APPLICATION OF MOLECULAR BEACON TECHNOLOGY FOR THE IDENTIFICATION OF BACTERIA

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Submitted to the Faculty of the
Graduate College of The
Oklahoma State University
In partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2003

APPLICATION OF MOLECULAR BEACON

TECHNOLOGY FOR THE

IDENTIFICATION OF

BACTERIA

Thesis Approved:

Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGEMENTS

I express my sincere appreciation to my mentor, Dr. Jerry Malayer, for his valuable advice and guidance and for creating a pleasant working environment in the lab. I thank all members of my advisory committee, Dr. Cyril Clarke, Dr. Kenneth Clinkenbeard and Dr. Rebecca Morton for their suggestions and advice during the course of my work. I am very much greatful to Dr. Ziad El Rassi for his valuable suggestions and help. I express my gratitude to Dr. Glenn Olah for his help and friendship. A special thanks is due to David Goad for all his help and advice. I thank all members of the lab, Mahesh Mohan, NagarajaThirumalapura, James Flinchbaugh, Leanne Wier, Amy Hurst, Shekher Mohan and Lori Green for their help.

I am grateful to my parents and brother for their constant love and support through out my career. Finally I thank my wife for her support, love and understanding.

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Chapter I

Introduction

Detection and identification of bacterial organisms is of great importance in disease diagnosis and environmental monitoring. The conventional methods for bacterial isolation and subsequent identification by biochemical or immuno detection require days to weeks for completion and may not be feasible in the case of highly pathogenic or fastidious organisms. The variability associated with bacterial enzymes and antigens speaks for an alternative and more definite standard procedure for bacterial identification. With the large number of bacterial genomes being sequenced, techniques targeting unique nucleic acid sequences have been developed for bacterial identification. Bacterial ribosomal RNA (ribonucleic acid) has attracted much attention as a target for identification (Kourentzi, Fox & Willson, 2001; Nelson et al., 2002). Prokaryotic ribosomes consist of two subunits. The small subunit is composed of 16S rRNA and protein while the large subunit is made up of 5S and 23S rRNA and protein. rRNA is characterized by regions that are highly conserved among bacterial organisms and regions that are variable. The 16S rRNA has been used for the phylogenetic classification of prokaryotes (Bintrim et al., 1997). Unlike genomic DNA, there are as many copies of rRNA as there are ribosomes. This innately amplified nature

makes it an attractive target for detection assays. In the present study molecular beacons have been used to detect unique sequences in the 16S rRNA and for identification of bacterial organisms. Molecular beacons are stem- loop shaped DNA molecules with an internally quenched fluorophore. Hybridization with target nucleic acid molecules will result in a conformational shift resulting in fluorescence emission. The presence of bacteria can be established based on this increase in fluorescence. The objectives of this study are as follows:

1. Develop molecular beacons uniquely targeting *Francisella tularensis* 16S rRNA
2. Evaluate selected parameters on the functioning of molecular beacons using synthetic targets in solution and on solid surface
3. Evaluate the sensitivity and specificity of molecular beacons using total RNA samples isolated from bacterial organisms.

Molecular beacons targeting the 16S rRNA of *Francisella tularensis* were used to study beacon reactions in solution and on a solid surface (objective 2). Being an organism that requires BSL-3 laboratory facilities, *Francisella tularensis* was not used for RNA experiments. Instead, molecular beacons targeting the 16S rRNA of *Escherichia coli* and *Mannheimia haemolytica* were developed and total RNA isolated from these organisms were used in bacterial identification experiments (objective 3).

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Chapter II

Review of Literature

Francisella tularensis

F. tularensis is a gram negative, coccobacillary bacterial organism (Dennis et al., 2001). It is a facultative intracellular pathogen and the causative agent of the zoonotic disease tularemia. The genus *Francisella* belongs to the family *Francisellacea* and comes under the class *gamma proteobacteria (Garcia Del Blanco et al., 2002). Francisella* has two recognized species, *F. tularensis* and *F. philomiragia*. *Francisella philomiragia* is not considered to be as clinically relevant as *F. tularensis* due to its lower virulence (Hollis et al., 1989). *Francisella tularensis* contains four subspecies, *F. tularensis* subspecies *tularensis* (or biovar A), *F. tularensis* subspecies *holartica* (or biovar B), *F. tularensis* subspecies *mediaasiatica* and *F. tularensis* subspecies *novicida.* Biovar A is the most virulent and is found in North America (Gurycova, 1998; Haristoy et al., 2003; Johansson et al., 2000). Biovar type B is less virulent and prevalent in Europe and Asia (Uhari, Syrjala & Salminen, 1990). *F. tularensis* subspecies *mediaasiatica* has

been reported from Central Asian countries (Sandstrom et al., 1992). Subspecies *novicida* is rarely isolated and considered to be less virulent (Hollis et al., 1989).

Diagnosis of tularemia is usually based on serological tests and clinical signs (Labayru et al., 1999). Serum antibodies can be demonstrated in blood only after two weeks of disease onset. Detection techniques based on ELISA and PCR have been developed to identify organisms in tissues (Ellis et al., 2002). *Francisella tularensis* are fastidious bacteria requiring cysteine and glucose for growth on culture media (Doern, 2000). They are highly infectious and as few as ten organisms can cause disease (Saslaw et al., 1961). Infection by *Francisella tularensis* can be initiated in many ways including ingestion of contaminated food or water, handling contaminated animal carcases or by inhalation (Anda et al., 2001). The respiratory form of tularemia resulting from inhalation of bacterial aerosols is the most deadly form of the disease (Conlan et al., 2002). The World Health Organization (WHO) report (World Health Organization., 1970) estimates that dispersal of fifty kilograms of *F. tularensis* could result in the death or incapacitation of about 155,000 people. The potential use of *F. tularensis* as a biological weapon has been recognized by many researchers (Cunha, 2002; Dennis et al., 2001; Katz et al., 2002).

Bioweapons

Bioweapons have been used throughout history in one form or the other, whether it be by early man to hunt for food or against the enemy in war. Probably the first use of a bacterial bioweapon on record occurred in 1346, when the Tartar army is said to have thrown plague infected bodies over the walls of the city of Kaffa (Ukraine), (Lederberg, 1999) and the latest incident of dispersal of a bioweapon occurred in 2001, when anthrax spores were dispersed through postal mails in the USA (Atlas, 2002). Organisms that can spread swiftly in a population and are capable of causing severe morbidity and mortality have the potential to be developed into bioweapons. Other suitable features for a potential bioweapon are their adaptability to large scale manufacturing, easy dispersion as aerosols and environmental stability (Robinson-Dunn, 2002). A number of organisms have been listed as potential bioweapon agents and some of the more likely bacterial organisms to be employed are *Bacillus anthracis, Yersinia pestis, Francisella tularensis* (Cunha, 2002; Robinson-Dunn, 2002). The timely detection and identification of biowarfare agents is of extreme importance to prevent the spread and subsequent effects on a population. Detection and identification of bacterial organisms can be achieved based on properties unique to the whole cell, bacterial proteins, nucleic acids and/or other cellular components.

Nucleic acid detection techniques

A number of techniques have evolved for the detection of nucleic acids that may be adapted for bacterial identification. Most techniques rely on the presence of unique nucleic acid sequences within the genome, plasmid or RNA fraction of the organism. Although targeting the RNA fractions has obvious disadvantages, considering the wide distribution of RNases, RNA-based detection can be successfully achieved by maintaining an RNase-free environment or synthesizing complementary DNAs (cDNAs) by reverse transcription. Furthermore, RNA targets in general will be single stranded and present in higher copy numbers than their DNA counterparts. This is especially true for ribosomal RNA (rRNA). DNA targets, although present in relatively smaller copy numbers, are more stable than RNA and are less prone to form secondary structures, thereby making the hybridization reaction less complicated. One of the main advantages of nucleic acid based bacterial detection/identification techniques is that target nucleic acids can be amplified by polymerase chain reaction (PCR) thereby increasing the sensitivity several fold. Many new methodologies that circumvent the PCR step, but still maintain a high sensitivity have been reported. Nucleic acid based techniques may be categorized as optical, electrochemical or pieozoelectric (Junhui, Hong & Ruifu, 1997).

Optical Techniques for nucleic acid detection: Optical techniques include the use of fluorescently tagged nucleotide probes, surface plasmon resonance,

Raman spectroscopy etc. Fluorescent probes targeting specific 16S rRNA sequences have been used to identify uncultured bacteria in mesophilic and thermophilic sludge granules using confocal laser scanning microscopy (CLSM (Sekigichi et al., 1999) The granules were fixed in formaldehyde and exposed to freeze thaw cycles to allow probe penetration into the granule. Sections of the granule mounted on glass slides were hybridized for 10 hours and observed using CLSM. Fluorescent-labeled probes were also used in a similar study (Neef et al., 1998) to monitor planctomycetes in sludge and wastewater habitat. A sandwich-type DNA sensor described by Chen et al. (1998) employed two probes; one was labeled with biotin and the other labeled with calf intestine alkaline phosphatase (CAP). Streptavidin coated magnetic beads were used to separate the probe-bound targets, and their presence was detected subsequently based on enzymatic chemiluminiscence by the action of CAP on its substrate.

Raman scattering is another optical technique that has been employed for nucleic acid detection. It is based on the principle that irradiation of molecules leads to scattering of light, and a fraction of the scattered light may have a frequency different from the incident light producing a pattern that can be used to identify the molecule. Due to the low sensitivity of conventional Raman spectroscopy (Junhui, Z *et al*, 1997), novel methods like surface enhanced Raman scattering (SERS) and surface enhanced resonance Raman scattering (SERRS) have been developed. In these methods, molecules are adsorbed onto

surfaces like gold, silver or copper. Sensitivity has been reported to increase by 10^5 to 10^6 times using SERS and up to 10^{12} times using SERRS (Bell et al., 1996). SERRS has been used to identify labeled DNA (Graham, Mallinder & Smith, 2000). A detection limit of 2×10^{-12} M labeled DNA was attained.

Surface plasmon resonance (SPR) has been used for the label-free detection of nucleic acid hybridization events based on changes in refractive index. Kai et al. (1999) used biotin labeled probes immobilized on the surface of a sensor chip via avidin-biotin linkage to capture products of a PCR reaction and the hybridization event was monitored by SPR. Conventional SPR techniques are limited in that it is not possible to simultaneously detect multiple molecular interactions. Using a method called SPR imaging, it was possible to monitor multiple molecular events (Nelson et al., 2002). In this study, DNA probes against the 16S rRNA were used to identify total RNA isolated from *Escherichia coli* and *Bacillus subtilis*. A detection limit of 2µg/mL was achieved. A method combining microfluidic networks and SPR imaging (Lee, Goodrich & Corn, 2001) was utilized to detect DNA hybridizations in small volumes. It was possible to reduce the sample volulme to 1uL and attain a sensitivity of 20 fmol. Nilsson et al. (1995) evaluated the use of SPR in studying DNA hybridization kinetics and strand separation by immobilizing DNA probes via an avidin – biotin linkage on to the sensor surface and measuring changes in refractive index over time. Ostroff et al. (1999) developed a "Thin Film Biosensor for Rapid Visual Detection of Nucleic Acid Targets". A unique sensor surface that was antireflective to certain wavelengths

of visible light was utilized. Probes immobilized on the surface, following exposure to target sequences and a biotin-labeled detector-oligonucleotide results in the formation of a thin film on the surface increasing the optical thickness of the surface producing a visible color change. The assay can be interpreted both qualitatively and quantitatively. Changes of 10 to 20 A° in thickness could be detected. The sensitivity of the technique was claimed to be in the range of attomols of target nucleic acids in a time period of less than 30 minutes.

Electrochemical techniques for nucleic acid detection: DNA detection by electrochemical methods involves the immobilization of a DNA probe onto electrodes. Exposure of the probe - immobilized electrode to target sequences results in a hybridization event which is detected using electroactive indicators. Probe immobilization onto electrodes may be achieved by adsorption (Hashimoto, Ito & Ishimori, 1994), avidin-biotin linkage (Marrazza, Chianella & Mascini, 1999) or covalent attachment (Millan & Mikkelsen, 1993). Different kinds of electrodes like carbon electrodes (Brett et al., 1997), gold electrodes (Pang & Abruna, 1998), mercury electrodes (Fojta & Palecek, 1997) etc, can be used. The electroactive indicators used to detect the hybridization event usually have a higher affinity for double stranded DNA. The electrochemical properties of different indicators have been studied (Hashimoto et al., 1994). Wang et al. (1997) developed an electrochemical method to detect *Mycobacterium tuberculosis* DNA using Co $(Phen)_3^{3+}$as an indicator, reaching a detection limit of

3.4nM. Using other indicators like Ferrocenyl naphthalene diimide, very low detection limits in the femtomolar range were achieved (Takenaka et al., 2000). Other types of electrochemical indicators, wherein hybridization reduces the interaction between the indicator and DNA, have been described (Erdem et al., 2000). Methylene blue is one such indicator that associates with single stranded DNA by virtue of its affinity to exposed guanine bases. Upon hybridization with a complementary strand, dye association with guanine bases is blocked, resulting in a reduction of the electrochemical signal. Label-free electrochemical detection of DNA based on the intrinsic redox activity of guanine residues in target DNA has been described (Wang & Kawde, 2001). Another method, based on changes in electrical properties of the sensor, was employed by Krull et al. (2000) for the label-free detection of DNA hybridization. This method used surface stabilized bilayer lipid membranes on silver wires as transducers; probe sequences were immobilized onto the lipid membranes and hybridization was monitored based on the reduction in ion currents through the surface stabilized bilayer lipid following hybrization. The alteration in ion current was dependent on target concentration.

Other electrochemical approaches based on charge conduction through DNA (Kelley et al., 1999; Umek et al., 2001) and through capacitance measurement (Wei et al., 2003) have also been employed for the detection of DNA hybridization.

Piezoelectric sensors for nucleic acid detection: Piezoelectric sensors generally utilize quartz crystals as the sensing device (Junhui, Z *et al* 1997). DNA probe molecules are linked to the crystal and target binding on the probe alters the mass on the crystal thereby changing its frequency of oscillation. This change in frequency can be monitored for the presence of target. Piezoelectric sensors offer label-free real time monitoring and the possibility to develop a solid surface based detection device. Use of quartz crystal microbalance biosensors for the detection of sequences in the genome of genetically modified organisms in solution has been reported (Mannelli et al., 2003). Thiol derivatised DNA probes immobilized on the sensor surface were used to detect PCR products. The sensor was reusable for 20 measurements. Detection of M13 phage DNA using oligonucleotide immobilized on gold electrodes of a Quartz crystal microbalance (QCM) with a sensitivity of 1 pM was reported (Okahata et al., 1992). It has also been suggested to measure impedence of quartz crystal oscillators to monitor DNA behavior (Yamaguchi & Shimomaura, 1993), since the change in frequency is dependent on factors such as viscosity, elasticity and surface roughness in addition to the change in mass.

Other novel techniques for nucleic acids detection based on magnetoresistance technology (Baselt et al., 1998), isothermal amplification (Baeumner et al., 2003), up-converting phosphor technology (Zuiderwijk et al., 2003) and array –based electrical detection (Park, Taton & Mirkin, 2002) have been successfully attempted.

Molecular Beacons

Molecular beacons (MBs) invented by Tyagi & Kramer (1996), are stem-loop (hair pin) shaped oligonucleotides dual labeled with a fluorophore and a quencher (see page 52). The proximity of the two moieties renders the fluorophore in a quenched status when the molecular beacon is in its native stem loop state. Upsetting the stem loop structure by hybridization with a complementary oligonucleotide or by increasing the temperature will interrupt energy transfer from the fluorophore to the quencher, resulting in the emission of fluorescence. Hybridization with excess of complementary target sequences results in several fold increase in fluorescence. The stability of molecular beacons depends on the stem length, G-C content of the stem and concentration of magnesium ions. Bonnet et al. (1999) studied the thermodynamic basis for the enhanced specificity of molecular beacons. It was shown that in the presence of targets, molecular beacons can exist in three different phases – double helix (molecular beacon – target hybrid), hairpin and random coil. The enhanced specificity of molecular beacons is attributed to the presence of a hairpin phase between the transitions of a double helix to a random coil. In another study, Tsourkas et al. (2003a) attributed the enhanced specificity of molecular beacons compared to conventional linear probes to the "competing reaction between the hairpin formation and target hybridization". It was also shown that, as the stem length of the molecular beacon increases, the discriminatory ability to distinguish between true and false targets increases owing to a decrease in the free energy,

which corresponds to better stability. On the other hand, increasing the loop length of the molecular bacon decreases the specificity but increases target binding affinity. The advantages of molecular beacons are not limited to the enhanced specificity to target molecules. Compared to conventional unilabeled nucleic acid probes used in hybridization assays, molecular beacons provide the advantage that unhybridized probes need not be washed away prior to data collection, as only hybridized molecular beacons will emit fluorescence. This allows hybridization to be carried out in a solution based assay. Molecular beacons also provide an opportunity for the real time monitoring of hybridization events. Similar to linear probes, molecular beacons can be employed in a surface immobilized fashion. Fang et al. (1999) described the functioning of a molecular beacon on a solid interface immobilized by an avidin – biotin linkage. Attaching the biotin moiety towards the quencher side of the stem resulted in minimal interference with fluorescence and hybridization kinetics. An avidin-biotin linkage has also been employed to immobilize molecular beacons onto ultra small optical fibre probes with submicrometer dimensions (Liu et al., 2000). Efficient target discrimination and a sensitivity of 10 nM were attained. Molecular beacon micro arrays printed on agarose film – coated glass slides were shown to discriminate targets differing by a single base (Wang et al., 2002). Immobilization of molecular beacons onto controlled pore glass beads have also been successfull (Brown et al., 2000).

The mechanism of fluorescence quenching in molecular beacons does not conform to typical fluorescence resonance energy transfer (FRET). One of the requirements for FRET to occur is that the acceptor molecule (quencher) should have a spectral overlap with the emission of the donor molecule. On exciting the donor molecule, energy is transferred to the quencher in a non-radiative form and then dissipated as heat. In the native hairpin shape of molecular beacons, quenching occurs in the absence of any spectral overlap. This was explained by Bernacchi & Mely (2001). They suggested that the formation of a heterodimer by the fluorophore and quencher leads to vast changes in the absorption spectra. Marras, Kramer & Tyagi (2002) suggested contact quenching as the mode for native state molecular beacons. It has also been suggested that binding of the fluorophore moiety to the quencher influences quenching efficiency.

Molecular beacons have been utilized in a wide range of applications. One of the most important has been their use to monitor in real time the progress of polymerase chain reaction (Tyagi, Bratu & Kramer, 1998). Molecular beacons are synthesized such that they form stable duplexes with the amplicon during the annealing phase of PCR. The amplicon concentration can be assessed based on the fluorescence intensity at the annealing phase. The melting temperature of the stem region of the molecular beacons are ideally designed to be 7 to 10 °C above the annealing temperature (Mhlanga & Malmberg, 2001). This will ensure that fluorescence increase occurs as a result of hybridization and not due to melting of the stem. Maintaining the loop region to contain 18 to 25 bases so that

the melting temperature on hybridization with the amplicon is slightly higher than the annealing temperature helps to retain a stable hybrid during the annealing phase. Use of molecular beacons in the detection of single nucleotide polymorphisms have been reported by many researchers (Frei et al., 2002; Giesendorf et al., 1998; Hodgson et al., 2002; Kostrikis et al., 1998; Marras, Kramer & Tyagi, 2003; Mhlanga & Malmberg, 2001; Täpp et al., 2000; Tyagi et al., 1998). In most cases, the detection technique was coupled to a PCR step to amplify the target concentration. Tyagi *et al* (1998) performed a multicolor molecular beacon assay wherein molecular beacons targeting different sequences were labeled differently, allowing detection of multiple targets to be carried out in a single tube. Molecular beacons have also been used in the genotypic studies of microorganisms (Piatek et al., 2000) and also to study drug resistance and virulence in bacterial and protozoal organisms (Durand et al., 2000; El-Hajj et al., 2001; Piatek et al., 1998; Rhee et al., 1999). Sokol *et al* (1998) microinjected molecular beacons into living cells and observed DNA.RNA hybridizations in real-time with the aid of fluorescence microscopy, attaining a detection limit of 10 molecules of mRNA. Arnold, Tims & McGrath (1999) used molecular beacons to study the expression of proteins in breast cancer cells based on reverse transcriptase – PCR. Molecular beacons have also been employed to detect point mutations in lung cancer (Clayton et al., 2000). Pierce et al. (2000) used molecular beacons to detect Y chromosomes in single human blastomeres. Molecular beacons were also successfully employed in the detection of transgenes in crop plants (Kota, Holton & Henry, 1999). Expression

levels of mRNA have been studied in bacteria using molecular beacons (Larsen et al., 2002; Manganelli et al., 1999). One of the major applications of molecular beacons is their use in the detection of microorganisms. Successful detection of different bacteria (Belanger et al., 2002; Chen, Martinez & Mulchandani, 2000; Fang et al., 2002; Fortin, Mulchandani & Chen, 2001; Harms et al., 2003; McKillip & Drake, 2000; Poddar & Le, 2001), viruses (de Baar et al., 2001; Eun & Wong, 2000; Hibbitts et al., 2003; Jebbink et al., 2003; Klerks et al., 2001; Lanciotti & Kerst, 2001; Lewin et al., 1999; Poddar, 1999; Szemes et al., 2002; Van Beuningen et al., 2001; Vet et al., 1999; Yang et al., 2002; Yates, 2001) and fungi (Nascimento et al., 2003; Park et al., 2000) have been achieved using molecular beacons. Enzyme interactions and activity have been quantified and monitored using molecular beacons (Fang, Li & Tan, 2000; Strouse et al., 2000; Summerer & Marx, 2002). The interaction between lactate dehydrogenase and single stranded DNA was studied by Fang et al (2000), demonstrating their potential use in protein – DNA interaction studies. Many researchers have come up with molecular beacons specifically interacting with proteins. Such molecular beacons called molecular aptamer beacons have been described (Li, Fang & Tan, 2002).

Improvements over conventional molecular beacons have been suggested for increasing the sensitivity of detection. Tyagi, Marras & Kramer (2000) described the development of wavelength-shifting molecular beacons. In addition to the fluorophore (primary fluorophore) and quencher molecule held in close proximity

by the native hairpin conformation, a secondary fluorophore is attached to the primary fluorophore at a distance of 20 to 100 A°, so that there is efficient fluorescence resonance energy transfer between the two fluorophores. On denaturation of the hairpin structure, the primary fluorophore is no longer quenched. Excitation of the primary fluorophore leads to emission from the secondary fluorophore due to FRET between the two moieties. A mixture of many such molecular beacons with different secondary fluorophores but the same primary fluorophore and quencher can be used in assays. Such wavelength shifting molecular beacons have the advantage that with a single monochromatic light source that excites the primary fluorophore, emissions can be achieved from a number of beacons that have different secondary fluorophores. Joshi & Tor (2001) reported the functioning of molecular beacons with metal moieties in place of organic fluorophores and quenchers. Metal moieties, apart from having higher photostability, are less sensitive to changes in pH and have a longer excited state. Tsourkas et al. (2003b) reported a novel experimental design to increase the fidelity of molecular beacon hybridization assays. Two molecular beacons that bind to adjacent regions of the target sequences were developed. Hybridization of these molecular beacons results in a FRET interaction between the fluorophores of the target-bound molecular beacons due to their proximity and this can be monitored for presence of target sequences. The advantage of this technique is that fluorescence increase resulting from molecular beacon degradation or nonspecific interaction with target can be distinguished from a true hybridization event.

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Chapter III

Capillary Electrophoresis and Fluorescence Studies on Molecular Beacon-Based Variable Length Oligonucleotide Target Discrimination

ABSTRACT

Molecular beacons (MBs) are oligonucleotide probes having a compact hairpin structure, with a fluorophore attached to one end and a quencher molecule attached to the other end. In its native state, the fluorophore is quenched by virtue of its proximity to the quencher molecule. Upon hybridization with its complementary oligonucleotide target, fluorescence is elicited due to a conformational change that results in separation of the fluorophore and quencher molecule. The present study describes the hybridization interaction of a MB to various complementary target sequences. The effects of temperature and length of complementary target sequences on hybridization were investigated using capillary electrophoresis and solution based fluorescence techniques. Hybridization efficiency was dependent on the ability of the target sequences to destabilize the stem region by binding directly to the stem region. Optimal

hybridization occurred between 40 and 50°C for all targets tested, with the true target forming a more stable hybrid complex.

Introduction

Molecular beacons (MBs), first described by Tyagi and Kramer in 1996 (Tyagi & Kramer, 1996), are important probes for quantitative and qualitative genomic studies. MBs are single-stranded DNA (ssDNA) molecules, composed of a hairpin-shaped oligonucleotide (i.e., stem-and-loop structure) that is tagged with a fluorophore and a quencher molecule at either end (see Fig. 1), held close to each other by a stem portion. MBs act as molecular switches that turn fluorescence "off" when the fluorophore and quencher are in proximity, and turn fluorescence "on" when the fluorophore and quencher are separated due to a conformational change (Tyagi & Kramer, 1996). These on/off fluorescing properties have made MBs useful in many applications, including biosensors (Liu et al., 2000; Liu & Tan, 1999), protein studies (Fang, Li & Tan, 2000b; Li et al., 2000a), monitoring of RNA/DNA amplification during PCR (Tyagi, Bratu & Kramer, 1998; Tyagi & Kramer, 1996), gene typing and mutation detection (Giesendorf et al., 1998; Rhee et al., 1999), enzymatic cleavage assays (Li, Geyer & Tan, 2000b), real time detection of DNA-RNA hybridization in living cells (Sokol et al., 1998), the detection of pathogenic retroviruses (Vet et al., 1999), etc.; for recent reviews see Refs (Broude, 2002; Fang et al., 2000a).

The most specific molecular recognition of a given MB is based on its hybridization to a complementary target oligonucleotide sequence, which induces a conformational change, causing a separation of the fluorophore from the

quencher, allowing it to fluoresce (Bonnet et al., 1999; Tyagi & Kramer, 1996). The resulting fluorescence will be of varying intensity depending on the spatial arrangement of the quencher with respect to the fluorophore. Molecular beacons are highly sensitive probes and have been reported to detect subnanomolar concentrations of target in real time (Liu et al., 2000), which makes them useful in the detection of highly infectious and virulent bacteria. As a prelude to using MBs for the actual detection of organisms *in situ*, and keeping in mind the eventual application of this research, we studied the conformational behavior of a MB designed to target a unique sequence in the 16S rRNA of *Francisella tularensis*, a highly infectious bacterial organism and a potential bioweapons agent (Christopher et al., 1997; Franz et al., 1999). The 16S rRNA forms the structural and functional unit of prokaryotic ribosomes, which are present on the order of approximately ten thousand per bacterial cell. In an average *Escherichia coli* cell, ribosomes vary in number from 6700 to 71,000 depending on the growth conditions (Neidhardt & Curtiss, 1996), making rRNA a favorable target for bacterial detection. Specificity and temperature-stability of the beacon were assessed using solution fluorescence and capillary electrophoresis (CE). While increased solution fluorescence implies an opening up of the hairpin structure upon hybridization; capillary electrophoresis provides additional evidence of such a structural event occurring.

Materials and Methods

Targets: All genebank entries for the 16s rRNA sequence of *Francisella* spp. were pooled and a consensus sequence was made using the software Vector NTI (Informax, Inc. Bethesda, MD, U.S.A.). Using the BLAST program (Altschul et al., 1990), four regions were identified in the consensus sequence that contained oligonucleotide sequences unique to *Francisella* organisms. A 20 base segment (CS11, see Table 1) derived from one of these sequences, (gccttgggggaggacgttac-Gene bank accession number Z21931, bases 454-473), was used as the true target in this study. Affinity of other sequences (CS12 to CS17, see Table 1) that were only partially complementary to the MB probe were also investigated.

Target sequences were synthesized at the core facility at Oklahoma State University, Stillwater, OK, U.S.A. For fluorescence assays, 800 nM solutions of the target were made in 10 mM Tris/3.5 mM $MgCl_2$ buffer, pH 8, and for CE studies, 160 μM solutions were used.

Molecular Beacon: A molecular beacon (HP1, Table 1) was designed to probe the true target sequence (CS11), using the mfold program (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/) (SantaLucia, 1998) and the DNA fold option in the RNA structure software (Mathews et al., 1999; SantaLucia, 1998) (http://128.151.176.70/RNAstructure.html). The MB was

designed to have a 20 base loop region that was complementary to the true target, a 4 bp stem, of which 2 bases were complementary to the true target, a Cy3 fluorescent group attached to the 5' end, and a black hole quencher (BHQ) molecule attached to the 3'end (see Fig. 1). The MB was obtained from IDT Inc. (Coralville, IA, U.S.A). For fluorescence studies, 400 nM solutions in 10mM Tris/3.5 mM $MgCl_2$ were used and roughly 80 μM solutions were used for capillary electrophoresis.

Modified beacons that had a fluorescent group (Cy3) but no quencher (IDT Inc.) were used to determine correction factors for temperature-dependent effects on fluorescence that are independent of hybridization.

Fluorescence studies: Hybridization was performed in triplicates in a 96-well microtiter plate and fluorescence was read using an ABI 7700 instrument (Applied Biosystems, Foster City, CA, U.S.A.). Volumes of 25 μL each of the molecular beacon and individual targets were mixed in a 96 well microtiter plate to give a final concentration of 200 nM and 400 nM, respectively, in a total volume of 50 μL per well. Three wells were also dedicated to the modified beacon (beacon without a quencher), which was dispensed in the same concentration as the beacon (200 nM) at a final volume of 50μL per well. Measurements were taken at 5°C decrements ranging from 90°C to 5°C at an exposure time of 25 ms. To account for the temperature-effect, fluorescence at different temperatures from all wells involving the molecular beacon was divided

by the average fluorescence from the modified beacon at the corresponding temperature.

Capillary electrophoresis: Two different instruments were used in the CE studies. One of the instruments was assembled in-house from commercially available components. It consisted of two 30 kV DC power supplies of positive and negative polarity, Models MJ30P400 and MJ30N400, respectively, from Glassman High Voltage (Whitehouse Station, NJ, U.S.A.) and a UV-Vis variable wavelength detector Model 200 from Linear Instrument (Reno, NV, U.S.A.) equipped with a cell for on-column detection. The electropherograms were recorded with a Shimadzu data processor Model CR6A (Kyoto, Japan). Fused-silica capillaries of 50 μm I.D. and 360 μm O.D. were from Polymicro Technologies (Phoenix, AZ, U.S.A.) The second CE instrument was a Model 5510 Beckman P/ACE instrument (Fullerton, CA, U.S.A.). The instrument was equipped with a diode array detector, a personal computer and P/ACE station software for data handling purposes.

Capillary electrophoresis studies were performed in capillaries maintained either at room temperature or at fixed temperatures ranging from 15 to 50°C in order to investigate conformational changes occurring in molecular beacons in the presence and absence of target sequences. In all cases, the final concentration of HP1 was ~40 μM and that of targets were ~80 μM in 10mM Tris/3.5 mM $MgCl_2$ solution. Specifications for the room-temperature run were as follows:

capillary, fused silica tube, 50 cm (inlet-to- detector distance), 65cm (total length) x 50 μm inner diameter; running buffer, 50mM phosphate, pH 7.0; running voltage, +25 kV; injection, 30s by hydrodynamic injection from a height of 20 cm above the outlet reservoir. Four targets were used in this study; namely, CS11, CS14, CS17 and CS21. CE was performed with each of the targets alone (target control), the MB alone (beacon control) and the reaction mix containing target and molecular beacon.

Specifications for the multiple-temperature runs were as follows: capillary, fused silica tube, 50cm (inlet-to-detector distance), 57 cm (total length) x 50μm inner diameter; running buffer, 50mM phosphate, pH 7.0; running voltage, +20 kV; pressure injection at 20 psi for 10s. The temperatures considered were 15, 20, 30, 40 and 50°C. Only two targets; namely, CS11 and CS14 were studied. Control runs were performed as for the CE experiments at room temperature.

Results and Discussion

Fluorescence studies: Fluorescence readings from the molecular beacon in the presence or absence of various targets at different temperatures were divided by the fluorescence readings from the modified beacon without a quencher at the corresponding temperatures to correct for temperature effect on fluorescence independent of the hybridization event. Figure 2 shows the fluorescence pattern of the molecular beacon observed at temperatures ranging from 5°C to 90°C

following hybridization with the different targets. Fluorescence produced as a result of the true target (i.e., CS11) hybridization was the highest. It is noteworthy that the sequence CS12 that binds to the loop sequence alone was ineffective in producing a high fluorescence, but the shorter sequence CS14, which binds to three bases in the stem region, produces a fluorescence that is comparable to the fluorescence resulting from binding of the true target CS11. CS15 which was of the same length as CS14 but complementary to only one base in the stem, and CS17 which was only 7 bases long but complementary to all bases but one in the stem, did not produce a significantly high fluorescence. It is believed that the chance of single stranded DNA folding on itself to form a secondary structure (i.e., stem-and-loop structure), which is an intramolecular event, is much higher than binding to other smaller DNA molecules. Moreover, the rigidity of the probe-target helix thus formed by the hybridization of a small DNA molecule to the stem region of the probe will not be sufficient to introduce a conformational shift of the quencher away from the fluorophore resulting only in a minimal emission of fluorescence. The cases with the other target sequences, CS13 and CS16, were the same, each giving a small signal comparable to the background signal. CS21 was a non-complementary sequence and was not expected to produce any fluorescence. Thus, the specificity of an MB towards target oligonucleotides comes from its loop-and-stem structure (Fang et al., 2000a). These results show that for a significant fluorescence to be produced, the hybridizing target molecules must not only bind to a significant portion of the loop, but must also destabilize the stem.

It is interesting to note that above 70°C, all the fluorescence lines converge more or less to the same fluorescence level including the molecular beacon HP1 by itself. This suggests that the steady increase in fluorescence is due to the melting of the stem with increasing temperature and the resultant displacement of the quencher away from the fluorophore. On the other hand, partial- and complete-complementary targets hybridize to the MB resulting in the beacon 'opening up' to various degrees at lower temperatures. As the temperature increases the fluorescence produced increases and reaches a maximum around 45°C for CS11 (the true target). For CS14, the fluorescence dips earlier, around 40°C, because the hybrid is less stable. All other sequences fail to produce as high a rise in fluorescence at any temperature. The optimum temperature for hybridization seems to be between 40 °C and 50 °C for this particular molecular beacon.

Capillary electrophoresis: Based on the fluorescence data, different target sequences were selected to be analyzed using CE and further understand the hybridization behavior. The migration rate of a particle through the capillary column is dependent on the charge-to-mass ratio and on the shape of the molecule. Since the charge-to-mass ratio is almost the same in all DNA the main factor here will be the alteration in the shape of the molecule. Therefore, in principle, CE should be a simple and useful technique that complements fluorescence studies for investigating changes upon probe binding to targets

requiring only minute amounts of materials. Moreover, the CE approach does not require labeling the MB with fluorophore and quencher, instead it relies directly on physical properties of the MB and hybridization products; thus, potentially greatly decreasing the expense for MB synthesis.

Hybridization studies by CE: Based on the fluorescence data, four targets were selected for CE. Figure 3 shows the electropherograms for the molecular beacon HP1 (Fig. 3a), the targets CS11, CS14, CS17 and CS21 (see Fig. 3b, 3c, 3d and 3e, respectively) and the respective MB-target interactions (see Fig. 3f through Fig 3i) obtained by UV detection (see materials and methods). Although the targets CS11, CS14, CS17 and CS21 are composed of 20, 16, 7 and 23 bases, respectively, they migrated within a narrow time window extending from 6.9 min to 8.8 min (see Figs 3b through Fig. 3e). This is because the target oligonucleotides have approximately the same charge-to-mass ratios in the electrophoretic medium used in this study. As expected, and in accordance with the fluorescence data, HP1 hybridizes almost completely with CS11 and CS14 as evidenced by the single peak obtained for the hybridization reactions in Figs 3f and 3g, respectively. For the CS17 and CS21 targets, little or no hybridization has taken place with HP1. This can be seen in Figs 3h and 3i, where no new peaks have appeared on the electropherograms. Under these conditions, the peak heights of HP1 and the targets in Figs 3a, 3d and 3e should in principle stay the same as their corresponding peaks in Figs 3h and 3i. The observed slight

deviation in the peak heights can be attributed to the imperfection in the sample introduction on the in-house built manual instrument used.

Effects of temperature: To gain further insight into the extent of hybridization, the effects of temperature on the hybridization of CS11 or CS14 with HP1 were studied by CE in the temperature range 15 to 50 °C. The theoretical melting temperatures of the probe-target hybrids (i.e., HP1-CS11 and HP1-CS14) as determined by the 'Hybridization Server' developed by Michael Zuker (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form6.cgi), was found to be around 72°C in both cases. The CE instrument used in this study had a temperature limit of 50°C. Hence we expected the probe and target to remain in the hybridized state at all temperatures tested. The migration patterns of the MB (i.e., HP1), targets and the hybridizations as well as the conformational changes occurring in each case with changes in temperature are shown in Fig. 4.

In order to correlate probable conformations and CE elution peaks, we first generated the possible secondary structure predictions for each oligonucleotide under investigation, namely, the target sequences, CS11 and CS14, as well as the probe HP1, using the DNA mfold server by Michael Zuker (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/) at 15 and 50 °C and a Na^+ concentration of 0.05 M using the default settings on the server. The results of the structural conformation predictions are shown in Fig. 5. While HP1 is most likely to exist in a single conformation at 15 °C (ΔG = -3.8 kcal/mole), a second

but less stable conformation seems to be possible at 50 °C (ΔG = 0.7 kcal/mole). In fact, a single and relatively sharp CE peak is obtained for HP1 at all temperatures studied except at 50 °C (see Fig. 4) where the peak becomes broader indicating the possibility of two conformations: the stem-and-loop structure (ΔG = -0.2 kcal/mole) and a more linear conformation due to the melting of the secondary structure at 50°C. The conservation of the stem-and-loop structure even at 50 °C may be viewed as an important feature of the molecular beacon HP1 in the sense that it conserves its molecular recognition conformational energetics towards complementary targets over a wide range of temperature.

The target CS11 seems to assume 4 stable conformations at 15 °C and 5 less stable conformations at 50 °C. This is well observed upon CE analysis at various temperatures as shown in Fig. 4. The few peaks observed at low temperatures converge into one major peak at 50 °C, indicating that CS11 may become linear at 50 °C due to the melting of the secondary structure at this relatively high temperature. This fact may explain why in fluorescence studies, the HP1-CS11 hybridization is most effective at about 40 °C, the temperature at which maximum fluorescence is observed. Also, the target CS14 can assume 4 conformations at 15 °C and 5 less stable conformations at 50 °C. Being a shorter molecule, the various conformations of CS14 appear similar to one another (see Fig. 5c). This may explain why only two distinct peaks are seen for CS14 at low temperature, which converge into one single major peak at 50 °C. Again, as the temperature

approaches 50 °C CS14 exists most probably as a linear molecule, thus explaining the higher fluorescence of the HP1-CS14 at 40 °C. Using the above-mentioned 'Hybridization Server' by Michael Zuker, the formation of stable target-target dimers in the case of both CS11 and CS14 were ruled out at the temperatures studied. As expected, in all cases and in the temperature range studied, the hybridization reactions yield one major peak as can be seen in Fig. 4. The linear configuration of the hybridization product makes the counter-directional mobility of HP1-CS11 or HP1-CS14 with respect to the electroosmotic flow (EOF) higher than the individual oligonucleotides HP1, CS11 or CS14, and consequently the apparent mobility of the hybridization product (i.e., HP1-CS11 and HP1-CS14) is slower. It should be noted that in all these hybridization reactions the molar ratio of target to HP1 was 2:1. However, very little residual of the target molecules (i.e., CS11 and CS14) remained after hybridization, which does not amount to half the initial amount offered. This may reflect two discrepancies: the original concentration as stated by the supplier of the oligonucleotides may not have been very accurate and/or sample evaporation during the experiment may have introduced some alteration to the actual concentration especially considering that only a few microliters (~ 25 µL) were available for the CE experiments.

In all cases, increasing the temperature of the electrolyte decreases the viscosity of the medium, thus increasing both the electrophoretic mobility of the analyte and the electroosmotic mobility of the medium and the net result is a decrease in

the migration time of the individual oligonucleotides and the hybridization products. From Fig. 4 it can be seen that the probe-target hybrid is stable at all temperatures and is represented by a single peak, which denotes the presence of only a single entity in accordance with the fluorescence data.

Conclusions

The results of the fluorescence and CE studies indicate that the molecular beacon can effectively discriminate between true and false targets. CE elution profiles correlated well with the different structural forms that the oligonucleotides may assume at various temperatures and they also validated the results obtained by fluorescence studies. This study has demonstrated the potential of CE in the field of molecular biology for assessing hybridization reactions and structural changes occurring in DNA molecules as well as performing simultaneous separation assays. CE also has the advantage that unlabelled molecular beacons can be used for hybridization studies, thereby reducing the expenses involved and the amount of material used.

Acknowledgements

We thank Dr. O.H. Spivey (Dept. of Biochemistry and Molecular Biology, OSU, Oklahoma) and Dr. S. Tyagi (PHRI, NY) for advising us on fluorescence intensity correction. We also thank Dr. Tyagi for his valued input on the use of the ABI prism 7700 SDS instrument for fluorescence detection. The technical assistance of William Wall in some CE measurements is greatly appreciated.

We acknowledge the financial support of the Oklahoma Center for the Advancement of Science and Technology (OARS Program), Grant No. 5849.

Figure 1a. Structure of Molecular Beacon (HP1)

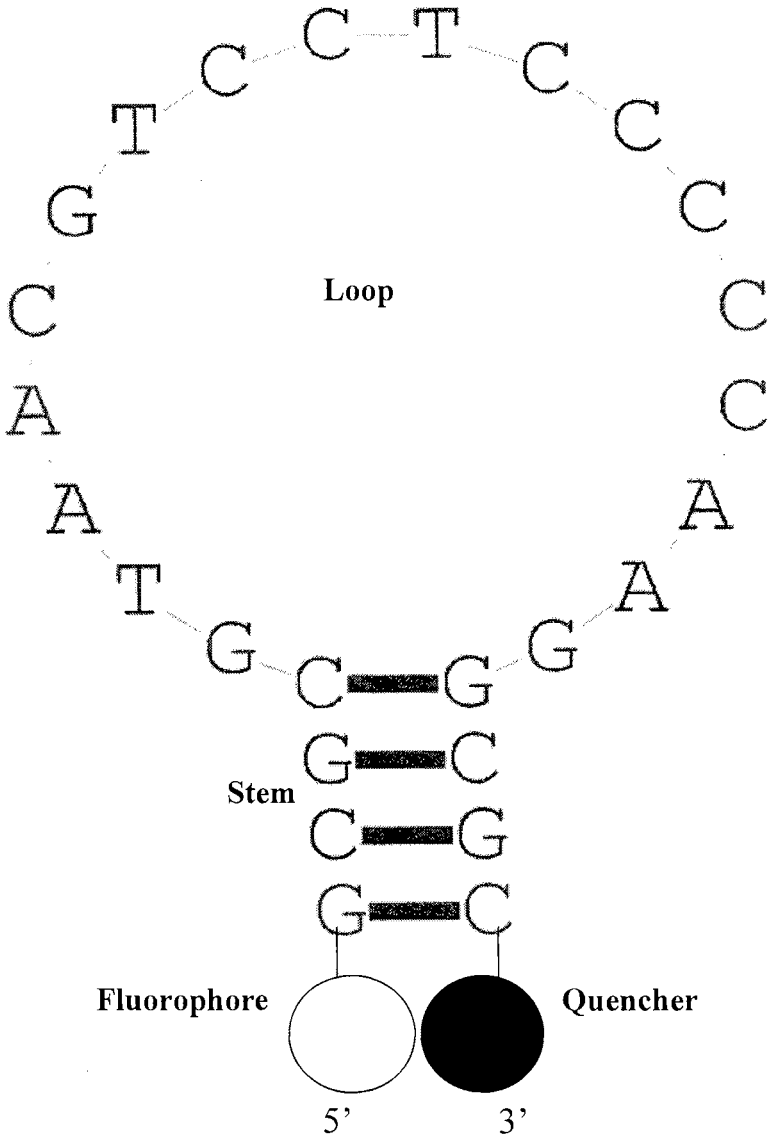

C C T C C C C C C A A A T G C G A A
Loop
Stem
G C
C G
G C
Fluorophore
Quencher
5'
3'

Figure1b. Mechanism of MB-DNA probe operation. Binding of MB to a target oligonucleotide sequence results in a conformational shift that moves the quencher (solid circle) away from the fluorophore (open circle) thereby allowing it to fluoresce.

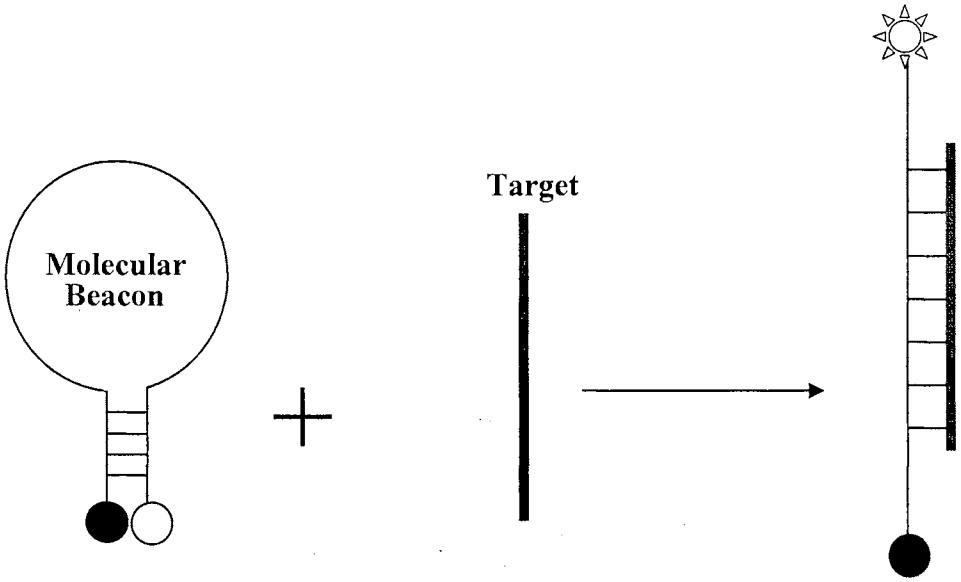

Molecular
Beacon
Target

Figure 2. Fluorescence signals of HP1 and its hybridization product with various oligonucleotide targets as a function of temperature.

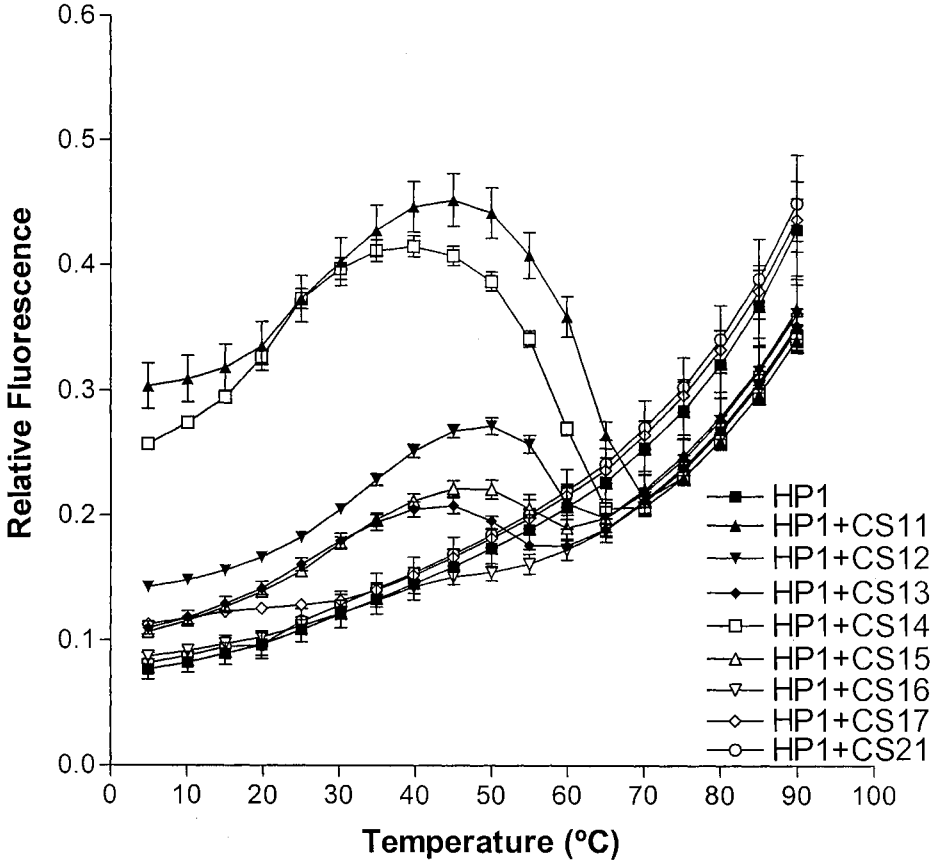

Relative Fluorescence
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100
Temperature (°C)
HP1
HP1+CS11
HP1+CS12
HP1+CS13
HP1+CS14
HP1+CS15
HP1+CS16
HP1+CS17
HP1+CS21

Figure 3. Electropherograms of the MB under investigation, the oligonucleotide targets, and their hybridization products. Conditions: fused-silica capillary, 50 cm (to detector), 65 cm (total length) x 50 μm i.d.; running buffer, 50 mM phosphate, pH 7.0; running voltage, +25 kV; 30 sec hydrodynamic injection from a height of 20 cm above the outlet reservoir. Detection wavelength 260nm. (a), HP1; (b), CS11; (c), CS14; (d) CS17; (e), CS21; (f), HP1+CS11; (g), HP1+CS14; (h), HP1+CS17; (i), HP1+CS21.

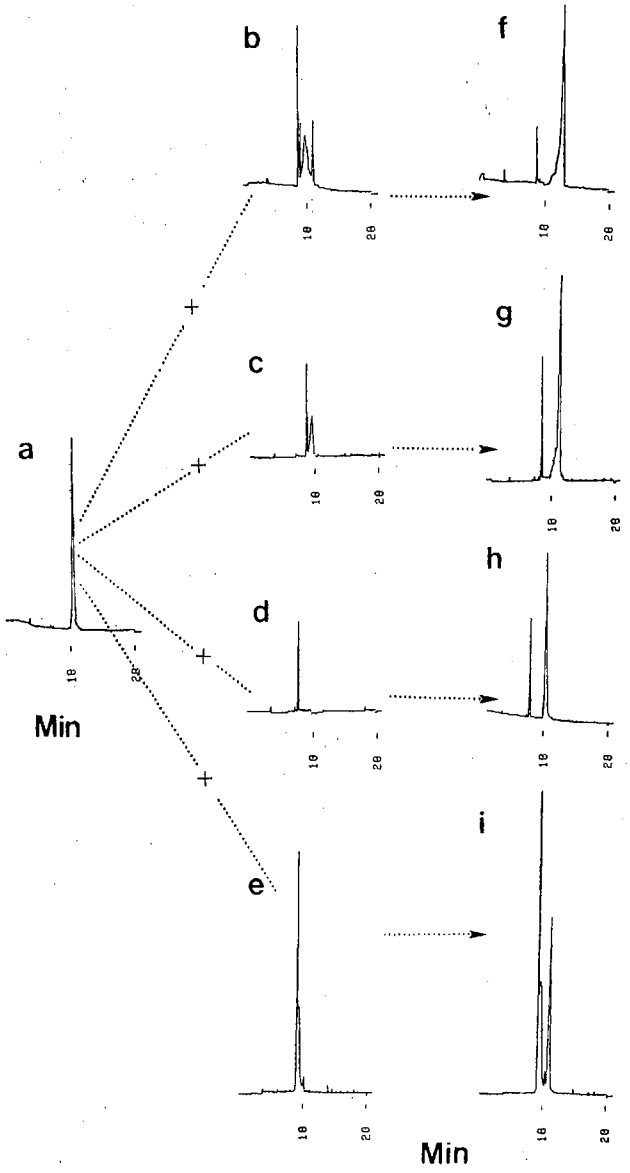
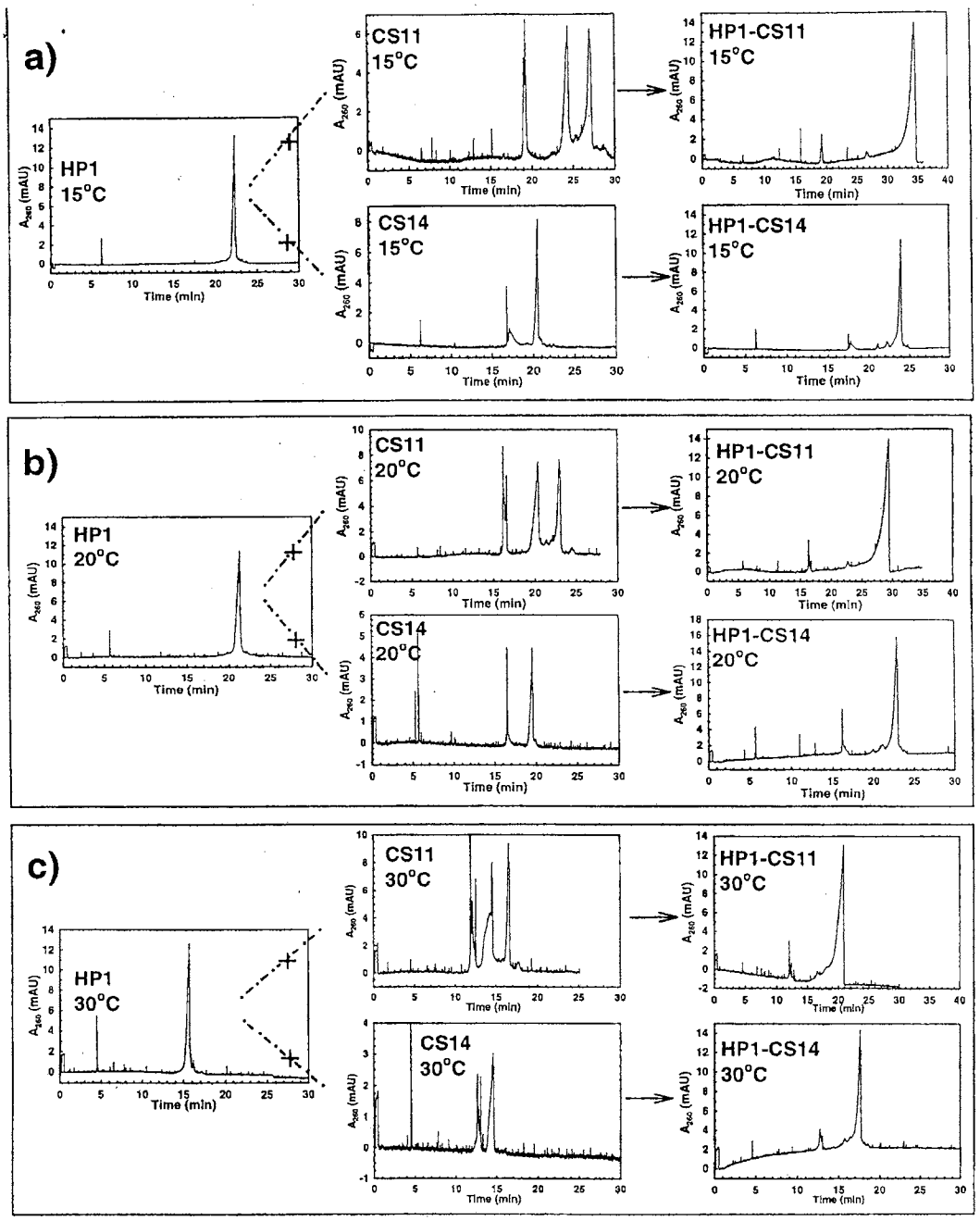

a
b
c
d
e
f
g
h
i
10
20
Min
Min

Figure 4. Electropherograms of the MB under investigation, the oligonucleotide targets and their hybridization products. Conditions: fused-silica capillary, 50 cm to detector, 57 cm (total length) x 50 μm i.d.; running buffer, 50 mM phosphate, pH 7.0; running voltage, +20 kV; pressure injection at 20 psi for 10 sec. Detection wavelength 260nm. (a), 15 °C; (b), 20 °C; (c), 30°C; (d), 40°C; (e), 50 °C.


a)
HP1
15°C
CS11
15°C
HP1-CS11
15°C
CS14
15°C
HP1-CS14
15°C
b)
HP1
20°C
CS11
20°C
HP1-CS11
20°C
CS14
20°C
HP1-CS14
20°C
c)
HP1
30°C
CS11
30°C
HP1-CS11
30°C
CS14
30°C
HP1-CS14
30°C
Time (min)
A260 (mAU)

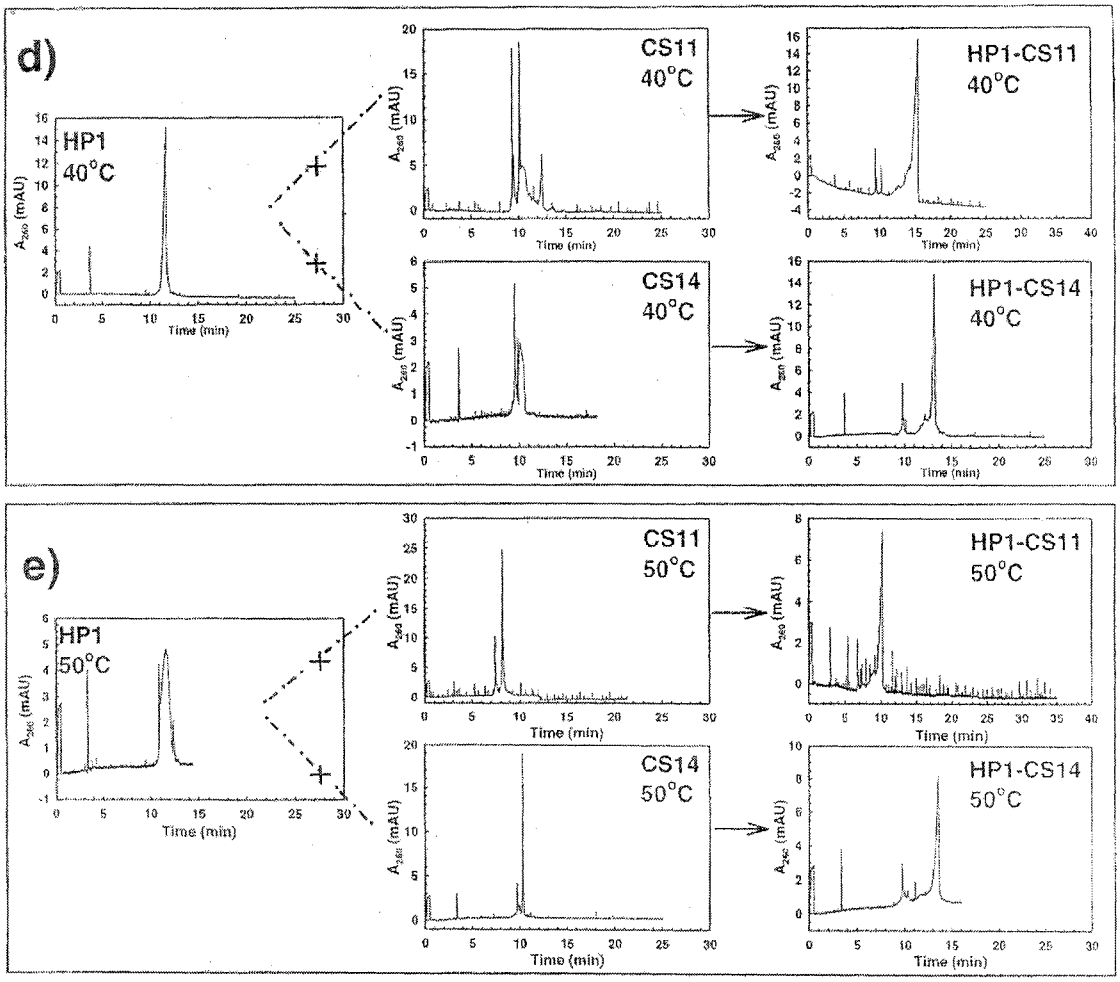
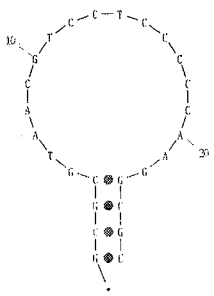
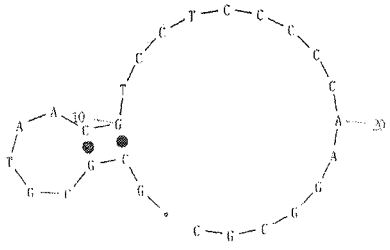

d)
HP1
40°C
CS11
40°C
HP1-CS11
40°C
CS14
40°C
HP1-CS14
40°C
e)
HP1
50°C
CS11
50°C
HP1-CS11
50°C
CS14
50°C
HP1-CS14
50°C
Time (min)

Figure 5. Secondary structure prediction of the probe HP1 (a) as well as that of the target sequences CS11 (b) and CS14 (c) at 15 and 50 °C and their associated free energy changes.

a)

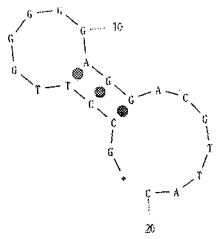


ΔG = -3.8 kcal/mole at 15 °C
ΔG = -0.2 kcal/mole at 50 °C

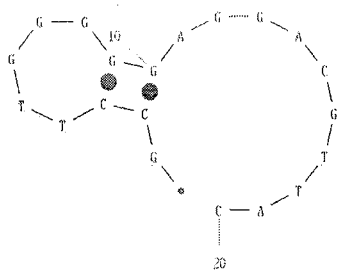


ΔG = 0.7 kcal/mole at 50 °C

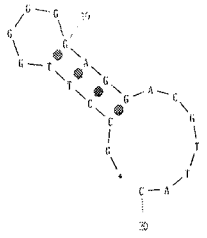
b)



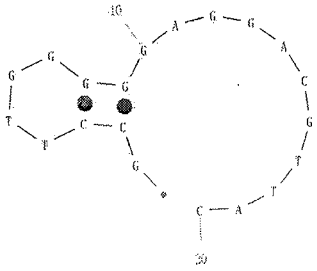
ΔG = -2.2 kcal/mole at 15 °C
ΔG = 0.8 kcal/mole at 50 °C



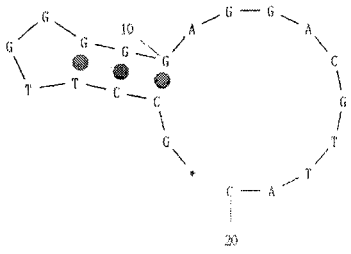
ΔG = -1.9 kcal/mole at 15 °C
ΔG = 0.5 kcal/mole at 50 °C



ΔG = -1.8 kcal/mole at 15 °C
ΔG = 1.0 kcal/mole at 50 °C

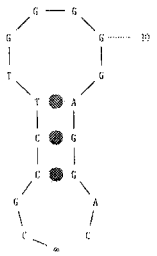


ΔG = -1.4 kcal/mole at15 °C
ΔG = 1.2 kcal/mole at 50 °C

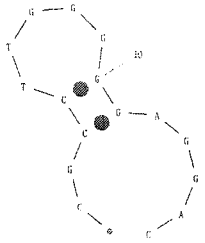


ΔG = 1.5 kcal/mole at 50 °C

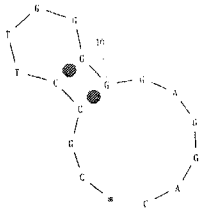
c)



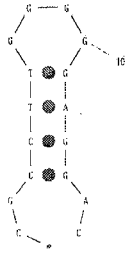
ΔG = -2.2 kcal/mole at 15 °C
ΔG = 0.8 kcal/mole at 50 °C



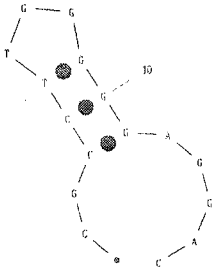
ΔG = -1.9 kcal/mole at15 °C
ΔG = 0.5 kcal/mole at 50 °C



ΔG = -1.4 kcal/mole at 15 °C
ΔG = 1.2 kcal/mole at 50 °C



ΔG = -1.8 kcal/mole at 15 °C
ΔG = 1.0 kcal/mole at 50 °C



ΔG = 1.5 kcal/mole at 50 °C

Table 1. Alignment of variable length target sequences across the length of beacon (HP1)

Description	Target sequence alignment with probe	No: of bases
HP1 probe *	3'-BHQ-**CGCG**GAACCCCCTCCTGCAATG**CGCG**-Cy3-5'	26
CS11 (True target)	5' GCCTTGGGGGAGGACGTTAC 3'	20
CS12	CTTGGGGGAGGACGTTAC	18
CS13	TTGGGGGAGGACGTTA	16
CS14	CGCCTTGGGGGAGGAC	16
CS15	CCTTGGGGGAGGACGT	16
CS16	GGGGGAGGACG	11
CS17	TTACGCG	7
CS21(non-complementary)	AGCTGTTGGATTCGGTGTAAAGG	23

* Bold underlined bases denote the stem of the molecular beacon. Italicized bases at the 3' end represents true-target binding sites within the stem

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Chapter IV

Target Discrimination by Surface-Immobilized Molecular Beacons Designed to Detect *Francisella tularensis*

Abstract

A molecular beacon array was designed based on unique regions of the 16s rRNA of the bacterium *Francisella tularensis*. Nucleic acid molecular beacons undergo a spontaneous fluorogenic conformational change when they hybridize to specific complementary targets. The array was printed on aldehyde glass or hydrogel slides and evaluated for functioning in presence of complementary oligonucleotide sequences, single nucleotide mismatch sequences and multiple nucleotide mismatch sequences. Discriminating true target from mismatched targets was found to be dependent on type, number, and location of mismatches within the beacon (i.e. located in the stem or loop regions). Optimal conditions for molecular beacon deposition, and target hybridization were determined for oligonucleotide target mismatch discrimination. The beacon array was stable upon recharging by exposure to an alkaline solution, and repeatedly used. In addition, performance of the beacon array biosensor was compared with molecular beacons in homogeneous solution.

Introduction

Management of and deterrents to disease outbreak require (Scheller et al., 2001) real-time *in situ* detection of suspect pathogens. Minimally, a detector should be sensitive to sub-infectious levels of various pathogens, possess high specificity, handle high throughput, and be reusable (Scheller et al., 2001). Tyagi and Kramer (Tyagi & Kramer, 1996) described a novel DNA molecular construction referred to as a molecular beacon (MB) that utilizes a novel design of fluorescence energy transfer, and is a good candidate for use at a pathogen-detector interface. MBs can recognize and report the presence of specific nucleic acids in homogeneous solutions, and more recently have been incorporated on various immobilizing platforms (Broude et al., 2001; Brown et al., 2000; Fang et al., 1999; Frutos et al., 2002; Liu et al., 2000; Liu & Tan, 1999; Riccelli et al., 2001; Steemers, Ferguson & Walt, 2000; Wang et al., 2002).Typically, molecular beacons are 15-25 nucleotides in length that energetically favor forming a hairpin (e.g. stem-loop) secondary structure (see Figure 1) with a fluorophore and quencher attached to opposing ends. In the absence of hybridization to a complementary target the fluorescence is quenched due to the close proximity of the fluorophore and quencher moieties. Upon hybridization of a complementary nucleic acid target to the MB probe a spontaneous conformational change disrupts the stem structure resulting in separation of the fluorophore and quencher, interrupting energy transfer with restoration of fluorescence. Various applications of MB have been described including real-time detection of DNA-

RNA hybridization in living cells (Sokol et al., 1998), detection of pathogenic retroviruses (Vet et al., 1999), monitoring enzymatic cleavage processes (Li, Geyer & Tan, 2000), probing interactions of single-stranded DNA binding to enzymes (Fang, Li & Tan, 2000), detection of DNA or RNA within integration amplification systems (Kaboev et al., 2000; Leone et al., 1998), and discrimination of alleles and single-nucleotide polymorphisms (Mhlanga & Malmberg, 2001; Tyagi, Bratu & Kramer, 1998).

For molecular beacons to be used at a pathogen-detector interface, it is convenient to first immobilize the probe on a solid platform. In recent years, miniaturized hybridization assays have been developed with positional separation and immobilization of nucleic acid fragments on microscope glass slides forming microarrays (Epstein & Butow, 2000 and references therein; Kato-Maeda, Gao & Small, 2001 and references therein). Microarrays allow multiplex processing, and simultaneous screening of thousands of unique nucleic acid fragments, and possibly may be adopted into a real-time reusable detector format. Researchers have made progress toward surface immobilization of molecular beacons on a microarray format using biotin-avidin coupling (Fang et al., 1999), or aldehyde coupling of free amino groups (Broude et al., 2001) (Wang et al., 2002). Approaches have been proposed to improve immobilized molecular beacon sensitivity by increasing the number of probes bound to the glass slide by first treating the slides; for example, treatment by photo- or persulfate-lithographic linked acrylamide-containing gels (Vasiliskov et al., 1999),

dendrimeric linker systems (Beier & Hoheisel, 1999), or agarose films (Afanassiev, Hanemann & Wolfl, 2000; Broude et al., 2001; Wang et al., 2002).

Typically, microarray methods have employed fluorescence labeling of the target to be detected. Instead, pathogen detection in the environment requires adoption of a platform in which the reporting fluorescence is associated with the immobilized probe, and fluorescence signaling elicited upon hybridization of the probe with specific unlabeled targets in real-time. Indeed, other researchers have also recognized possible application of immobilized molecular beacons for detecting unlabeled cognate oligonucleotide targets (Brown et al., 2000; Fang et al., 1999; Steemers et al., 2000; Wang et al., 2002). Miniaturized beacon immobilization combined with fiber-optic techniques (Liu & Tan, 1999; Steemers et al., 2000) has been shown to selectively detect genomic cystic fibrosis targets, and has been applied to the analysis of specific gamma-actin mRNA sequences.

In this paper, we evaluate a molecular beacon designed to detect *Francisella tularensis* based on a unique 16S rRNA subunit nucleotide sequence. Ribosomal RNA being naturally amplified sequences, are ideal target molecules for a probe based detection system. *Francisella tularensis* is a potential biological weapons agent and a highly infectious bacterium known to cause severe systemic illness in a variety of mammals including humans. In a previous work (Ramachandran et al., 2003), we evaluated the specificity of the molecular beacon to variable length targets in solution using capillary electrophoresis and solution based

fluorescence experiments. The MB probe, which possesses a primary amine attached to its 3' -end *via* a C_6 linker strand, was adapted to a microarray platform. Immobilization on commercially available aldehyde coated glass slides and hydrogel-aldehyde slides was achieved *via* a covalent linkage of the primary amino groups with the reactive aldehyde groups resulting in a Schiff base formation. Fluorescence discrimination ratios and specificity of the true target against targets with one, two, three, or four mismatches were investigated on the microarray platforms and compared to beacon performance in homogeneous solution.

Materials and methods

Molecular Beacon and Target Designs Based on *Francisella* spp: All genebank entries for the 16S rRNA subunit sequence of *Francisella* spp. were pooled and a consensus sequence was made using the software Vector NTI (Informax, Inc. Bethesda, MD, U.S.A.). Using the BLAST program (Altschul et al., 1990), four regions were identified that contained oligonucleotide sequences unique to *Francisella* organisms. Two sequences (HP1 and HP2) derived from two of these regions were shown to energetically favor a stem-loop structure as predicted by mfold program, and RNA/DNA structure software (Barciszewski & Clark, 1999; Mathews et al., 1999; SantaLucia, 1998) (http://bioinfo.math.rpi.edu/~zukerm). The HP1 MB was designed with an 18 base

loop region that was complementary to a true target, a 4 bp stem of which 2 bases were complementary to the true target on the 3' end, and a Cy3 fluorescent group attached to the 5' end. An additional thymidine was attached to the 3' end of the oligonucleotide probe to which a black hole quencher (BHQ-2) molecule was covalently linked to the 3' position of the deoxyribose, and a C_6 linker strand ending in a primary amino group was covalently linked to the 5-methyl group of the thymine aromatic ring (see Figure 1 and Table 1). The C_6 linker strand was omitted for hybridization studies in solution. Molecular beacons (HP1 and HP2 with and without the C_6 linker strand) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A). True target oligonucleotides (CS11 and CS27), and various mismatch targets against HP1 were obtained from the Core Facility at Oklahoma State University, OK, U.S.A. CS11 and CS27 are complementary in sequence to HP1 and HP2, respectively (Table 1). HP2 and CS27 were used as controls. Single, double, triple, and quadruple target mismatches were constructed as given in Tables 1 and 2. Mismatches were strategically placed in or near regions corresponding to the probe stem segment to which the true target was also designed to partially hybridize, or in the center of the loop region. Purines were replaced by other purines (G to A, A to G), and pyrimidines by other pyrimidines (C to T, T to C). Molecular beacons were synthesized in 250 nmole and targets in 100nmole scales. Molecular beacon stock solutions with a concentration of 200 μM were prepared using doubly distilled sterile water, divided into 10 μL aliquots, and stored at -20°C until use. Target oligonucleotides were similarly prepared and

stored, except 400 μM stock solutions were prepared using 10 mM Tris/3.5 mM $MgCl_2$ buffer (pH 8).

Printing Molecular Beacon Microarrays: SuperAldehyde slides were purchased from TeleChem, International, Inc. (Sunnyvale, CA), and hydrogel-aldehyde activated slides were obtained from NoAb BioDiscoveries (Mississauga, Ontario, Canada). A robotic printer (PixSys5500, Genomic Solutions, Inc., Ann Arbor, MI, U.S.A.) equipped with a quill pin was used to print molecular beacon arrays on to the superaldehyde or hydrogel-aldehyde slides. Molecular beacon stock solutions were first thawed, and then diluted to a concentration of 50μM for printing. Five microliter aliquots of each 50 μM molecular beacon solution (HP1 and HP2) were transferred to alternate wells of a 384 well plate for pin pick-up. Printing was done at room temperature (~ 24°C), and relative humidity of 60%. Each beacon was printed in triplicate in order to evaluate variability. After printing, the slides were allowed to air-dry for 1 h, blocked by dipping in 1% BSA (FisherBiotech, Fair Lawn, NJ, U.S.A) for 30 s, rinsed twice in a coplin jar containing 10mM Tris buffer, and again allowed to air-dry for approximately 15 min.

Hybridization of Immobilized Molecular Beacons: Before use in immobilized beacon hybridization experiments, target sequences were thawed, and further diluted in either 3.5mM or 5mM $MgCl_2$, and 10mM Tris buffer (pH 8) to a concentration of 200nM. Two micro liters of the 200nM target in $MgCl_2$-Tris buffer was deposited on a 22mm x 22mm hybrislip cover slip (Sigma, St. Louis, U.S.A).

The cover slip was inverted on to the surface of the glass slide where the beacons were spotted allowing a thin film of the target solution to form between the glass slide and the cover slip. Hybridization was done at room temperature. Length of time for hybridization was optimized, and five minutes was determined adequate for maximal fluorescence emission. Therefore, after hybridization for five minutes, the cover slip was removed by dipping the slide in a coplin jar filled with 10mM Tris buffer, and the slide was allowed to dry for approximately 15 min. Fluorescence was measured with a ScanArray 3000 laser scanner (PerkinElmer Life Sciences, Inc., Boston, MA, U.S.A.). A helium-neon laser excitation wavelength of 543 nm was used, and fluorescence detected in a 360° configuration at a wavelength of 572 nm corresponding to Cy3 peak emission. The fluorescence data output from the scanner was processed using Genepix software (Axon Instruments, Inc., Union City, CA, U.S.A). Reproducibility was assessed by repeating experiments on different occasions starting with the three spot depositions of beacons on new slides.

Hybridization of HP1 Molecular Beacon in Solution: The HP1 beacon and target designs used in the microarray studies were also used in these studies, except for the omission of the MB 3'-end C_6 linker strand and terminal primary amine. In addition, MB and targets were prepared in 400 nM and 800 nM stock solutions, respectively, using 3.5mM or 5mM $MgCl_2$/10mM Tris buffer (pH 8), and stored at -20°C until use. Modified-HP1 beacons (IDT, Coralville, Inc., IA, U.S.A.) were also constructed with a fluorescent group (Cy3) attached to the 5'

end of the oligonucleotide, but no quencher. Modified-HP1 beacons were used to correct for temperature effects on fluorescence that are independent of the hybridization event.

Hybridization was performed in a 96 well microtiter plate, and fluorescence was read using an ABI 7700 instrument (Applied Biosystems, Foster City, CA, U.S.A.). Measurements were taken in 5°C decrements ranging from 90 to 5°C. Samples were allowed to equilibrate for one minute at each temperature before a 25 milliseconds measurement time. Volumes of 25 μL each of the molecular beacon and individual targets were mixed to give a final concentration of 200 nM and 400 nM, respectively, in a total reaction volume of 50 μL per well. Three wells were always dedicated for each molecular beacon-target combination tested, and experiments were performed on three different occasions for reproducibility assessment. HP1 molecular beacon or modified-HP1 beacon (beacon without a quencher) alone at concentrations of 200 nM, targets alone at concentrations of 400 nM, and buffer alone were also dispensed in three wells giving final reaction volumes of 50μL per well. To account for hybridization independent temperature-effects, fluorescence at different temperatures from all wells involving the molecular beacon was divided by the average fluorescence from the modified beacon at the corresponding temperatures.

The sensitivity and specificity of DNA hybridization events are known to be dependent on magnesium chloride concentration. Solution hybridization

experiments were run in which the magnesium chloride concentration was varied from 0 to 500 mM. Optimal hybridization was obtained at $MgCl_2$ concentrations between 5 – 10 mM for the HP1 beacon. Data presented for solution hybridization experiments has 3.5 mM or 5 mM $MgCl_2$, highlighting the effect of $MgCl_2$ concentration on target specificity for the HP1 beacon in solution. On the other hand, no difference in fluorescence amplitude was observed on the microarray platform upon target-probe hybridization in 3.5 mM or 5 mM $MgCl_2$ concentration and the data were combined.

Beacon array Recharging Experiment: Two sets of molecular beacons, a test beacon (HP1) and a control beacon (HP2), were printed on an aldehyde-modified slide. Hybridization was carried out at room temperature using 2 μL of 200nM true target (CS11) in 3.5mM MgCl2 /10mM Tris buffer, pH 8 for 10 minutes. The fluorescence was recorded after washing the slide in 10mM Tris buffer as described before. The slide was treated with 0.4 M sodium hydroxide solution for one minute, washed in 10mM Tris buffer and the decrease in fluorescence was recorded. The molecular beacons were then rehybridized with 200nM true target, washed in Tris buffer and the fluorescence recorded again.

Results and Discussion

The average fluorescence of three replicas of the spotted beacons (HP1 and HP2) on microarray platform before and after hybridization with various targets

were determined and plotted as illustrated in Figure 2 for the hybridization of CS11 and CS27 true targets. Hybridization with CS11 produced an increase in fluorescence of HP1 probe, but not HP2 probe, and vice versa for CS27. The slight difference in fluorescence of the samples before hybridization could be a function of the variability in sample deposited during printing or due to the inherent lack of uniformity of the aldehyde coating on the slides. For evaluating the effectiveness of the HP1 probe to differentiate true target from various mismatch targets, discrimination ratios for each target tested were defined as follows:

$$R = 1 - \frac{B_T \cdot (\alpha \cdot S_M - B_M)}{B_M \cdot (\alpha \cdot S_T - B_T)} \qquad (1)$$

where

R = discrimination ratio,

B_T = Background fluorescence signal from spotted molecular beacon probes before hybridization with true target

S_T = fluorescence signal from spotted molecular beacon probes after hybridization with true target true target (CS11)

B_M = Background fluorescence signal from spotted molecular beacon probes before hybridization with mismatch target

S_M = fluorescence signal from spotted molecular beacon probes after hybridization with mismatch target

α= scaling factor.

According to equation (1), the R value for CS11 (true target) will be zero. For mismatch targets, R values greater than zero indicate discrimination from CS11, while R values less than or equal to zero indicate poor or no discrimination. In addition, signals on each microscope slide were normalized by α, the ratio of the fluorescence signals before and after hybridization from the HP2 probe run on the same slide.

We found background fluorescence signal from hydrogel-aldehyde slides to be approximately 2x lower than the superaldehyde slides. This could be attributed to the aqueous environment of hydrogel slides which better stabilizes the hairpin structure of molecular beacons and a higher density of aldehyde groups on hydrogel slides. Otherwise target detection was similar on both platforms. Data presented in this paper was scaled and combined from both platforms. Histograms and discrimination ratios for single nucleotide target mismatches against HP1 probe on microarray platform are given in Figure 3. Discrimination ratios presented are the average between three and six different runs in triplicate performed on different occasions. We intentionally designed and worked with targets that were slightly longer than the probe loop region and complementary to 2 additional base pairs in the stem region in order to assess affects on specific location of each mismatch. Targets that bind the stem portion in addition to the loop of a molecular beacon form more stable duplexes compared to those that bind the loop region only (Tsourkas, Behlke & Bao, 2002), and would possibly be

more effective on a sensor type platform. No discrimination was noted for the single mismatch targets tested; CS111 (R = -0.12±0.31), CS112 (R = -0.15±0.37), CS113 (R = 0.06±0.19), and CS114 (R= -0.10±0.19). It is not surprising that CS112, which has a single mismatch at the 5' end, is not readily discriminated from CS11. However, target CS114, which has a single mismatch in the center of the probe loop region, is also not discriminated. Hybridization energy analysis using mfold software gives relative Gibb's free energy values for each target-probe duplex that decreases as the single mismatch moves from the end toward the center of the target. This analysis predicts poor stability of CS114*HP1 duplex which is not borne out in our data. It should be kept in mind that this analysis gives hybridization free energy values representative of whole duplexes, and it is conceivable that local interactions due to target binding to the stem portion of the probe may be sufficient for disrupting the stem portion of the probe only, or may directly interfere with fluorescence quenching and, thus, fluorescence amplitude when measured far below the duplex melting temperature. Indeed, a target with the same loop-centered mismatch as CS114, but spanning only the loop region of the probe gave a lower fluorescence signal relative to CS114 (data not shown). CS27 (R = 0.72±0.07) was easily discriminated as expected, and served as a negative control.

HP1 probe discrimination of multiple mismatch targets on microarray platform was investigated, and histograms and discrimination ratios from this study are given in Figure 4. Combinations of two, three or four mismatches were chosen

based primarily on location including loop only, stem only and loop-stem mismatch combinations (see Table 2). Not surprisingly, two targets, CS123 (R = 0.00±0.16) and CS131 (R = -.27±0.42), with two and three adjacent mismatches at the 5' end, respectively, were not readily distinguished from CS11. Target CS120 (R = 0.05±0.12) with two adjacent mismatches in the center of the loop region was also not distinguished from CS11, as similarly observed for the single mismatch target CS114. The second mismatch in CS120 was A to G, and a somewhat stable unconventional hybridization of G:T would be possible. The same arguments stated earlier for the lack of discrimination for CS114 could also apply to CS120. Target CS130 (R = 0.41±0.06) with three adjacent mismatches in the center of the loop region, however, was better discriminated than CS114 and CS120. The remaining mismatch targets, CS121(R = 0.27±0.23), CS122 (R = 0.47±0.15), CS132 (R = 0.76±0.09) and CS140 (R = 0.64±0.04), having two to four non-adjacent mismatches were discriminated from CS11, with a trend of improved discrimination as the number of target mismatches increases.

Figure 5 shows the fluorescence signal with respect to temperature of the single nucleotide mismatch targets hybridized with HP1 probe in solution at two different magnesium chloride concentrations. Discrimination ratios were again used to evaluate target specificity of the HP1 probe. True target CS11 was discriminated from all single nucleotide mismatch targets tested, with improved differentiation in solutions containing 5 mM $MgCl_2$. However, discrimination of CS111 target was poor relative to the other targets tested, especially in 3.5 mM

$MgCl_2$ solution. Gibb's free energy calculations predict that CS111, although possessing a G:T unconventional mismatch should still be better differentiated from CS11 than CS112 or CS113. This disparity between measurement and calculation could be explained if the measured fluorescence amplitude is not solely a property of the whole duplex as is the case of free energy calculations. The melting temperature is an additional variable that can be measured, and it is a property of the whole duplex related to duplex dissociation. Melting temperatures derived from our data agree with the trend predicted by hybridization energy calculations; namely, CS11 (72°C) > CS112 = CS113 = CS111 (~ 67°C) >> CS114 (60°C). In addition, target discrimination in solution compared to that on the microarray emphasizes the difference between beacon fluorescence behaviors in these two environments. It can be argued that, on the microarray surface interactions, steric crowding of bound probes, altered accessibility of target to probes, and possibly the addition of the thymidine plus C_6-linker affect hybridization kinetics.

The same multiple mismatch targets investigated on the microarray platform were also studied with HP1 probes in a solution environment. Fluorescence measurements and discrimination ratios with respect to temperature are given in Figure 6. With respect to maximum fluorescence signal only, targets CS120 (fluorescence signal max. = 0.64) and CS122 (0.46) gave signals approximately 98% and 70% of the true target, CS11 (0.65), respectively, while the remaining targets were readily discriminated over the entire temperature range below the

theoretical melting temperature of CS11*HP1 duplex (72°C). Interestingly, CS120 has two adjacent mismatches located centrally in the loop, but still preserving stem interaction. CS122 contains a mismatch in the loop, and one in the stem region of the probe; in effect, it forms a combination of single mismatch targets, CS113 and CS114. While CS113 and CS114 are discriminated (in solution) over the entire temperature range below ~ 70°C, it is surprising that CS122 is not. If we consider the trend of the duplex melting temperatures, we find CS11 (72°C) > CS123 (64°C) > CS120 = CS131 (62°C) > CS130 (58°C) > CS122 (56°C) > CS132 (40°C) > CS140 (38°C); fairly consistent with hybridization energy calculation predictions as similarly found for the single mismatch targets. When considering fluorescence signals only, it is evident that the HP1 probe in solution can discriminate three and four mismatch targets regardless of mismatch locations, with immensely improved discrimination (>80%) in the temperature range between 50°C and 65°C. Furthermore, double mismatch target discrimination (~ 50 – 90 %, depending on mismatch location and number) was also improved in a temperature range between 55°C and 65°C just below the melting temperature of the CS11*HP1 duplex where the mismatch targets would be expected to favor dissociation from the probe. However, in this temperature range, the fluorescence signal with respect to temperature is fairly steep for all tested targets, and would make quantification of the amount of probe bound target present based on fluorescence signal alone difficult.

Reusability is a characteristic that may be in certain applications for practical employment of a biosensor in the field. Since effective denaturation of duplex DNA occurs with alkaline pH, low ionic strength, or heating, we initiated a preliminary investigation regarding regeneration of the MB microarray by exposure to high pH sodium hydroxide solution, and repeated the hybridization step. Figure 7 shows the results of recharging the beacon array whereby an increase in fluorescence was obtained after rehybridization of the probe (HP1) to true target. The fluorescence of the control beacon remained more or less the same before and after the second hybridization step (data not shown). The fluorescence intensity of the test beacon after the second hybridization was approximately 75% of the first hybridization. The reduction in fluorescence intensity could be attributed to the effect of NaOH on the fluorophore or possibly the beacon being physically removed from the slide surface following successive washes. This preliminary recharging attempt shows the covalently linked MB probes to be fairly stable to harsh alkaline treatment, the targets can be washed off, and the MB probe recharged for subsequent reuse. Recharging the beacon array while potentially problematic is promising and warrants further study.

The objective of the present study was not to optimize the sensitivity of the beacon array, but to evaluate the probe specificity that is essential for pathogen discrimination. If we define hybridization specificity of a probe for a particular target as $f = \exp(-\frac{\Delta G_{M-MM}}{R \cdot T})$, where $\Delta G_{M-MM} = (G_M - G_{MM})$ is the difference in Gibb's free energy between binding of the (true) perfect target and a mismatch

target (Broude, 2002), then free energy calculations using mfold software predict the poorest discrimination would occur with the dangling 5'-end single mismatch target CS112. When we consider melting temperature trends, binding stability of the various targets is consistent with predictions. However, we have shown in this paper that when the only measurement parameter is the induced-fluorescence upon interaction of a molecular beacon with a target, discrimination is highly dependent on number and location of target mismatches, suggesting at least in part that fluorescence amplitude upon hybridization is target-dependent. In addition, immobilized molecular beacon fluorescence response can differ from that of molecular beacons in solution for reasons previously discussed. We suggest that fluorescence elicited upon target interaction is a property of direct or local effects, in addition to global probe-target interactions. Obviously, discrimination less than ~100 % would greatly limit confidence in true target detection; therefore, these effects must be considered if molecular beacons are to be used at a pathogen-detector interface. We also showed that these immobilized MB probes have the potential for reuse lending to their possible practical application.

Further studies are required to determine whether increasing the stem length of the HP1 probe could improve specificity, and to what cost to sensitivity this would have. Also, probe discrimination of targets with variable nucleotide lengths needs investigations. For example, contaminate DNA sequences in the field with complementary sequences shorter than the total probe length may be

problematic. In any event, these DNA probes still hold promise, whereby it is conceivable that multiple MB probes specific for different nucleotide sequences of a particular pathogen can be deposited on a single substrate, allowing parallel processing to circumvent the specificity limitation of a single MB probe.

Acknowledgements

We thank Dr. Alain Stintzi (Dept. of Pathobiology, College of Veterinary Medicine, Oklahoma State University) for his input and help on the microarray experiments. We thank David W. Goad (Dept. of Physiology, College of Veterinary Medicine, Oklahoma State University) for his valuable advice and help during the course of this work.

We acknowledge the financial support of the Oklahoma City National Memorial Institute for the Prevention of Terrorism (Grant No. MIPT106-113-2000-33) and of the Oklahoma Center for the Advancement of Science and Technology (OARS Program Grant No. 5849).

Figure 1a. Attachment of a molecular beacon (HP1) containing a primary amino group to the surface of aldehyde modified glass substrates *via* the formation of Schiff base

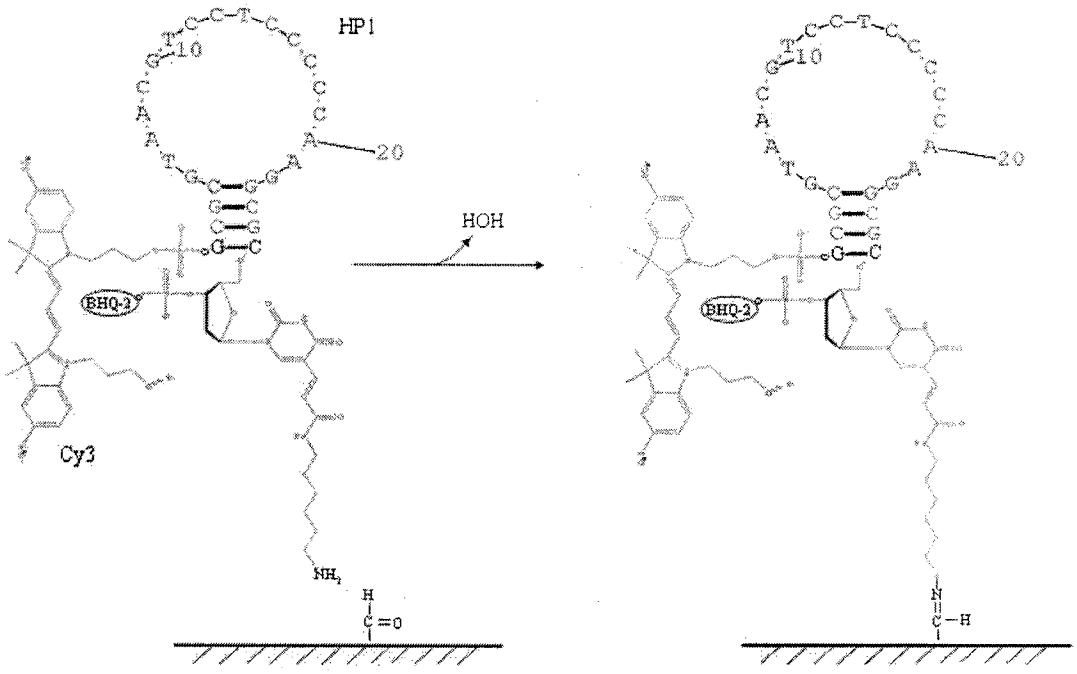

HP1
10
20
HOH
BHQ-2
Cy3
NH2
H
C=O
BHQ-2
N
C-H

Figure 1b. Hybridization of the beacon probe with a target (CS11) results in spatial separation of the Cy3 fluorophore and quencher resulting in fluorescence emission .

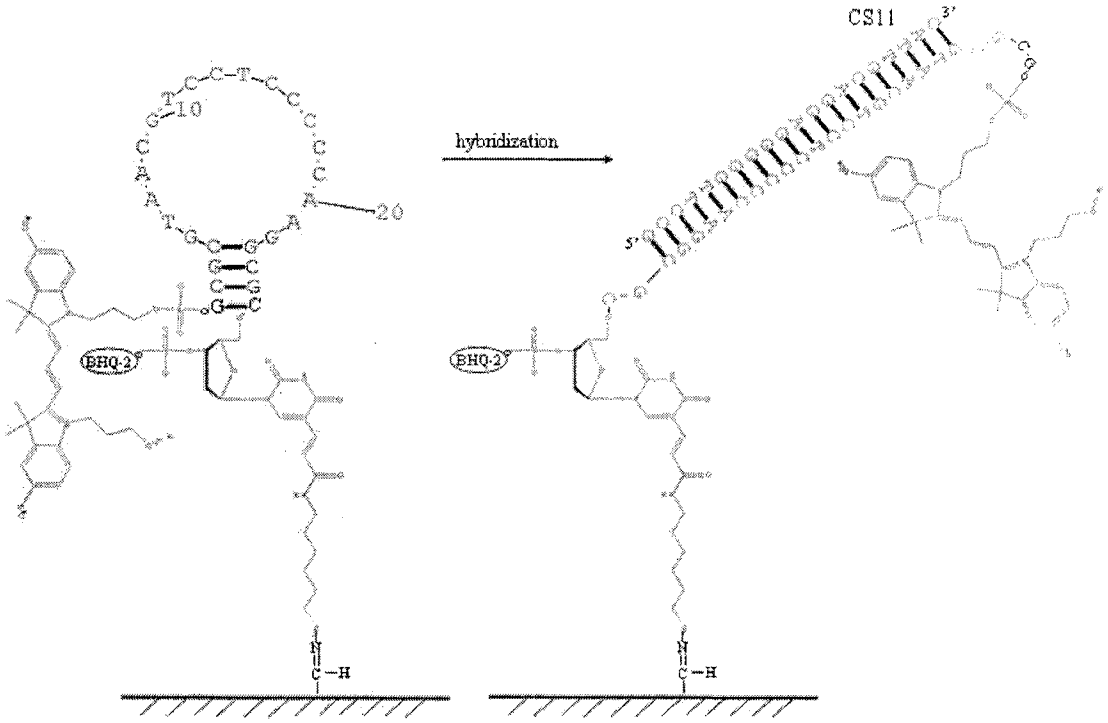

CS11
hybridization
BHQ-2
BHQ-2

Figure 2a. Typical molecular beacon microarray background fluorescence before hybridization (i), and fluorescence after hybridization to specific targets, CS11 (ii) and CS27 (iii). The three spots in the left column are HP2 molecular beacon deposits, and those in the right column are HP1 molecular beacon deposits

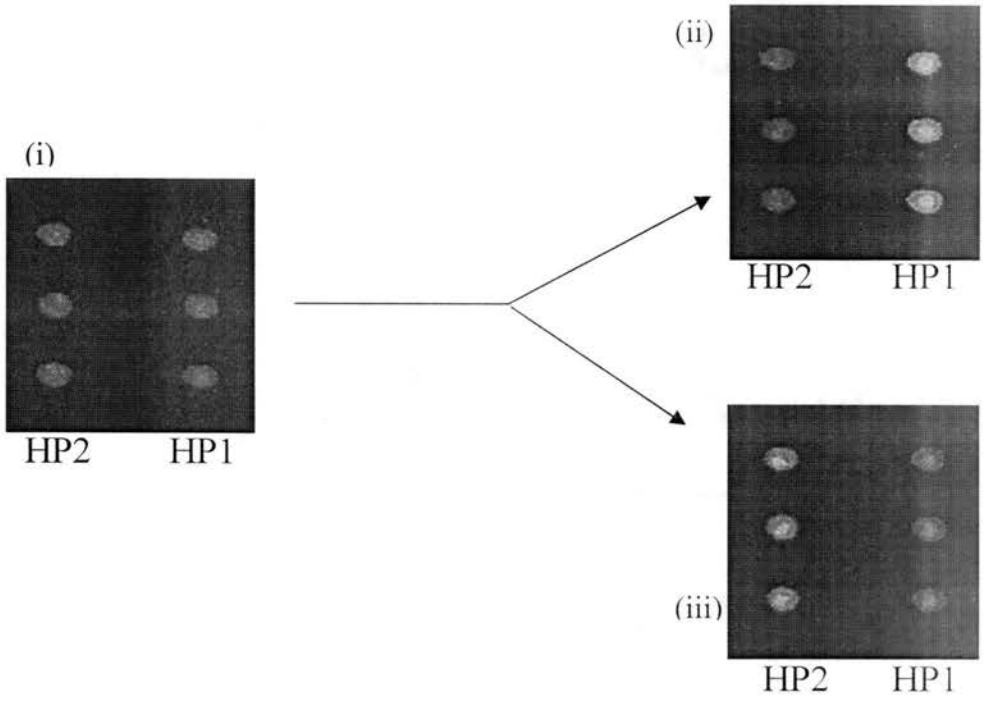

(i)
HP2
HP1
(ii)
HP2
HP1
(iii)
HP2
HP1

Figure 2b. Increase in fluorescence upon binding of molecular beacon probes to specific target sequences.

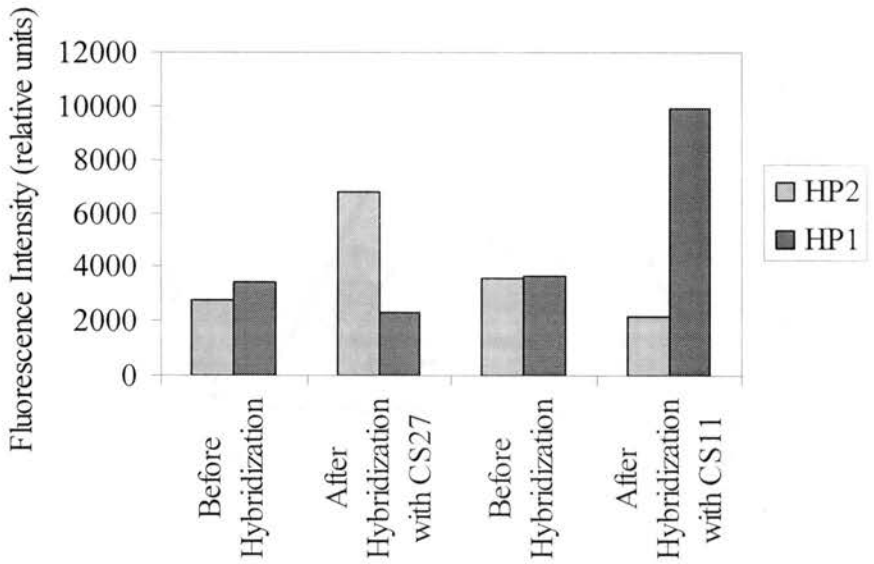

Fluorescence Intensity (relative units)
12000
10000
8000
6000
4000
2000
0
Before Hybridization
After Hybridization with CS27
Before Hybridization
After Hybridization with CS11
HP2
HP1

Figure 3a. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and single-nucleotide mismatch targets on a microarray platform. White bars denote fluorescence before hybridization and Black bars denote fluorescence after hybridization.

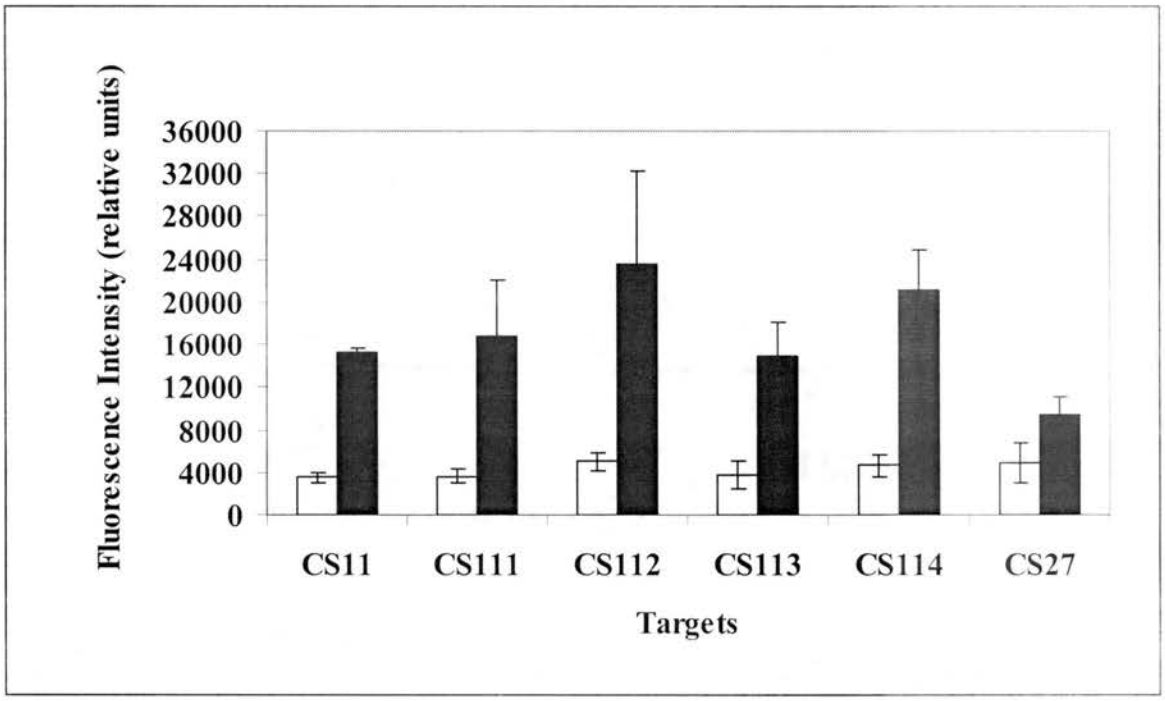

Fluorescence Intensity (relative units)
36000
32000
28000
24000
20000
16000
12000
8000
4000
0
CS11
CS111
CS112
CS113
CS114
CS27
Targets

Figure 3b. Discrimination Ratios - true and single-nucleotide mismatch targets on a microarray platform

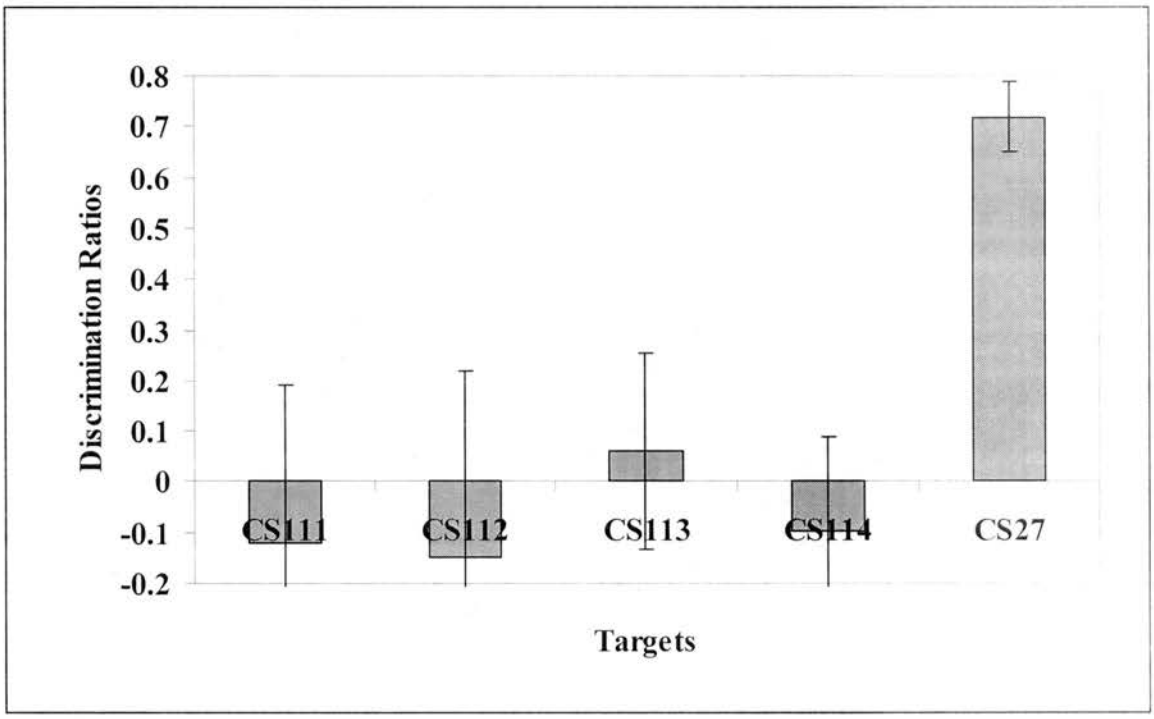

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
Discrimination Ratios
CS111
CS112
CS113
CS114
CS27
Targets

Figure 4a. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and multiple-nucleotide mismatch targets on a microarray platform. White bars denote fluorescence before hybridization and black bars denote fluorescence after hybridization

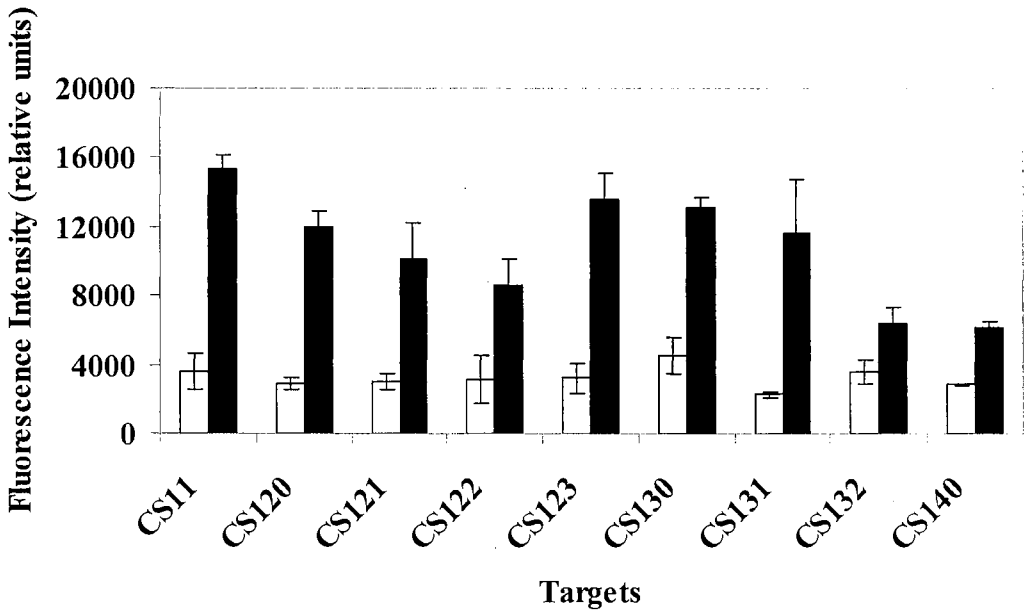

Fluorescence Intensity (relative units)
20000
16000
12000
8000
4000
0
CS11
CS120
CS121
CS122
CS123
CS130
CS131
CS132
CS140
Targets

Figure 4b. Discrimination ratios.- true and multiple-nucleotide mismatch targets on a microarray platform

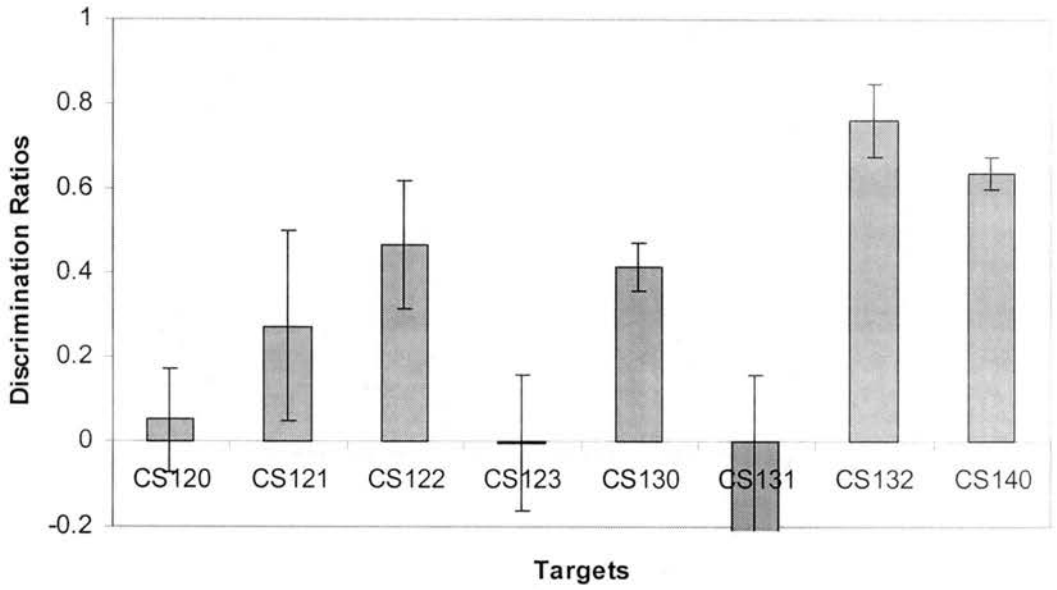

1
0.8
0.6
0.4
0.2
0
-0.2
Discrimination Ratios
CS120
CS121
CS122
CS123
CS130
CS131
CS132
CS140
Targets

Figure 5. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and single nucleotide mismatch targets in solution, with respect to temperature, are shown at 3.5 mM $MgCl_2$ (A) and 5.0 mM $MgCl_2$ (C). Dicrimination ratios corresponding to (A) and (C) are shown in panels (B) and (D) respectively.

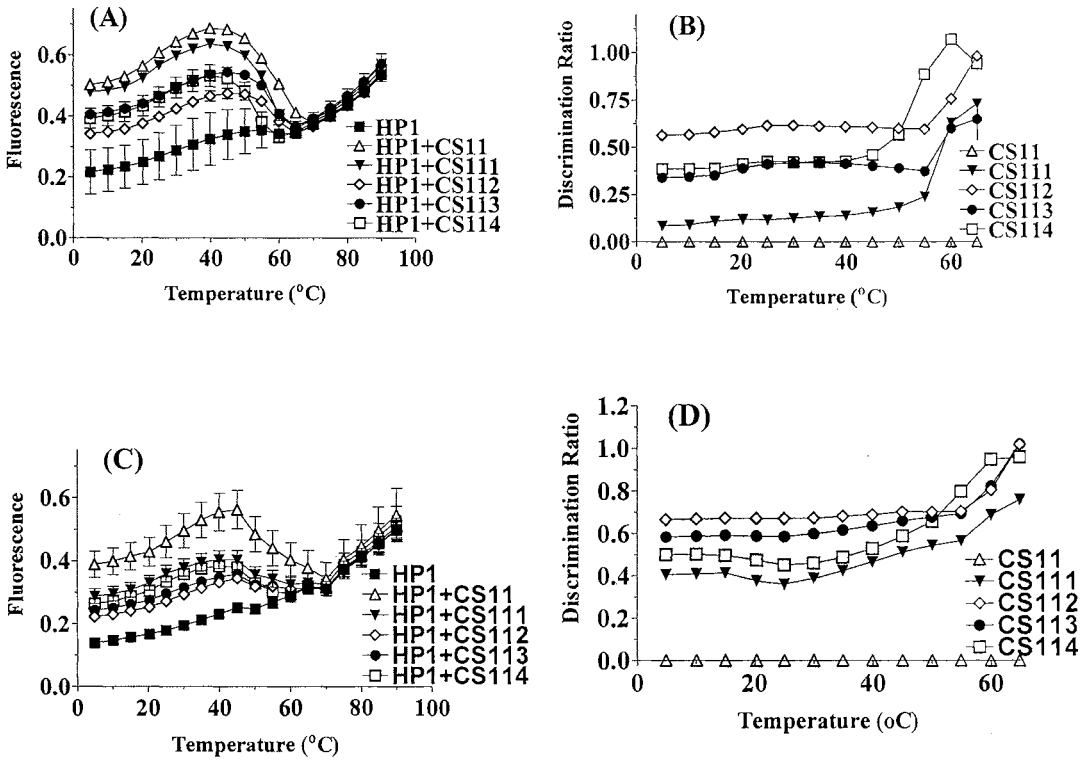
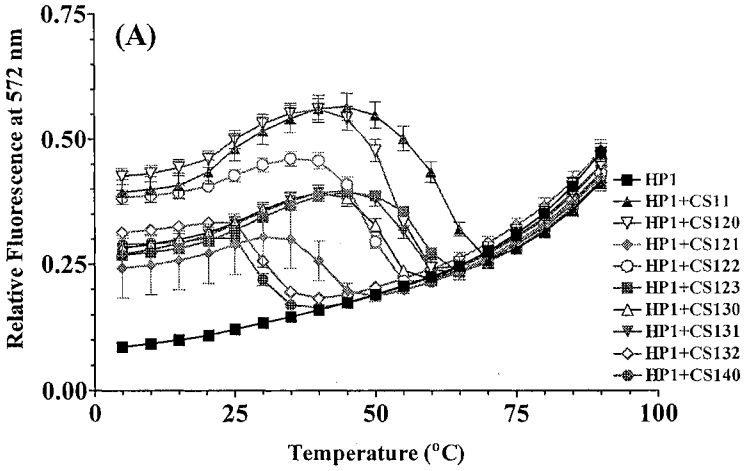

(A)
Fluorescence
Temperature (°C)
HP1
HP1+CS11
HP1+CS111
HP1+CS112
HP1+CS113
HP1+CS114
(B)
Discrimination Ratio
Temperature (°C)
CS11
CS111
CS112
CS113
CS114
(C)
Fluorescence
Temperature (°C)
HP1
HP1+CS11
HP1+CS111
HP1+CS112
HP1+CS113
HP1+CS114
(D)
Discrimination Ratio
Temperature (oC)
CS11
CS111
CS112
CS113
CS114

Figure 6. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and multiple nucleotide mismatch targets in solution, with respect to temperature, at 5mM $MgCl_2$ (A) and corresponding discrimination ratios (B).


(A)
Relative Fluorescence at 572 nm
0.75
0.50
0.25
0.00
0
25
50
75
100
Temperature (°C)
HP1
HP1+CS11
HP1+CS120
HP1+CS121
HP1+CS122
HP1+CS123
HP1+CS130
HP1+CS131
HP1+CS132
HP1+CS140

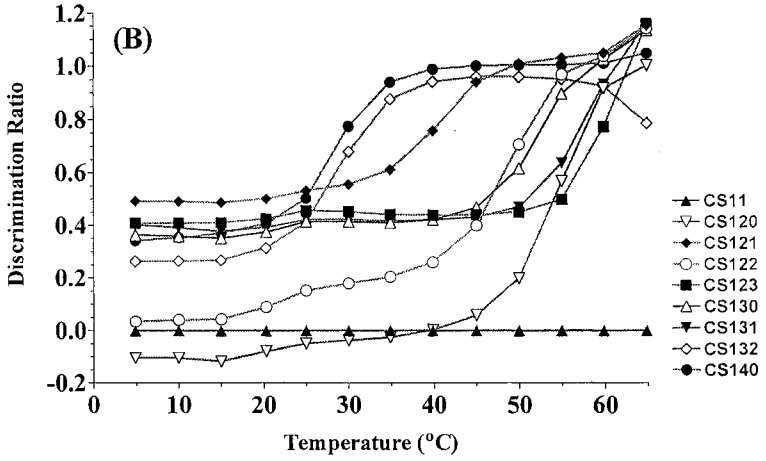

(B)
Discrimination Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
10
20
30
40
50
60
Temperature (°C)
CS11
CS120
CS121
CS122
CS123
CS130
CS131
CS132
CS140

Figure 7. Recharging of the molecular beacon (HP1) array by NaOH treatment. The white bars correspond to pre hybridization fluorescence and the black bars correspond to post hybridization fluorescence for both the initial and post recharge-hybridzations.

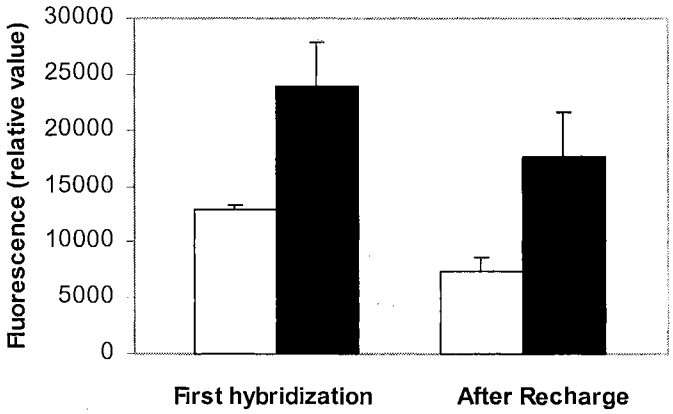

Fluorescence (relative value)
30000
25000
20000
15000
10000
5000
0
First hybridization
After Recharge

Table 1. Alignment of single mismatch target sequences across the length of beacon (HP1)

Description	Target sequence alignment with probe
HP1 probe *	3'-BHQ-T($C_6H_{12}NH_2$)**<u>CGCG</u>**GAACCCCCTCCTGCAATG**<u>CGCG</u>**-Cy3-5'
CS11 (True target to HP1)	5' GCCTTGGGGGAGGACGTTAC 3'
CS111	5' GC<u>T</u>TTGGGGGAGGACGTTAC 3'
CS112	5' <u>A</u>CCTTGGGGGAGGACGTTAC 3'
CS113	5' G<u>T</u>CTTGGGGGAGGACGTTAC 3'
CS114	5' GCCTTGGGG<u>A</u>AGGACGTTAC 3'
HP2 probe *	3'-BHQ-T($C_6H_{12}NH_2$)**<u>GC*TCG*</u>**ACAACCTCAGCCACATTTC**<u>*CGA*GC</u>**-Cy3-5'
CS27(True target to HP2)	5' AGCTGTTGGAGTCGGTGTAAAGGCTC 3'

* Bold underlined bases denote the stem of the molecular beacon. Italicized bases at the 3' side represent target binding sites within the stem. B HQ = blackhole-2 quencher from IDT, Inc. (Coraville, IA, USA).
Mismatch bases in the target sequences are underlined.

Table 2. Alignment of multiple mismatch target sequences across the length of beacon (HP1)

Description	Target sequence alignment with probe
HP1 probe *	3'-BHQ-T($C_6H_{12}NH_2$)**CGCG**GAACCCCCTCCTGCAATG**CGCG**-Cy3-5'
CS11 (True target to HP1)	5' GCCTTGGGGGAGGACGTTAC 3'
CS120	5' GCCTTGGGGAGGGACGTTAC 3'
CS121	5' GCCTTGAGGGAGGACATTAC 3'
CS122	5' GTCTTGGGGAAGGACGTTAC 3'
CS123	5' ATCTTGGGGGAGGACGTTAC 3'
CS130	5' GCCTTGGGGAGAGACGTTAC 3'
CS131	5' ATTTTGGGGGAGGACGTTAC 3'
CS132	5' GCCTTAGGGGAAGACGTCAC 3'
CS140	5' GCCTCGGGGAAGGATGTTAT 3'

* Bold underlined bases denote the stem of the molecular beacon. Italicized bases at the 3' side represent target binding sites within the stem. BHQ = blackhole-2 quencher from IDT, Inc. (Coraville, IA, USA).
Mismatch bases in the target sequences are underlined.

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Chapter V

Bacterial Identification Based on 16S rRNA Sequence Using Molecular Beacons

Abstract

Gene bank entries for the 16S rRNA of *Mannheimia haemolytica* and *Escherichia coli* were analysed for unique sequences and molecular beacon probes targeting one such unique sequence in each species were designed. Molecular beacons are hairpin shaped DNA sequences attached to a flourophore and quencher molecule that are held in close proximity there by quenching the fluorescence in the native state. Fluorescence is emitted upon hybridization of the molecular beacon to target sequences due to a conformational change that separates the fluorophore from the quencher. Hybridization reactions conducted using total RNA isolated from each bacterial species with the molecular beacons successfully discriminated between the targets and were found to be specific. The effect of target concentration on molecular beacon hybridization was also studied.

Introduction

Identification of bacterial organisms is a very important aspect of disease diagnosis and environmental monitoring. The common methods employed in bacterial identification like staining, biochemical tests and culture characteristics may not be useful in the identification of all organisms unless they have unique features with respect to the specific method of identification. Many of the biochemical tests are long and laborious and only a few bacteria have unique cultural characteristics. Novel bacterial identification methods based on vibrational spectroscopic techniques (Kirschner et al., 2001) and fatty acid composition (Leclercq et al., 2000) have been reported. Antibody based tests like ELISA or agglutination tests, even though useful and widely used for microbial identification, could be restricted in their use by the presence of cross reacting antigens. There is a need for the development of a simple standard technique that can be used for the identification of bacterial organisms.

With the advances in genome sequencing and the large amount of sequences available in databases, it has now become possible to identify bacterial organisms based on their nucleic acid sequences. We have described the use of molecular beacons to detect unique sequences in the 16S ribosomal RNA of bacteria. Ribosomal RNAs are the molecules of choice for the phylogenetic

classification of bacteria (Fox et al., 1980) and have been employed in the study of bacterial evolution (Woese, 1987). Due to their high copy number per cell and single stranded nature, rRNA serve as attractive targets for a probe based detection system.

In the present study we have designed molecular beacon probes complementary to unique sequences in the 16S rRNA of *Escherichia coli* and *Mannheimia haemolytica*. Molecular beacons were first developed by Tyagi and Kramer (Tyagi & Kramer, 1996), following which various applications for molecular beacons have been reported, such as, studying DNA binding enzyme activity (Fang, Li & Tan, 2000), detection of single nucleotide polymorphisms (Tyagi, Bratu & Kramer, 1998), real time detection of DNA-RNA hybridization in living cells (Sokol et al., 1998) and detection of pathogenic retroviruses (Vet et al., 1999), to name a few. Molecular beacons are hairpin shaped deoxyoligonucleotide sequences that have a quencher moiety and a fluorophore at opposing ends and are quenched in the native state. Upon hybridization with a target nucleic acid sequence, the native hairpin structure of the molecular beacon is disrupted resulting in de-quenching and fluorescence emission (Fig1). RNA isolated from *E.coli and M. haemolytica* was positively identified by hybridization reactions with the respective molecular beacons. We further studied the specificity of the *M. haemolytica* molecular beacon by testing its ability to discriminate RNA from *Pasteurella multocida*. *M. haemolytica* was formerly named *Pasteurella haemolytica* and recently was re-classified into a genus of its

own based on rRNA sequences (Angen et al., 1999). Dependence of molecular beacon function on RNA concentration was also studied.

Materials and Methods

RNA extraction: RNA was extracted following a hot-phenol method published previously (Chuang, Daniels & Blattner, 1993; Stintzi, 2003). Briefly, overnight cultures of bacteria pelleted by centrifugation were resuspended in 1mL of TE buffer containing 0.5mg/mL lysozyme and then mixed with 10% SDS and 1M sodium acetate. This was mixed with an equal volume of water saturated phenol (Invitrogen Corp. CA, USA) and incubated at 64°C. The aqueous layer was separated by centrifugation at 4°C and extracted with an equal volume of chloroform. The aqueous layer was separated and treated with cold ethanol to precipitate RNA. The RNA pellet was treated with RNase inhibitor – RNasin (Promega Corp. WI, USA) and RNase free DNase (Promega Corp. WI, USA) to remove any RNase or DNA contamination. The RNA sample was further cleaned up using an RNA isolation kit (Qiagen Inc. CA, USA), following manufacturer recommendations and dissolved in RNase free sterile double distilled water and stored at -80°C. The concentration and purity of RNA samples were assessed by measuring absorption at 260 nM and 280 nm in a spectrometer. RNA samples were also run on a gel to assess their quality.

Molecular beacon design: Using the software Vector NTI (Informax, Inc. Bethesda, MD, U.S.A.), published sequences of 16S ribosomal RNA from *E.coli* and *M.haemolytica* were aligned and regions that were unique to each species were identified (Table 1). Two molecular beacons *viz.* ManHP1 and EcoHP1 (Table 2) were designed complementary to unique regions of the 16S rRNA from *M heamolytica* and *E. coli,* respectively, using the mfold software (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/form1.cgi) (Zuker, 2003). Molecular beacons were synthesized by IDT DNA Inc. with Cy3 as the fluorophore and BHQ2 as the quencher. The molecular beacon samples were stored as 200μM aliquots at -20°C until further use.

Target discrimination studies: Hybridization was carried out in 96 well plates using the ABI 7700 machine. The amount of total RNA used was determined by UV absorption at 260nm and was approximately 55 μg of *E.coli* RNA (EcoRNA), 40 μg *of M. haemolytica* RNA (ManRNA) and 45 μg of *P. multocida* RNA (PastRNA). 25 μL of 2 μM solution of molecular beacon made in 20mM Tris/200mM MgCl2 buffer (pH 8) was mixed with 25 μL of RNA sample in sterile double distilled water treated with DEPC so that the final $MgCl_2$ concentration in the reaction mix was 100mM. Controls were also maintained in which 25 μL of 2 μM molecular beacon in 20 mMTris/200 mM $MgCl_2$ was mixed with 25 μL of sterile double distilled DEPC water. The samples were heated to 95 °C for 10minutes and cooled slowly to 25 °C and fluorescence measured.

Concentration dependence studies: Total RNA isolated (~50 µg) as described above was serially diluted two fold in sterile DEPC treated water. An equal volume (25 µL) of 2 µM ManHP1 beacon was added to each of the serial RNA dilutions. Hybridization was carried out as before by heating the RNA-molecular beacon reaction mix to 95 °C and allowing it to cool. Fluorescence was measured at 25 °C.

Results and Discussion

Target discrimination experiments: It is known that hybridization reactions between nucleic acid molecules are dependent on the concentration of cations in the the hybridization mixture. The negative charges on the nucleic acid molecules are shielded by cations in the hybridization buffer to facilitate an optimal hybridization. The potential of RNA molecules especially rRNA to form secondary structures is another factor to be considered in a hybridization event. Usually, denaturing agents like formamide may be used to disrupt secondary structures. This was not possible in the current experiment since it would also disrupt base pairing in the stem region of the molecular beacon and denature the stem loop structure of the molecular beacon probe. In this study we heated the total RNA-molecular beacon mixture to 95 °C for ten minutes and then cooled the mixture to 25 °C to effect hybridization. Hybridization was carried out, with both synthetic oligonucleotide targets (data not shown) and total RNA isolated from

the different bacterial species, at different concentrations of $MgCl_2$ in the hybridization buffer. It was found that the molecular beacons successfully hybridized to synthetic targets at low concentrations of $MgCl_2$ around 5 mM but failed to hybridize to the total RNA isolated at a similar $MgCl_2$ concentration. Hybridization to the total RNA targets occurred at a high $MgCl_2$ concentration of 100 mM in the buffer. Fig 2 shows the results of the hybridization experiment with ManHP1 and EcoHP1 beacons. The percentage increase in fluorescence was calculated with respect to samples of control molecular beacons under identical conditions in the absence of any target RNA. It can be seen that fluorescence of the molecular beacon resulting from hybridization with total RNA isolated from the corresponding species was greater than that produced in the reaction with RNA from the other species. From table 1 it can be noted that there is a 10 base difference between the 16S rRNA target sequences of *E. coli* and *M. haemolytica*. To further test the specificity of the hybridization reaction, ManHP1 beacon was tested against RNA from *M. haemolytica* and *P. multocida* (Fig3). Both these organisms belong to the same family (Pasteurellaceae) and were once grouped under the same genus of *Pasteurella*. Incubation of ManHP1 with total RNA isolated from *M. haemolytica* produced an increase in fluorescence whereas incubation with total RNA from *P. multocida* failed to produce any increase in fluorescence. The negative value corresponding to the percentage fluorescence increase on incubation with the *P. multocida* RNA is an artifact resulting from minor differences in the background fluorescence between different wells of the 96 well plate.

Concentration studies: Fig 4 shows the effect of target concentration on fluorescence. A positive association of the fluorescence with the concentration of target was observed. The fluorescence resulting from hybridization with the first three concentrations appeared variable but as the concentration further decreased the fluorescence showed a downward trend. The lowest concentration of RNA that resulted in fluorescence above background was 1650 ng. This low sensitivity should not be a problem in bacterial identification experiments since the amount of RNA that can be extracted is not restricted when isolating RNA from overnight cultures. Even though studies have shown that very low concentrations of target can be detected using molecular beacons (Vet et al., 1999), in the present study, the target molecule being rRNA, there could be steric limitations to the molecular beacon probe in accessing its target sequences in the secondary structure mesh of rRNA. Problems arising from the secondary structure of target rRNA molecules may be overcome by fragmentation of the RNA sample (Nelson et al., 2002). Optimal buffer conditions and target presentation modes should be studied further. Sensitivity is an important factor to be considered in a detection or biosensor platform. A higher sensitivity may be achieved as researchers (Fortin, Mulchandani & Chen, 2001; Park et al., 2000) have already shown by coupling the molecular beacon technology with PCR.

Conclusion

The present study shows the potential application of molecular beacons in the field of bacterial identification and disease diagnosis. Further studies should be conducted to increase the sensitivity of the assay. Developing molecular beacons against regions of the rRNA molecule that are more accessible (Fuchs et al., 1998) in the whole ribosome will help in the development of *in situ* hybridization assays on tissue samples.

Figure 1. Diagrammatic representation of molecular beacon functioning. The fluorophore and quencher are held in close proximity in the native state (a) resulting in minimal fluorescence. On hybridization with a target nucleic acid there is a conformational change resulting in fluorescence emission (b).

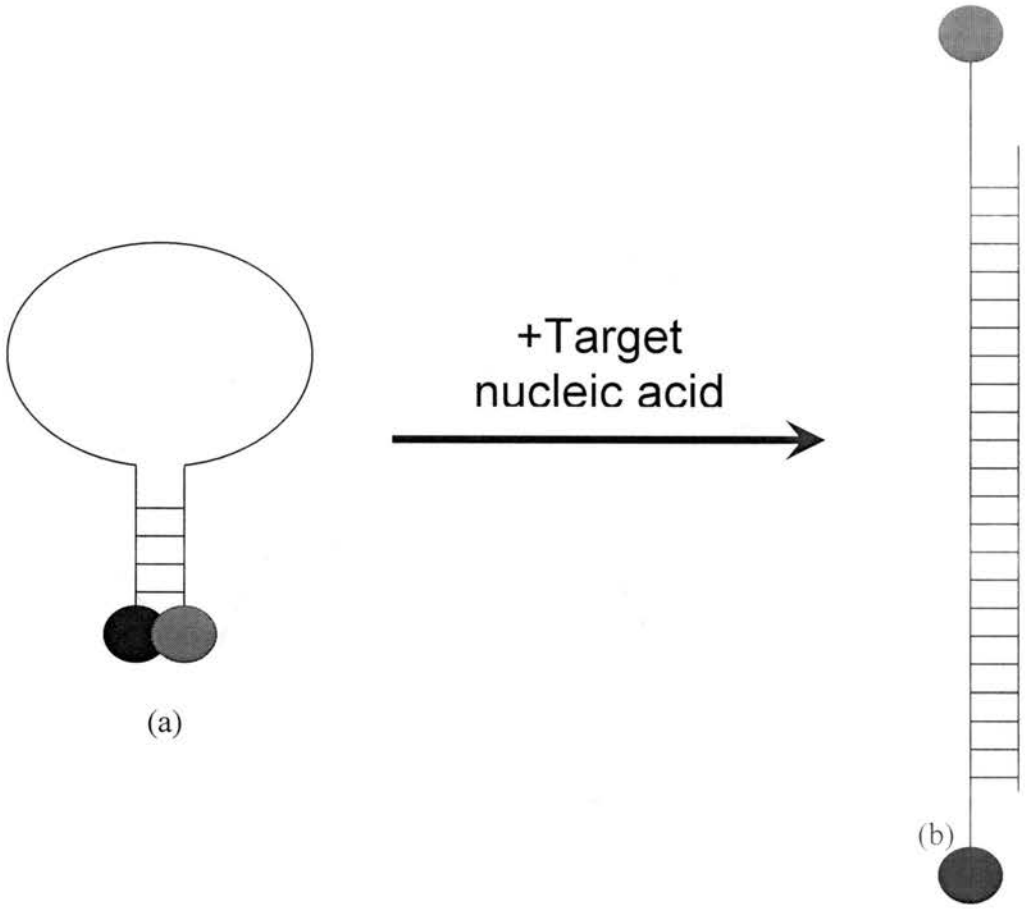
+Target
nucleic acid
(a)
(b)

Figure 2. Fluorescence response of molecular beacons EcoHP1 and ManHP1 on hybridization to total RNA sample isolated from *E. coli* or *M. haemolytica*. Fluorescence was measured at 25°C.

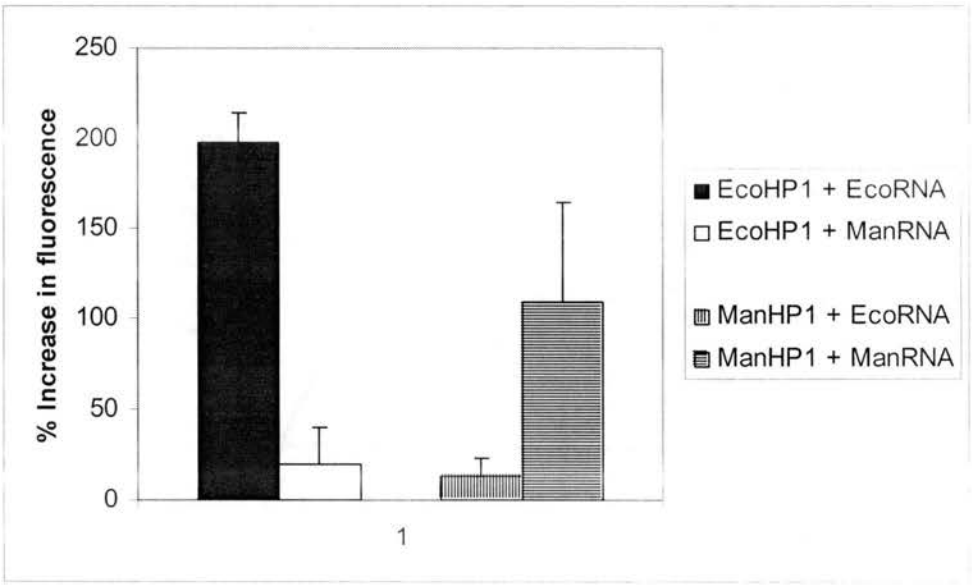
250
200
150
100
50
0
% Increase in fluorescence
1
EcoHP1 + EcoRNA
EcoHP1 + ManRNA
ManHP1 + EcoRNA
ManHP1 + ManRNA

Figure 3. Fluorescence increase on hybridization of ManHP1 beacon to total RNA isolated from *M. haemolytica* and *P. multocida*. Fluorescence was measured at 25°C.

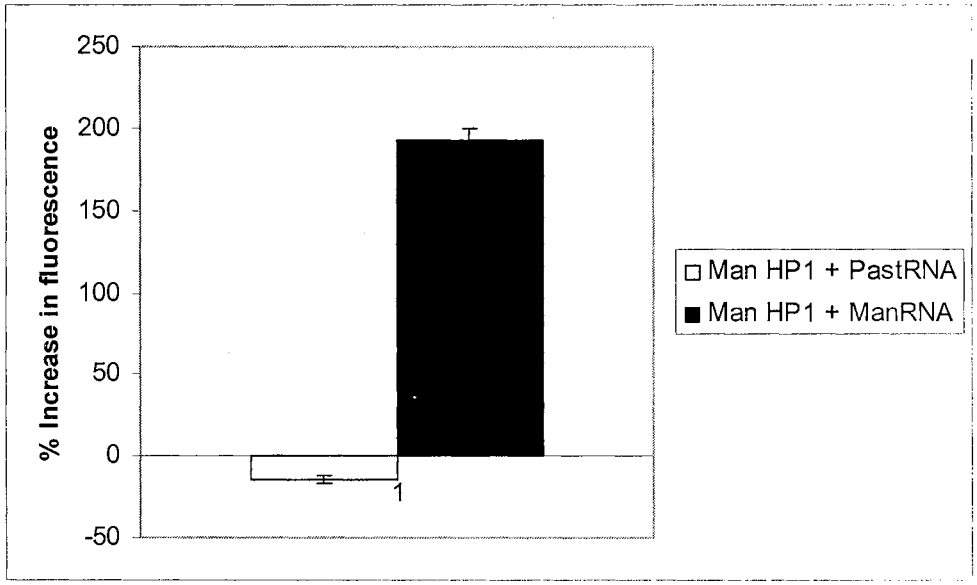
% Increase in fluorescence
250
200
150
100
50
0
-50
1
Man HP1 + PastRNA
Man HP1 + ManRNA

Figure 4. Fluorescence response of ManHP1 molecular beacon on incubation with a serial two fold dilution of total RNA sample isolated from *M. haemolytica.*

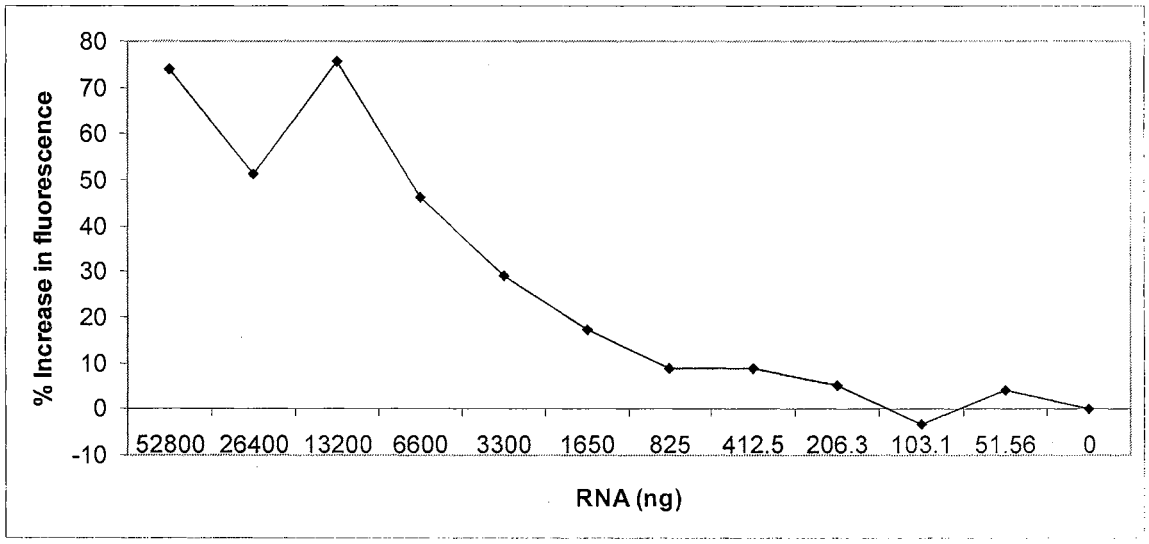
80
70
60
50
40
30
20
10
0
-10
% Increase in fluorescence
52800 26400 13200 6600 3300 1650 825 412.5 206.3 103.1 51.56 0
RNA (ng)

Table 1 – Target sequences in the 16S rRNA of *E. coli* and *M. haemolytica*

Organism	Target Sequence
E.coli	CCUUUGCUCAUUGACGUUAC
M. haemolytica	GACAAAGCAAUUGACGUUAG

Table2 – Molecular beacon probe sequences for E. coli (EcoHP1) and M. haemolytica (ManHP1)

Beacon Name	Probe Sequence*
EcoHP1	***GCCC***GTAACGTCAATGAGCAAA***GGGC***
ManHP1	***GCGAC***TAACGTCAATTGCTTT***GTCGC***

* Italicized bases indicate the stem region of the molecular beacon

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Chapter VI

Conclusions

The purpose of this research was to evaluate the applicability of molecular beacon technology for bacterial identification. Molecular beacon probes were developed to detect unique rRNA sequences of *Francisella tularensis*, *Escherichia coli* and *Mannheimia haemolytica,* based on available Genbank data. It was found that beacon functioning was dependent on cation concentration. Variable length targets could be discriminated in a solution based hybridization assay, as was shown both in fluorescence and capillary electrophoresis studies. Molecular beacons immobilized on glass slides were also efficient in discriminating double or triple base mismatch targets, although it was not possible to discriminate single base mismatch targets. The reduced discriminatory behavior of molecular beacons immobilized on glass slides could be due to the reduced stability of the stem loop structure of the molecular beacon in the absence of hydrophobic interactions. Probably, increasing the stem length may help increase the stability of molecular beacons on solid platforms and thus provide better target discrimination. One advantage of surface immobilized molecular beacons is that they have the potential to be reused. It has been shown in this study that treatment with alkali will help to remove probe-bound target and the probe reforms the stem loop structure to be used in a second

hybridization. There was a drop in fluorescence following subsequent hybridization as compared to the first hybridization. This could be the result of photobleaching or probe wash-off from the slide surface.

For the purpose of bacterial identification experiments, molecular beacons were developed against unique sequences in the 16S rRNA of two bacterial species, *E. coli* and *M. haemolytica*. Successful discrimination was achieved on exposing the probes to total RNA isolated from different species. The sensitivity of the reaction was low and further studies to increase the sensitivity should be conducted. A probable reason for the low sensitivity could be the secondary structure formations in the rRNA molecule that prevent optimal probe access to the target sequences. Fragmentation of RNA by treatment with specific buffers may help solve the problem of secondary structure formation and steric hindrances to probe access. Increase in sensitivity may also be achieved by developing multiple probes against unique sequences in the same or different target molecules present in the organism.

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