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A REVIEW

Molecular beacon: a multitask probe

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1. SUMMARY

The specificity of hybridization of complementary sequences in DNA is the basic strategy for identifying target genes. For this, stem loop oligonucleotide probes have been developed in order to enhance the specificity and selectivity to the target DNA. Among stem loop oligonucleotides, molecular beacons are the recent probes used for biomolecular recognition reactions. Molecular beacon-based assays are fast, simple, inexpensive, and enable real-time monitoring of nucleic acid reactions both, *in vivo* and *in vitro*. This review has been designed to provide a better understanding of the different aspects of molecular beacons, e.g. structure, designing and applications in real-time monitoring of nucleic acid amplification, detection of pathogens, nucleic acid–protein interaction, genetic analysis and array technology.

2. INTRODUCTION

Hybridization between complementary sequences forms the base of recent molecular approaches for the identification and detection of a particular gene. These hybridization techniques use specific labelled probes of small ssRNA or DNA fragments that can recognize and hybridize complementary sequence in the target RNA or DNA. Traditionally, there are different methods of radioactive/nonradioactive and fluorescent probe labelling. The hybridization steps constitute the labelling of probes, immobilization of target molecule on solid surface, hybridization to labelled probes, removal of un-hybridized probes and finally the detection of bound probes. Removal of unbound probes and further washing steps usually disturb the equilibrium of nucleic acid hybridization event. Besides, due to the toxic effects of radioactive chemicals, the radiolabelled probes cannot be used to monitor real-time amplification of DNA during PCR and for *in vivo* DNA synthesis. Because of these difficulties encountered with conventional probe labelling methods, research has been diverted towards oligonucleotide probe designing that enable dynamic, real-time detection of nucleic acid amplification both *in vivo* and *in vitro* (Tyagi and Kramer 1996; Kostrikis *et al.* 1998; Tyagi *et al.* 1998). Molecular beacons were first developed by Tyagi and Kramer (1996) at the Public Health Research Institute, New York, USA. These are the fluorescent probes that produce fluorescence on hybridization with the complementary target. Molecular beacon probes can be used to monitor real-time amplification during PCR, genetic analysis, detection of pathogens, gene mutation and in various other biological contexts. Research is still in progress for the modification of conventional molecular beacon probe to enhance the specificity and sensitivity. Scorpion probe is one of the variant of such modification that functions simultaneously as a PCR primer and a beacon probe (Whitcombe *et al.* 1999). Catalytic molecular beacons (Stojanovic *et al.*

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2001) are another variant that can detect the target sequences without amplification. Another modification includes the construction of PNA-DNA (Peptide Nucleic Acid) loop with molecular beacon, stemless beacon (Kuhn *et al.* 2002). PNA molecular beacons were also reported to be superior than conventional molecular beacon probes because of their faster hybridization kinetics, high signal to background ratio and much better specificity (Xi *et al.* 2003).

3. STRUCTURE OF MOLECULAR BEACON

Molecular beacon is a single-stranded oligonucleotide probe with a special loop stem structure (Tyagi and Kramer 1996). The loop portion is an oligodeoxyribonucleotide probe for a complementary target. The stem is constructed of annealed complementary arm sequences at the ends of probe sequence. It is a dual labelled oligonucleotide having a fluorescent reporter group at 3′ and fluorescent quencher group at 5′ end of the arm (Fig. 1). The annealing of stem causes intramolecular energy transfer from fluorophore to quencher. The process of energy transfer is much more efficient at shorter distances.

3.1 Fluorophore

Many different fluorophore dyes have been tested for their efficiency depending on the quencher group. Among them the most commonly used dyes are 5-(2′-aminoethyl) aminonapthalene-1-sulfonic acid (EDNAS), fluorescein (Fam), tetrachloro-6-carboxyfluorescein (Tet), hexachloro-6-carboxy fluorescein (Hex) and tetramethylrhodamine (Tamra) and 5-carboxyrhodamine-X (Rox). The flexibility in reporter dye offers its use in multiplex molecular beacon detection reactions, i.e. enabling multiple targets to be distinguished in the same solution. Proper selection of fluorophore is critical for improved signal to background ratio.

3.2 Quencher

Capture and transfer of light energy from an excited fluorophore is referred as quenching and the substances involved in it are termed as quenchers. The commonly used quencher, 4-(4′-dimethylaminophenylazo) benzoic acid dabcyl, is a nonfluorescent chromophore. Dabcyl, a neutral and hydrophobic molecule can serve as a universal quencher for variety of fluorophores (Tyagi *et al.* 1998). It optimally quenches the fluorescein, but its quenching efficiency decreases to 93–98% for dyes emitting longer wavelength. The use of metals has opened up a new era to use different qualities of quenchers with different properties. Now gold nanoparticles (1·4 nm in diam.), having higher quenching efficiency are being used as quenchers (Dubertret *et al.* 2001). The gold-quenched beacons offer greater sensitivity to single-nucleotide mismatches of DNA sequence.

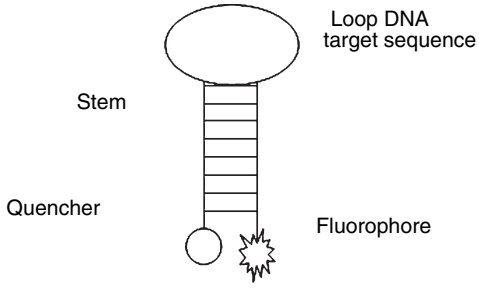

Fig. 1 Structure of a molecular beacon probe

3.3 Probe sequence

The sequence of probe is the real determinant of the specificity of the molecular beacon and the length of the probe sequence should be such that it dissociates itself from the target at a 7–10°C higher temperature than that of PCR annealing temperature. The melting temperature of the probe-target hybrid can be predicted using percentage GC (guanine and cytosine) content, available with commercial software packages like Oligo 6.0 (Molecular Biology Insight, Inc., Cascade, CO, USA). Generally, the length of the probe sequence should range from 15 to 30 nucleotides and should not form any secondary structure (Tyagi and Kramer 1996; Kostrikis *et al.* 1998; Ortiz *et al.* 1998). If it happens, then 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 leads to reduced specificity.

3.4 Stem sequence

The criteria for stem sequence include the length, sequence and GC content. The stem should have a melting temperature of 7–10°C more than the detection temperature (Tyagi and Kramer 1996) that could be predicted by Zuker DNA folding program available at http://www.bioinfo.rpi.edu/applications/mfold/. This program predicts the free energy of formation of stem hybrid, thus helps in determining the melting temperature. Most beacon studies (Tyagi and Kramer 1996; Kostrikis *et al.* 1998; Ortiz *et al.* 1998; Tyagi *et al.* 1998) indicate that the maximum stability with target is obtained with 15–25 base sequences together with 5–7 bp in the stem. Molecular beacons with short stem length have faster hybridization kinetics but suffer from lower signal to background ratio. The nucleotide positioned just before the fluorophore should be chosen carefully as nucleotides exhibit a variable degree of quenching in order of G>A>C>T (Drake and Tan 2004).

4. SPECIFICITY OF MOLECULAR BEACON PROBE

The specificity of a particular probe lies in the stem loop structure. The specificity and sensitivity can be optimized for specific application by adjusting GC content. Hybridization to the complementary target results in a detectable conformational change that has low background and thus eliminates the removal of unbound probe. The probe is so designed that in the absence of a complementary target, the molecule forms the hairpin structure. It brings the reporter and quencher in close proximity where they share electrons and form a nonfluorescent complex that absorbs energy of the light and loses that energy as heat. Thereby, the proximity results in efficient quenching of the reporter. However, in presence of specific target, the probe anneals to the complementary sequence, the longer and stronger probe-target duplex overcomes the hairpin structure leading to disruption of unimolecular stem-loop conformation. The rigidity of the probe-target sequence forces the hairpin stem to unfold, leading to fluorophore and quencher separation and thus emitting the fluorescence of a characteristic wavelength (Fig. 2). In closed state, there is radiationless transfer of electronic excitation energy from fluorophore to quencher, known as static or contact quenching which is significantly different from Fluorescence Resonance Energy Transfer (FRET) (Marras *et al.* 2002). Contact quenching is the leading mechanism in molecular beacon probes because of short distances between the dyes. During this phenomenon, the fluorophore and chromophore form a ground state heterodimer which allows strong coupling between the transition dipoles of dyes (Bernacchi and Mely 2001). Whereas, FRET requires two conditions that the fluorophore and the quencher interact within a distance of 20–100 Å and there should be a significant overlap between the emission spectrum of fluorophore and absorption spectrum of quencher (Stryer 1978). However, the specificity is limited during single nucleotide mismatches.

5. DESIGNING OF MOLECULAR BEACON PROBE

The most important design parameters for molecular beacon are the length and sequence of probe and stem, as they participate in the three different conformational states: bound-to-target, stem-loop and random-coil. The general design can be altered to fit in with specific application, e.g. single mismatches can be better discriminated with the use of shorter loop sequences. The molecular beacons can be synthesized as soluble or glass bound probes (Tyagi *et al.* 1998; Brown *et al.* 2000). Both types of probes have equivalent properties but the advantage in using glass bound probes lies in easy isolation and analysis of fluorescent beads (Brown *et al.* 2000). The probe is synthesized from the modified oligonucleotides that have a primary amino group at the 3′ end and a trityl protected sulfahydral group at the 5′ end. The sulfahydral group is covalently attached to 5′ phosphate via a $(CH_2)_6$ spacer and amino group is linked to 3′ hydroxyl moiety via a $(CH_2)_7$ spacer. Then the quencher and fluorophore are attached to stem by two consecutive coupling reactions. Static quenching efficiencies for different pairs of fluorophore and quencher should be considered for better sensitivity. This primary product is then purified by HPLC to remove the unreacted quencher units. The protective trityl moiety is then removed from the sulfahydral group and fluorophore is introduced to the reduced thiol group. The mature probe is then purified by gel exclusion chromatography and HPLC. The probe can be prepared in bound form as biotinylated (Fang *et al.* 1999) or controlled pore glass bound probes (Tyagi and Kramer 1996; Fang *et al.* 1999). The detailed protocols for the synthesis of beacon probes are available at: http://www.molecular-beacons.org and http://www.phri.org.

5.1 Thermal denaturation profile of molecular beacon

For determining the effect of temperature on the secondary structure, a thermal denaturation profile is obtained after

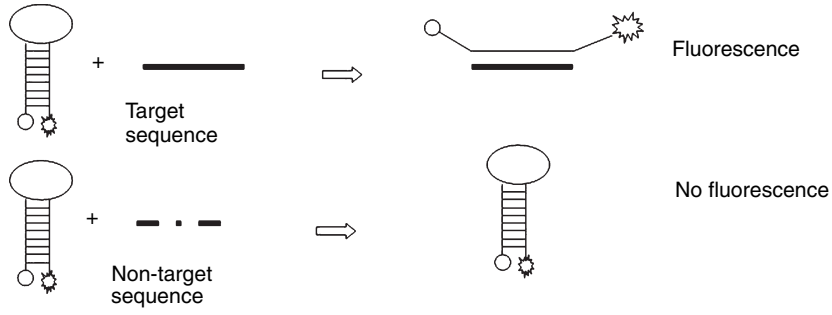

Fig. 2 Specificity of molecular beacon probe

synthesizing the beacon. The fluorescence of the probe is measured as a function of temperature and the phase transition is used for the determination of the thermal denaturation profile. At low temperature the probe-target duplexes form spontaneously. As hairpin stem is less stable than the probe-target helix, hence it unwinds to allow probe-target binding. Thus, molecular beacon in duplex opens up and results in fluorescence. As the temperature is raised probe-target destabilizes, releasing the beacon and finally return to the original closed conformation resulting in decreased fluorescence. Bonnet *et al.* (1999) reported a transition temperature of 42°C while at 54°C they return to random coil conformation.

6. APPLICATIONS OF MOLECULAR BEACON

The use of molecular beacons probes to explore gene identification at a global level has revolutionized the area of probe labelling and it seems fit to become more powerful tool for different applications in human health aspects. Some of the important applications of these probes are summarized below.

6.1 Real-time PCR monitoring

A wider application of molecular beacons is in the real-time detection of PCR reaction (Tyagi and Kramer 1996; Tyagi *et al.* 1998). The monitoring is possible at lower temperature because at higher temperature the beacon itself becomes dissociated. When the temperature is lowered for annealing of primers, the probe will not fluoresce because of their stem loop hybrids. However, some of the molecules of the probe will bind to the target amplicons to generate a fluorescent signal. This fluorescence will indicate the degree of amplification taking place at each annealing step in the closed tube. The real-time PCR monitoring has the ability to process many samples at a time with relatively high speed. The confirmation of the amplified product in the same tube with minimum risk of contamination is another advantage of this technique over time-consuming gel electrophoresis and Southern blotting. Ortiz *et al.* (1998) have developed surface immobilized PNA-DNA hybrid probes that detect PCR amplicons by simply adding a PCR reaction to a microtitre well containing the previously immobilized probe and reading the generated fluorescence. The PNA-DNA probes have been used for detection of rDNA from *Entamoeba histolytica* (Ortiz *et al.* 1998). The technique has been used by Whitcombe *et al.* (1999) and Nilsson *et al.* (2002) for PCR products and rolling circle amplification. Quantification of viruses has also been achieved using multiplex PCR by Weusten *et al.* (2002) and Yates *et al.* (2001). Replication of Hepatitis virus was studied by strand specific real-time amplification (Komurian-Pradel *et al.* 2004).

6.2 Genetic analysis

Currently, the determination of genetic variation involves automated procedures like sequencing but it demands high throughput methods. The beacon probes serves well to discriminate single-base mismatches. The probes are so selected that at a wide range of temperatures only perfectly complementary probe-target hybrids are more stable and force the stem hybrid to open. Tyagi *et al.* (1998) found that mismatched hairpin probe duplexes were less stable than mismatched linear probe duplexes at all target concentrations. This stability is affected by a number of factors such as the length of the hybridization sequence, GC content, location of the mismatch bases in the sequence and the hybridization temperature. The stability of the hairpin stem can be deduced from the DNA folding program. This program can discriminate between targets that have a single base pair change, making them ideal for investigating single nucleotide polymorphism (Kostrikis *et al.* 1998; Tyagi *et al.* 1998; Marras *et al.* 1999). Therefore, careful designing of the probe and optimization of the reaction conditions are required. As the molecular beacons have a high selectivity with one base mismatch identification, therefore, they serve as promising probes for genetic analysis, i.e. 'Spectral genotyping' (Kostrikis *et al.* 1998). The molecular beacon approach has been used to analyse 81 bp region of the *rpoB* gene for mutation providing rifampin resistance in *Mycobacterium tuberculosis* (Piatek *et al.* 1998). Giesendorf *et al.* (1998) have designed molecular beacon to detect point mutation in methylenetetrahydrofolate reductase gene that increases the risk for cardiovascular diseases and neural tube defects. This approach has also been used to analyse the alleles of β-chemokine receptor 5 gene which determine the susceptibility to HIV-I (Kostrikis *et al.* 1998).

6.3 Molecular beacon and array technology

DNA probes are used as gene-based biosensors that specifically bind to DNA-DNA and DNA-hybrids at the surface of biosensor. Fluorescence-based biosensors need fluorescent stains/reagents and cannot be used for real-time hybridization studies; therefore, molecular beacon DNA biosensors have been developed (Liu and Tan 1999; Brown *et al.* 2000; Liu *et al.* 2000; Steemers *et al.* 2000). The advantage behind the use of molecular beacon biosensors is that these are stable, reproducible, do not need competitive assay and capable of single nucleotide identification. Li *et al.* (2001) have used a surface-immobilized biotinylated ssDNA molecular beacon on ultra small optical fibre probes using avidin–biotin binding. But the limits are 0·3 nm and 15 amol for 105 μm biosensor and 10 nm and 0·27 amol for a submicrometre biosensor. This biosensor reaction was used for quantification of specific rat γ-actin mRNA sequence amplified by PCR. The

hybridization was found to be affected by ionic strength, i.e. divalent ions which reduce the repulsive forces between the anionic chains of molecular beacon on surface and target nucleic acid in high concentration of $MgCl_2$. The molecular beacon-based biosensors are useful in detection of specific RNA/DNA inside living cells, detection of free or nonlabelled nucleic acid targets in real-time with exceptional sensitivity and selectivity with rapid, regenerable and durable results. Molecular beacon biosensors have been developed by Du *et al.* (2003) using DNA hairpins on gold surfaces as it has ability to quench fluorescent molecules.

6.4 Detection of pathogens

The inability of some pathogens to grow on many selective media necessitates the use of molecular approaches for identification. Among these techniques, the widely used technique is the heterogeneous gel electrophoresis method with amplified nucleic acid product. But the homogenous methods like molecular beacons possess the distinguished advantages over other methods. The high sensitivity and specificity makes this technique more suitable to detect most common pathogens (Table 1).

Table 1 Application of molecular beacons in the detection of pathogens

Pathogen	References
Adenovirus	Poddar (1999)
Bordetella pertussis	Poddar and Le (2001)
Candida dubliniensis	Park *et al.* (2000)
Chlamydophila felis	Helps *et al.* (2001)
Chlamydia trachomatis	Li *et al.* (2000c), Zhang *et al.* (2002)
Clostridium difficile	Belanger *et al.* (2003)
Epstein–Barr virus and cytomegalovirus	Jebbink *et al.* (2003)
Escherichia coli O157:H7	McKillip and Drake (2000), Fortin *et al.* (2001)
Francisella tularensis	Ramachandran *et al.* (2004)
Four retroviruses	Vet *et al.* (1999)
Group B streptococci	Bergeron and Ke (2001)
Hepatitis B virus	Yates *et al.* (2001)
HIV-I	Saha *et al.* (2001), Kostrikis *et al.* (2002)
Legionella pneumophila	Templeton *et al.* (2003a)
Mycobacterium tuberculosis	Li *et al.* (2000c)
Mycobacterium avium subsp. *paratuberculosis*	Fang *et al.* (2002)
Mycoplasma pneumoniae	Templeton *et al.* (2003b), Loens *et al.* (2003)
Neisseria gonorrhea	Li *et al.* (2000c)
Papillomavirus	Szuhai *et al.* (2001)
Salmonella	Chen *et al.* (2000)

Besides molecular detection of pathogens the technique has also been used to identify the drug resistant marker in the pathogens such as rifampin resistance in *M. tuberculosis* because of mutation associated with 81 bp region of *rpo* gene (El-Hajj *et al.* 2001), chloroquine-resistant associated with Pfcrt K76T point mutation in *Plasmodium falciparum* (Durand *et al.* 2002), methicillin resistance in *mec*A and *nuc* genes of *Staphylococcus aureus* (Elsayed *et al.* 2003), itraconazole resistance associated with mutation in CYP 51A gene in *Aspergillus fumigatus* (Nascimento *et al.* 2003).

6.5 Nucleic acid–protein interactions

The proteins having sequence specificity in binding to DNA can be studied by time-consuming gel shifting assays and DNA footprinting assays. Molecular beacon probe labelling was firstly used for studying interactions between Single Strand Binding (SSB) proteins and DNA (Tan *et al.* 2000). The interaction between the SSB protein and DNA produced pronounced fluorescence when beacon probe was used (Li *et al.* 2000b). It can detect SSB at a concentration as low as 2×10^{-10} mol l^{-1} by conventional spectrophotometer. The study indicated that there are significant differences in molecular binding affinity by different proteins and therefore imparts high specificity and selectivity in bioassays of proteins. Heyduk and Heyduk (2002) have used this approach to detect the p53 protein that is involved in the development of more than 50% cancers. The same technique has also been used to study the interaction between lactate dehydrogenase and ssDNA (Fang *et al.* 2000). Li *et al.* (2000a) and Tan *et al.* (2000) have studied the enzymatic cleavage of ssDNA. Molecular beacons have engineered to incorporate single-stranded DNA or RNA molecules, called aptamers as the target binding region of the probe.

6.6 *In vivo* RNA detection

The RNA-targeted hybridization assays have been very useful for studying cellular metabolism but real-time monitoring of the hybridization of mRNA to its complementary strands and changes thereof to translational processes under various cellular conditions are still in mystery. The molecular beacons proved its potential in decoding the messages coded on mRNA. The use of molecular beacon for *in vivo* studies depends upon the combination of rational sequence design, efficient probe insertion in the cell and choice of target mRNA sequence. The mRNA transcripts that arise from mutated genes in the living cells can be detected by the use of molecular beacon and thus is a novel way of detecting cancer at an early stage. A 15-nucleotide-long antisense sequence for human basic fibroblast growth factor has been developed for detection of

mRNA corresponding to the protein (Matsuo 1998). Fluorescent confocal microscopy of microinjected molecular beacon probe into K562 human leukaemia cells reveals the real-time visualization and interaction of mRNA (Giesendorf *et al.* 1998; Bratu *et al.* 2003). Molecular beacon probes are being used for rRNA detection and quantification in solutions and cells. Xi *et al.* (2003) reported that the use of PNA molecular beacons instead of traditional fluorescent *in situ* hybridization probes or DNA molecular beacons would be more helpful in detecting cells under a wide range of environmental conditions.

7. CONCLUSION AND FUTURE PROSPECTS

The stem loop oligonucleotide sequences such as molecular beacons, peptide nucleic acid constructs have a wide range of application in structural and molecular biology because of their enhanced specificity and real-time monitoring of amplification reactions. Scorpion probes, catalytic molecular beacons, PNA-DNA molecular beacon and stemless beacon are the modified beacon probes used in different applications. Existing modifications and others still to come will definitely increase the sensitivity and specificity of the conventional probes. More improvement can also be achieved in other areas such as in probe designing for improved signal to background ratio, improvement in resistance to cellular enzymatic activities for their effective use in *in vivo* studies. Conclusively, a molecular beacon probe proves its potential in the modern era of genomics and proteomics involving DNA–protein interactions, microarray technology, molecular beacon aptamers and biosensors.

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