as, excellent selectivity, high sensitivity, and no separation detection. In addition, this modification could significantly increase the nuclease resistance of MB, reduce the false-positive signal and improve the accuracy of living cell mRNA detection. Various improvements of MBs design have been done to produce nuclease resistant MB with improved stability at the cytoplasm, such as 2′-*O*-methylated MBs which offer good nuclease resistance and also resist RNase activity (Monroy-Contreras and Vaca 2011). These new approaches allow for the ultrasensitive detection of target molecules in a way that could be readily applied to real-time imaging of gene expression in living cells.

Conclusions and Future Prospects

Molecular beacons are single-stranded, fluorophore-labeled nucleic acid probes that are capable of generating a fluorescent signal in the presence of target, but are dark in the absence of target. Although it still has some limitations, it has been widely applied in many fields for its specificity and sensitivity, such as biology, basic medical science, and clinical medicine. And in order to enrich its application, researchers are working on improving its structure constantly by many ways. The important new fashions of MBs are wavelength-shifting MBs, dual FRET MBs and permeation peptide-conjugated MBs. The biochip technology based on MBs can be used for high throughput screening. MBs technology will be perfected if it contact with nanometer technology, confocal laser technology and other advanced technology. It is no doubt that MBs technology has promising perspective with the reform of design approach and progress of prepared method. However, there is still a long way to go for us before making MBs more accurate, safer and faster in diseases diagnosis.

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References

Antony T, Subramaniam V (2001) Molecular beacons: nucleic acid hybridization and emerging applications. J Biomol Struct Dyn 19:497–504

Banerjee R, Allen J, Lin SY et al (2010) Rapid drug susceptibility testing with a molecular beacon assay is associated with earlier diagnosis and treatment of multidrug-resistant tuberculosis in California. J Clin Microbiol 48:3779–3781

Barton GM, Medzhitov R (2002) Retroviral delivery of small interfering RNA into primary cells. Proc Natl Acad Sci USA 99:14943–14945

Bonnet G, Tyagi S, Libchaber A et al (1999) Thermodynamic basis of the enhanced specificity of structured DNA probes. Proc Natl Acad Sci USA 96:6171–6176

Chen L, Shopsin B, Zhao Y et al (2012) Real-time nucleic acid sequence-based amplification assay for rapid detection and quantification of agr functionality in clinical *Staphylococcus aureus* isolates. J Clin Microbiol 50:657–661

Deiman B, van Aarle P, Sillekens P (2002) Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol 20:163–179

Deiman B, Schrover C, Moore C et al (2007) Rapid and highly sensitive qualitative real-time assay for detection of respiratory syncytial virus A and B using NASBA and molecular beacon technology. J Virol Methods 146:29–35

Dubertret B, Calame M, Libchaber AJ (2001) Single-mismatch detection using gold-quenched fluorescent oligonucleotides. Nat Biotechnol 19:365–370

Epstein JR, Leung AP, Lee KH et al (2003) High-density, microsphere-based fiber optic DNA microarrays. Biosens Bioelectron 18:541–546

Hadjinicolaou AV, Farcas GA et al (2011) Development of a molecular-beacon-based multi-allelic real-time RT-PCR assay for the detection of human coronavirus causing severe acute respiratory syndrome (SARS-CoV): a general methodology for detecting rapidly mutating viruses. Arch Virol 156:671–680

Haldar S, Chakravorty S, Bhalla M et al (2007) Simplified detection of Mycobacterium tuberculosis in sputum using smear microscopy and PCR with molecular beacons. J Med Microbiol 56(Pt10):1356–1362

Kim H, Kane MD, Kim S et al (2007) A molecular beacon DNA microarray system for rapid detection of *E. coli* O157:H7 that eliminates the risk of a false negative signal. Biosens Bioelectron 22:1041–1047

Lai CL, Ratziu V, Yuen MF et al (2003) Viral hepatitis B. Lancet 362:2089–2094

Landry ML, Garner R, Ferguson D (2005) Real-time nucleic acid sequence-based amplification using molecular beacons for detection of enterovirus RNA in clinical specimens. J Clin Microbiol 43:3136–3139

Li H, Rothberg LJ (2004) DNA sequence detection using selective fluorescence quenching of tagged oligonucleotide probes by gold nanoparticles. Anal Chem 76:5414–5417

Li X, Huang Y, Song C et al (2007) Several concerns about the primer design in the universal molecular beacon real-time PCR assay and its application in HBV DNA detection. Anal Bioanal Chem 388:979–985

Li Y, Zhou X, Ye D (2008) Molecular beacons: an optimal multifunctional biological probe. Biochem Biophys Res Commun 373:457–461

Liang XF, Chen YS, Wang XJ et al (2005) A study on the sero-epidemiology of hepatitis B in chinese population a aged over 3 years old. Zhonghua Liu Xing Bing Xue Za Zhi 26: 618–655

Liao N, Gao JH, Zeng WS et al (2011) Establishment of molecular beacon real-time PCR for detecting p53 gene single nucleotide polymorphism. Nan Fang Yi Ke Da Xue Xue Bao 31:1875–1878

Liu B, Yang XH, Wang KM et al (2010a) Quantitative detection of p21 mRNA in tumor cells based on molecular beacon. Acta Chim Sinica 68:1303–1307

Liu XP, Hou JL, Liu JH (2010b) A novel single nucleotide polymorphism detection of a double-stranded DNA target by a ribonucleotide-carrying molecular beacon and thermostable RNase HII. Anal Biochem 398:83–92

Lockett MR, Shortreed MR, Smith LM (2007) Molecular beacon-style hybridization assay for quantitative analysis of surface invasive cleavage reactions. Anal Chem 79:6031–6036

McDonald D, Vodicka MA, Lucero G et al (2002) Visualization of the intracellular behavior of HIV in living cells. J Cell Biol 159:441–452

Mitchell P (2001) Turning the spotlight on cellular imaging. Nat Biotechnol 19:1013–1018

Molden T, Kraus I, Skomedal H et al (2007) PreTect (TM) HPV-proofer: real-time detection and typing of E6/E7 mRNA from carcinogenic human papillomaviruses. J Virol Methods 142: 204–212

Monroy-Contreras R, Vaca L (2011) Molecular beacons: powerful tools for imaging RNA in living cells. J Nucleic Acids 2011: 741723

Morandi L, Ferrari D, Lombardo C et al (2007) Monitoring HCV RNA viral load by locked nucleic acid molecular beacons real time PCR. J Virol Methods 140:148–154

Morozumi M, Nakayama E, Iwata S et al (2006) Simultaneous detection of pathogens in clinical samples from patients with community-acquired pneumonia by real-time PCR with pathogen-specific molecular beacon probes. J Clin Microbiol 44: 1440–1446

Nguyen C, Grimes J, Gerasimova YV et al (2011) Molecular-beacon-based tricomponent probe for SNP analysis in folded nucleic acids. Chemistry 17:13052–13058

Nitin N, Santangelo PJ, Kim G et al (2004) Peptide linked molecular beacons for efficient delivery and rapid mRNA detection in living cells. Nucleic Acids Res 32:e58

Ortiz E, Estrada G, Lizardi P (1998) PNA molecular beacons for rapid detection of PCR amplicons. Mol Cell Probes 12:219–226

Park S, Wong M, Marras SA et al (2000) Rapid identification of *Candida dubliniensis* using a species-specific molecular beacon. J Clin Microbiol 38:2829–2836

Peng XH, Cao ZH, Xia JT et al (2005) Real-time detection of gene expression in cancer cells using molecular beacon imaging: new strategies for cancer research. Cancer Res 65:1909–1917

Perlette J, Tan W (2001) Real-time monitoring of intracellular mRNA hybridization inside single living cells. Anal Chem 73:5544–5550

Poddar SK (1999) Detection of adenovirus using PCR and molecular beacon. J Virol Methods 82:19–26

Polstra A, Goudsmit J, Cornelissen M (2002) Development of real-time NASBA assays with molecular beacon detection to quantify mRNA coding for HHV-8 lytic and latent genes. BMC Infect Dis 2:18

Rehman S, Kapur N, Durgapal H et al (2008) Subcellular localization of hepatitis E virus (HEV) replicase. Virology 370:77–92

Santangelo PJ, Nix B, Tsourkas A et al (2004) Dual FRET molecular beacons for mRNA detection in living cells. Nucleic Acids Res 32:e57

Santangelo P, Nitin N, Bao G (2006) Nanostructured probes for RNA detection in living cells. Ann Biomed Eng 34:39–50

Shah R, El-Deiry WS (2004) p53-dependent activation of a molecular beacon in tumor cells following exposure to doxorubicin chemotherapy. Cancer Biol Ther 3:871–875

Sidoti F, Bergallo M, Terlizzi ME et al (2012) Development of a quantitative real-time nucleic acid sequence-based amplification assay with an internal control using molecular beacon probes for selective and sensitive detection of human rhinovirus serotypes. Mol Biotechnol 50:221–228

Sokol DL, Zhang X, Lu P et al (1998) Real time detection of DNA RNA hybridization in living cells. Proc Natl Acad Sci USA 95:11538–11543

Span PN, Manders P, Heuvel JJ et al (2003) Molecular beacon reverse transcription-PCR of human chorionic gonadotropin-beta-3, -5, and -8 mRNAs has prognostic value in breast cancer. Clin Chem 49:1074–1080

Sum SS, Wong DK, Yuen MF et al (2004) Real-time PCR assay using molecular beacon for quantitation of hepatitis B virus DNA. J Clin Microbiol 42:3438–3440

Takacs T, Jeney C, Kovacs L et al (2008) Molecular beacon-based real-time PCR method for detection of 15 high-risk and 5 low-risk HPV types. J Virol Methods 149:153–162

Tan W, Fang X, Li J et al (2000) Molecular beacons: a novel DNA probe for nucleic acid and protein studies. Chemistry 6:1107–1111

Tan L, Li Y, Drake TJ et al (2005) Molecular beacons for bioanalytical applications. Analyst 130:1002–1005

Tang HX, Yang XH, Wang KM et al (2008) Monitoring p21 mRNA expression in living cell based on molecular beacon fluorescence increasing rate. Chin. Sci Bull 53:357–361

Tsourkas A, Behlke MA, Xu Y et al (2003) Spectroscopic features of dual fluorescence/luminescence resonance energy-transfer molecular beacons. Anal Chem 75:3697–3703

Tsuji A, Koshimoto H, Sato Y et al (2000) Direct observation of specific messenger RNA in a single living cell under a fluorescence microscope. Biophys J 78:3260–3274

Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14:303–308

Tyagi S, Marras SA, Kramer FR (2000) Wavelength-shifting molecular beacons. Nat Biotechnol 18:1191–1196

Venkatesan N, Seo YJ, Kim BH (2008) Quencher-free molecular beacons: a new strategy in fluorescence based nucleic acid analysis. Chem Soc Rev 37:648–663

Vet JA, Marras SA, Herdewijn P (2004) Design and optimization of molecular beacon real-time polymerase chain reaction assays. Methods Mol Biol 288:273–290

Waltz TL, Marras S, Rochford G et al (2005) Development of a molecular-beacon assay to detect the G1896A precore mutation in hepatitis B virus-infected individuals. J Clin Microbiol 43: 254–258

Wang Y, Wang H, Gao L et al (2005) Polyacrylamide gel film immobilized molecular beacon array for single nucleotide mismatch detection. J Nanosci Nanotechnol 5:653–658

Wang W, Cui ZQ, Han H et al (2008) Imaging and characterizing influenza A virus mRNA transport in living cells. Nucleic Acids Res 36:4913–4928

Wang C, Wu R, Li FD et al (2011) Expression patterns of prion protein gene in differential genotypes sheep: quantification using molecular beacon real-time RT-PCR. Virus Genes 42:457–462

Wu ZS, Jiang JH, Fu L et al (2006) Optical detection of DNA hybridization based on fluorescence quenching of tagged oligonucleotide probes by gold nanoparticles. Anal Biochem 353: 22–29

Xu Y, Yu RM, Zhang X et al (2006) Effects of PCBs and MeSO2-PCBs on adrenocortical steroidogenesis in H295R human adrenocortical carcinoma cells. Chemosphere 63:772–784

Yang CJ, Medley CD, Tan W (2005) Monitoring nucleic acids using molecular beacons. Curr Pharm Biotechnol 6:445–452

Yates S, Penning M, Goudsmit J et al (2001) Quantitative detection of hepatitis B virus DNA by real-time nucleic acid sequence-based amplification with molecular beacon detection. J Clin Microbiol 39:3656–3665

Yeh HY, Hwang YC, Yates MV et al (2008) Detection of hepatitis A virus by using a combined cell culture-molecular beacon assay. Appl Environ Microbiol 74:2239–2243

Yeo AC, Chan KP, Kumarasinghe G et al (2005) Rapid detection of codon 460 mutations in the UL97 gene of ganciclovir-resistant cytomegalovirus clinical isolates by real-time PCR using molecular beacons. Mol Cell Probes 19:389–393

Zhao J, He D, He H et al (2007) Primary application study in early diagnosis of bladder cancer by survivin molecular beacons. Urology 70:60–64

Zhou C, Xu S, Yang J et al (2009) Detection of Toxoplasma gondii with a DNA molecular beacon probe. In: International symposium on photoelectronic detection and imaging 2009: material and device technology for sensors (Proceedings Volume). Proceedings of SPIE, vol 7381. doi:10.1117/12.835543