

Target discrimination by surface-immobilized molecular beacons designed to detect *Francisella tularensis*

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Received 30 January 2003; received in revised form 18 July 2003; accepted 10 August 2003

Abstract

A molecular beacon (MB) 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.

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Keywords: Target discrimination; Surface-immobilized molecular beacons; *Francisella tularensis*

1. Introduction

Management of and deterrents to disease outbreak require 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 (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 (Fang et al., 1999; Liu and Tan, 1999; Brown et al., 2000; Liu et al., 2000; Steemer et al., 2000; Broude et al., 2001; Riccelli

et al., 2001; Frutos et al., 2002; 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 Fig. 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 et al., 2000), probing interactions of single-stranded DNA binding to enzymes (Fang et al., 2000), detection of DNA or RNA within integration amplification systems (Leone et al., 1998; Kaboev et al., 2000), and discrimination of alleles and single-nucleotide polymorphisms (Tyagi et al., 1998; Mhlanga and Malmberg, 2001), to name a few.

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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 and Butow, 2000, and references therein; Kato-Maeda et al., 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 and Hoheisel, 1999), or agarose films (Broude et al., 2001; Wang et al., 2002; Afanassiev et al., 2000).

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 (Fang et al., 1999; Brown et al., 2000; Steemer et al., 2000; Frutos et al., 2002; Wang et al., 2002). Miniaturized beacon immobilization combined with fiber-optic techniques (Liu and Tan, 1999; Steemer 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. rRNA, being naturally amplified sequences, are ideal target molecules for a probe based detection system. *F. 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 C₆ 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.

2. Materials and methods

2.1. 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, USA). 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 (SantaLucia, 1998; Mathews et al., 1999; Zuker et al., 1999; <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 two 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 C₆ linker strand ending in a primary amino group was covalently linked to the 5-methyl group of the thymine aromatic ring (see Fig. 1 and Table 1). The C₆ linker strand was omitted for hybridization studies in solution. Molecular beacons (HP1 and HP2 with and without the C₆ linker strand) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). True target oligonucleotides (CS11 and CS27), and various mismatch targets against HP1 were obtained from the Core

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

Description	Target sequence alignment with probe
HP1 probe ^a	3'-BHQ-T(C ₆ H ₁₂ NH ₂) <u>CGCG</u> GAAAC CCCCTCCTGCAATG <u>CGCG</u> -Cy3-5'
CS11 (true target to HP1)	5' GCCTTGGGGGAGGACGTTAC 3'
CS111	5' GCTTTGGGGGAGGACGTTAC 3'
CS112	5' <u>AC</u> CTTGGGGGAGGACGTTAC 3'
CS113	5' <u>GT</u> CTTGGGGGAGGACGTTAC 3'
CS114	5' GCCTTGGGG <u>A</u> AGGACGTTAC 3'
HP2 probe ^a	3'-BHQ-T(C ₆ H ₁₂ NH ₂) <u>GCTCG</u> AACA CCTCAGCCACATTT <u>C</u> CGAGC-Cy3-5'
CS27(true target to HP2)	5' AGCTGTTGGAGTCCGGTGTAAAGGCTC 3'

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

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

Description	Target sequence alignment with probe
HP1 probe ^a	3'-BHQ-T(C ₆ H ₁₂ NH ₂) CGCG GAACC CCCTCCTGCAATG CGCG -Cy3-5'
CS11 (true target to HP1)	5' GCCTTGGGGAGGACGTTAC 3'
CS120	5' GCCTTGGGGAGGGACGTTAC 3'
CS121	5' GCCTTGA GGG AGGACATTAC 3'
CS122	5' G T CTTGGGG A AGGACGTTAC 3'
CS123	5' <u>ATT</u> CTTGGGGGAGGACGTTAC 3'
CS130	5' GCCTTGGGGAGAGACGTTAC 3'
CS131	5' <u>ATTT</u> TGGGGGAGGACGTTAC 3'
CS132	5' GCCTT A GGGG A AGACGTAC 3'
CS140	5' GCCT C GGGG A AGGATGTTAT 3'

^a Italicized underlined bases denote the stem of the molecular beacon. Bold 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.

Facility at Oklahoma State University, OK, USA. 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 nmol and targets in 100 nmol scales. Molecular beacon stock solutions with a concentration of 200 μ M were prepared using doubly distilled sterile water, divided into 10 μ l aliquots, and stored at -20°C until use. Target oligonucleotides were similarly prepared and stored, except 400 μ M stock solutions were prepared using 10 mM Tris/3.5 mM MgCl₂ buffer (pH 8).

2.2. 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, Ont., Canada). A robotic printer (PixSys 5500, Genomic Solutions, Inc., Ann Arbor, MI, USA) 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 50 μ M for printing. Five-microliter aliquots of each 50 μ M 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 ($\sim 24^{\circ}\text{C}$), 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, USA) for 30 s, rinsed twice in a coplin jar containing

10 mM Tris buffer, and again allowed to air dry for approximately 15 min.

2.3. Hybridization of immobilized molecular beacons

Before use in immobilized beacon hybridization experiments, target sequences were thawed, and further diluted in either 3.5 or 5 mM MgCl₂, and 10 mM Tris buffer (pH 8) to a concentration of 200 nM. Two microliters of the 200 nM target in MgCl₂-Tris buffer was deposited on a 22 mm \times 22 mm hybridlip cover slip (Sigma, St. Louis, USA). 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 5 min was determined adequate for maximal fluorescence emission. Therefore, after hybridization for 5 min, the cover slip was removed by dipping the slide in a coplin jar filled with 10 mM Tris buffer, and the slide was allowed to dry for approximately 15 min. Fluorescence was measured with a ScanArray 3000 laser scanner (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA). A helium-neon laser excitation wavelength of 543 nm was used, and fluorescence detected in a 360 $^{\circ}$ 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, USA). Reproducibility was assessed by repeating experiments on different occasions starting with the three spot depositions of beacons on new slides.

2.4. 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 C₆ linker strand and terminal primary amine. In addition, MB and targets were prepared in 400 and 800 nM stock solutions, respectively, using 3.5 or 5 mM MgCl₂/10 mM Tris buffer (pH 8), and stored at -20°C until use. Modified-HP1 beacons (IDT, Coralville, Inc., IA, USA) 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, USA). Measurements were taken in 5 $^{\circ}\text{C}$ decrements ranging from 90 to 5 $^{\circ}\text{C}$. Samples were allowed to equilibrate for 1 min at each temperature before a 25 ms measurement time. Volumes of 25 μ l each of the molecular beacon and individual targets were mixed to give a final concentration of 200 and 400 nM, respectively, in a total reaction volume of 50 μ l 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 50 μ l 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 MgCl₂ concentrations between 5 and 10 mM for the HP1 beacon. Data presented for solution hybridiza-

tion experiments has 3.5 or 5 mM MgCl₂, highlighting the effect of MgCl₂ 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 or 5 mM MgCl₂ concentration and the data were combined.

2.5. 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 μ l of 200 nM true target (CS11) in 3.5 mM MgCl₂/10 mM Tris buffer, pH 8 for 10 min. The fluorescence was recorded after washing the slide in 10 mM Tris buffer as described before. The slide was treated with 0.4 M sodium hydroxide solution for 1 min, washed in 10 mM Tris buffer and the decrease in fluorescence was recorded. The

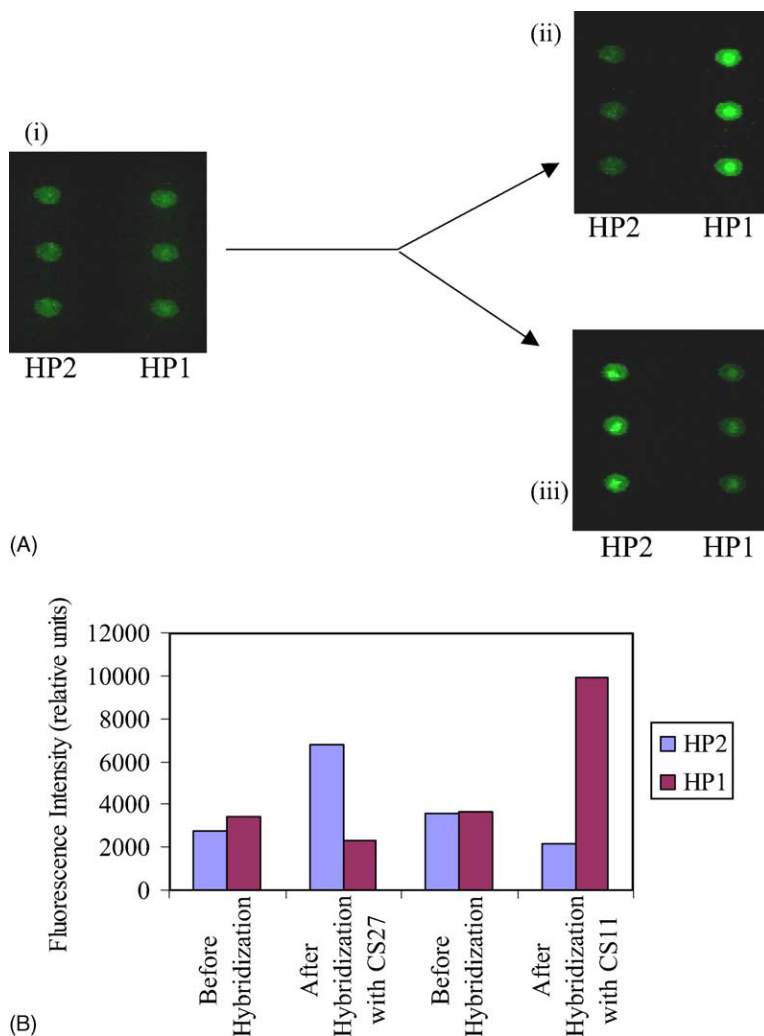


Fig. 2. Typical molecular beacon microarray background fluorescence before hybridization (i), and fluorescence after hybridization to specific targets, CS11 (ii) and CS27 (iii). The three spots in the left column are HP2 molecular beacon deposits, and those in the right column are HP1 molecular beacon deposits (A). Increase in fluorescence upon binding of molecular beacon probes to specific target sequences.

molecular beacons were then rehybridized with 200 nM true target, washed in Tris buffer and the fluorescence recorded again.

3. 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 Fig. 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:

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

where, R is the discrimination ratio, S_T the true target (CS11) signal, B_T the true target background signal, S_M the mismatch target signal, B_M the mismatch target background signal and α the scaling factor.

According to Eq. (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 two times 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 Fig. 3. Discrimination ratios presented are the average between three and six different runs in triplicate performed on different occasions. We intentionally designed

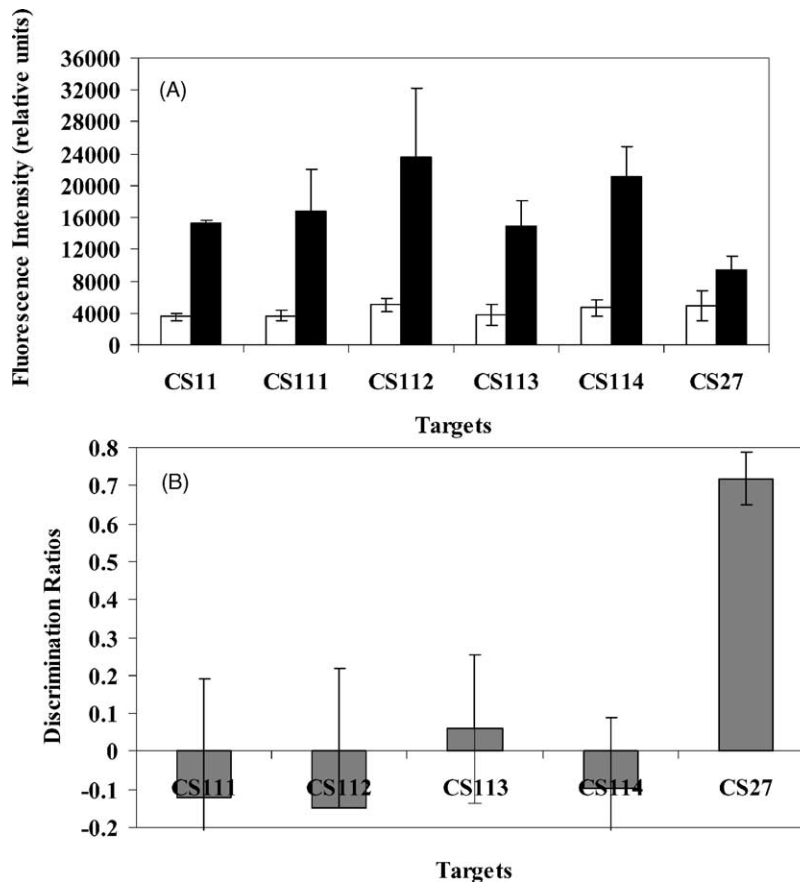


Fig. 3. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and single-nucleotide mismatch targets on a microarray platform. White bars denote fluorescence before hybridization and Black bars denote fluorescence after hybridization (A). Discrimination ratios (B).

and worked with targets that were slightly longer than the probe loop region and complementary to two additional base pairs in the stem region in order to assess effects 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 et al., 2002), and would possibly be more effective on a sensor type platform. No discrimination was noted for the single mismatch targets tested; CS111 ($R = -0.12 \pm 0.31$), CS112 ($R = -0.15 \pm 0.37$), CS113 ($R = 0.06 \pm 0.19$) and CS114 ($R = -0.10 \pm 0.19$). 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 Gibbs 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 ($R = 0.72 \pm 0.07$) 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 Fig. 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 ($R = 0.00 \pm 0.16$) and CS131 ($R = -0.27 \pm 0.42$), with two and three adjacent mismatches at the 5' end, respectively, were not readily distinguished from CS11. Target CS120 ($R = 0.05 \pm 0.12$) 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

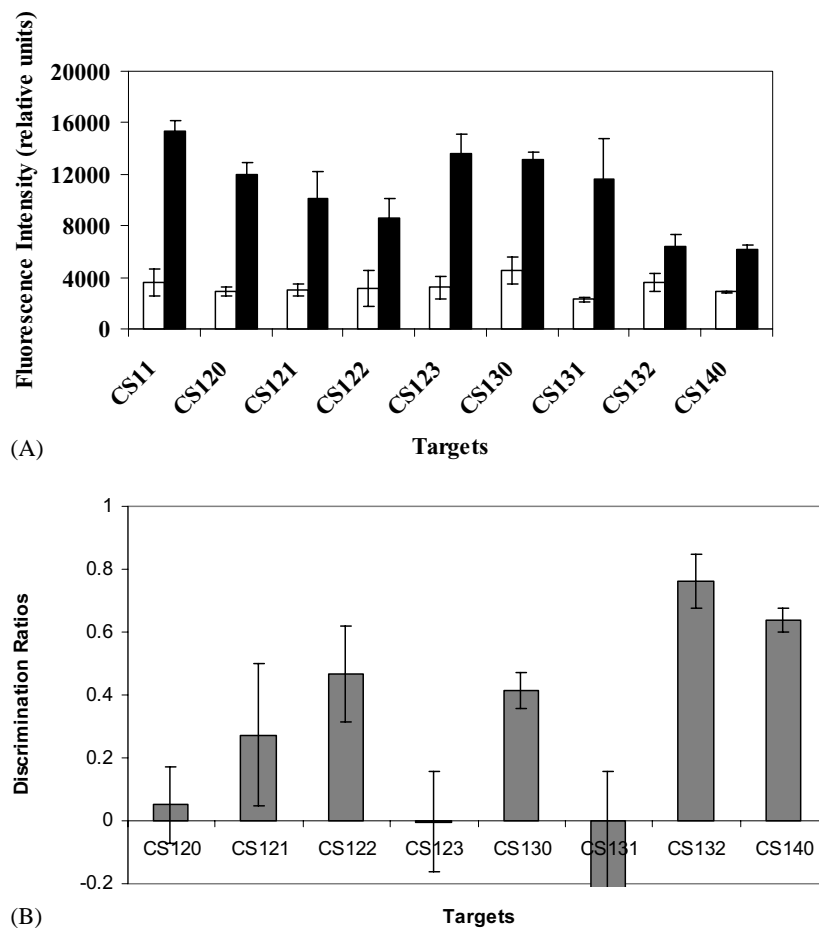


Fig. 4. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and multiple-nucleotide mismatch targets on a microarray platform. White bars denote fluorescence before hybridization and black bars denote fluorescence after hybridization (A). Discrimination ratios (B).

also apply to CS120. Target CS130 ($R = 0.41 \pm 0.06$) with three adjacent mismatches in the center of the loop region, however, was better discriminated than CS114 and CS120. The remaining mismatch targets, CS121 ($R = 0.27 \pm 0.23$), CS122 ($R = 0.47 \pm 0.15$), CS132 ($R = 0.76 \pm 0.09$) and CS140 ($R = 0.64 \pm 0.04$), having two to four non-adjacent mismatches were discriminated from CS11, with a trend of improved discrimination as the number of target mismatches increases.

Fig. 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. Gibbs 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 calcu-

lations; namely, CS11 ($72^\circ C$) > CS112 = CS113 = CS111 ($\sim 67^\circ C$) \gg CS114 ($60^\circ C$). 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 Fig. 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 \times HP1 duplex ($72^\circ C$). 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 $\sim 70^\circ C$, it is surprising that CS122 is not. If we consider the trend of the duplex melting temperatures, we find CS11 ($72^\circ C$) > CS123 ($64^\circ C$) > CS120 = CS131 ($62^\circ C$) > CS130 ($58^\circ C$) > CS122 ($56^\circ C$) > CS132 ($40^\circ C$) > CS140 ($38^\circ C$); fairly consistent

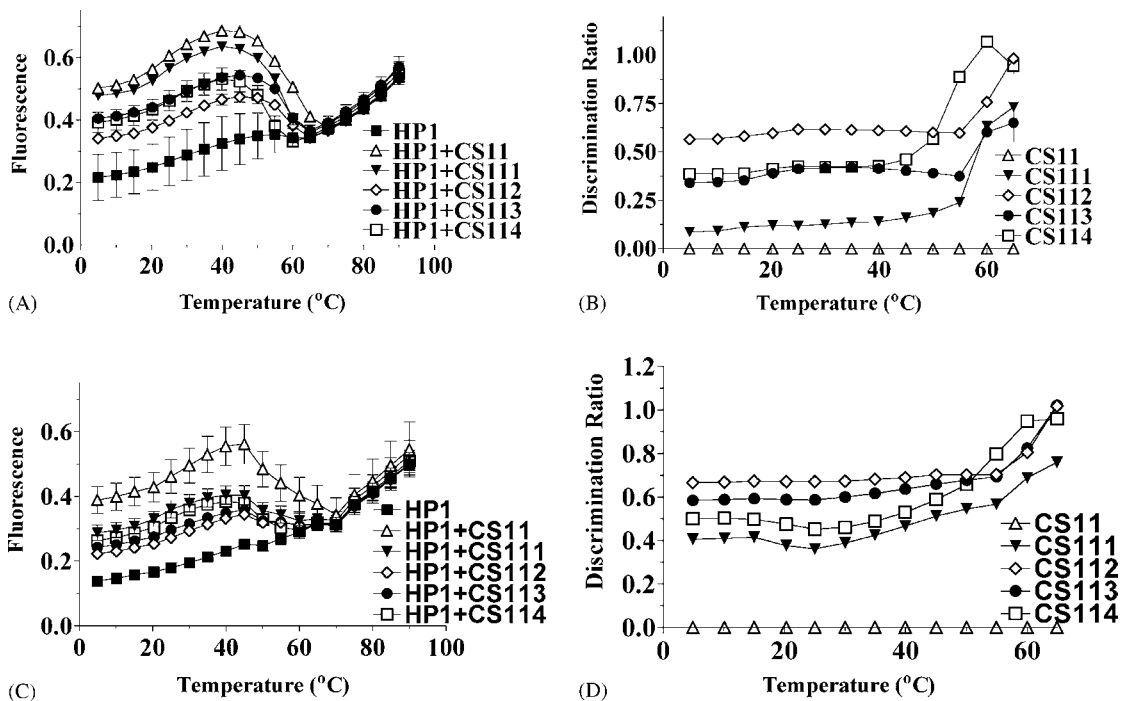


Fig. 5. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and single-nucleotide mismatch targets in solution, with respect to temperature, are shown at 3.5 mM $MgCl_2$ (A) and 5.0 mM $MgCl_2$ (C). Discrimination ratios corresponding to (A and C) are shown in panels (B and D),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

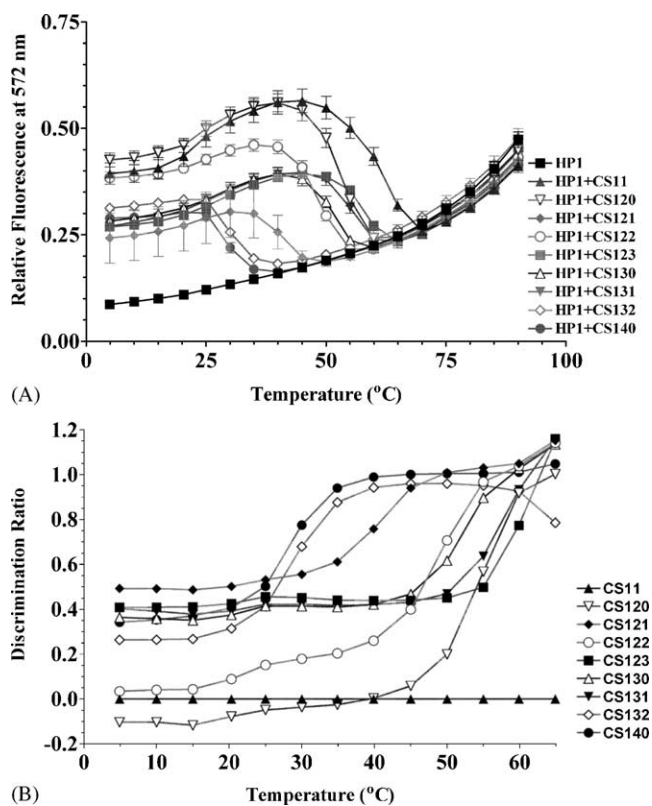


Fig. 6. Fluorescence changes upon hybridization of molecular beacon (HP1) with true and multiple nucleotide mismatch targets in solution, with respect to temperature, at 5 mM $MgCl_2$ (A) and corresponding discrimination ratios (B).

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 and 65 °C. Furthermore, double mismatch target discrimination ($\sim 50\text{--}90\%$, depending on mismatch location and number) was also improved in a temperature range between 55 and 65 °C just below the melting temperature of the CS11 \times 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 desirable 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. Fig. 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

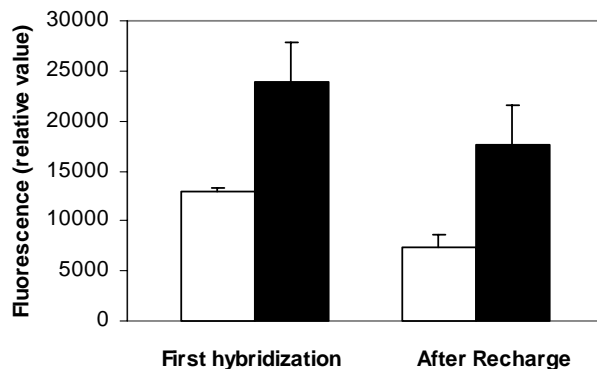


Fig. 7. Recharging of the molecular beacon (HP1) array by NaOH treatment. The white bars correspond to pre-hybridization fluorescence and the black bars correspond to post-hybridization fluorescence for both the initial and post recharge hybridizations.

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 $f = \exp(-\Delta G_{M-MM}/RT)$, where $\Delta G_{M-MM} = G_M - G_{MM}$ is the difference in Gibbs 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 $\sim 100\%$ 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 leading 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 (Department of Pathobiology, College of Veterinary Medicine, Oklahoma State University) for his input and help on the microarray experiments. We thank David W. Goad (Department 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).

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