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Molecular-beacon-based array for sensitive DNA analysis

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Abstract

Molecular beacon (MB) DNA probes provide a new way for sensitive label-free DNA/protein detection in homogeneous solution and biosensor development. However, a relatively low fluorescence enhancement after the hybridization of the surface-immobilized MB hinders its effective biotechnological applications. We have designed new molecular beacon probes to enable a larger separation between the surface and the surface-bound MBs. Using these MB probes, we have developed a DNA array on avidin-coated cover slips and have improved analytical sensitivity. A home-built wide-field optical setup was used for imaging the array. Our results show that linker length, pH, and ionic strength have obvious effects on the performance of the surface-bound MBs. The fluorescence enhancement of the new MBs after hybridization has been increased from 2 to 5.5. The MB-based DNA array could be used for DNA detection with high sensitivity, enabling simultaneous multiple-target bioanalysis in a variety of biotechnological applications.
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In the past decade, DNA arrays have become one of the leading methods for the investigation of biomolecular interactions, diagnosis of disease, and discovery of genes [1–9]. The major advantages of DNA arrays over conventional methods are their capability of simultaneous detection of different targets, their virtual automation, and their functional integration for high-throughput screening [4]. DNA arrays are mostly based on the hybridization of a target DNA with probe DNA strands immobilized on a chip surface. Generally, DNA fragments can be prepared on the substrate either through on-chip synthesis of nucleic acids or by attachment of presynthesized oligonucleotides [1,10]. Although each has its own advantages, presynthesized oligonucleotides are preferred and commonly used in DNA array production [1].

A variety of presynthesized DNA probes, including molecular beacons (MBs)[1] for DNA arrays, have been reported [11–14]. Molecular beacons are single-stranded oligonucleotide probes that possess a stem-and-loop structure [15–18]. The loop portion of an MB is complementary to a target single-stranded DNA, while the stem is formed by 5 to 7 bp from two complementary arm sequences that are on either end of the MB. A fluorophore is attached to the end of one arm, while a quencher is attached to the end of the other arm. The stem maintains a close proximity of the two moieties, causing fluorescence to be quenched by energy transfer. When an MB hybridizes with its complementary DNA (cDNA), the beacon undergoes a spontaneous conformational reorganization with the opening of the stem, leading to a fluorescence restoration. This built-in signaling property makes the MB a highly sensitive and selective DNA probe to report label-free targets. There are three primary advantages to the use of MBs over traditional fluorescent probes for the detection of specific gene targets [11–19]: (i) an inherent signal transduction mechanism for high sensitivity [15], (ii) the ability to detect target hybridization without separation of hybridized and nonhybridized probes, i.e., detection without separation [18], and (iii) an enhanced specificity over traditional linear DNA probes, i.e., single base mismatch distinguishing capability [15,18]. All three advantages are critical for DNA array development and applications. With the ability to increase its fluorescence intensity up to 200 times after hybridizing to its target

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[1] *Abbreviation used:* MBs, molecular beacons.

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[15], the MB serves as a highly sensitive, extremely selective, nonradioactive, and easily detectable probe for monitoring DNA hybridization and protein interactions. MBs have provided many new opportunities in DNA and protein studies [11–18].

We first reported the immobilization of an MB on a solid surface for biosensor development [11,12]. However, the immobilized MB had a fluorescence enhancement of only about 2, much less than that in a solution (best reported ~200×; generally around 25× [15–19]). This low enhancement factor has impeded exploration the full potential of MBs in applications in DNA arrays and biosensors. It is well known that an immobilized MB has a much lower activity than the same MB in solution [11–14], causing a greatly reduced bioanalytical sensitivity (fluorescence enhancement in analytical measurements) of the immobilized MBs after hybridization. Speculations on reasons for the reduced fluorescence enhancement focus on higher background signal and lower MB reactivity. Some surface-immobilized MBs are absorbed on the surface, thus destabilizing the MB stem-and-loop structure. These MBs will have much higher background fluorescence than closed MBs in solution. In addition, the liquid/solid surface interfacial effect can hinder the hybridization of an MB with cDNA if the MB is on or near the surface. It was thus our intent in this study to investigate the surface immobilization effects on DNA hybridization and to search for a better usage of MBs in DNA chip and biosensor development. We have designed new MB probes with different lengths of spacing between the MB and the solid support surface, investigated MB surface immobilization, studied the effects of surface linker length, pH, and ionic strength on the hybridization of immobilized MBs, and used the new array for sensitive DNA bioanalysis.

Materials and methods

Reagents

Molecular beacons, biotinylated linkers, complementary DNA, and noncomplementary DNA (ncDNA) were synthesized as summarized in Table 1. They were custom designed and then synthesized and purified by Integrated DNA Technologies Inc (Coralville, IA). Avidin was purchased from Molecular Probes (Eugene, OR). All chemicals were used without further purification. Tris–HCl buffer and ultrapure water (>18.3 MΩ/cm) were used in all experiments reported in this paper.

Preparation of molecular beacon array

The MBs were mixed with the biotinylated linker at a 1:1 ratio in a pH 8.0, 20 mM Tris–HCl, 3 mM $MgCl_2$ buffer solution to prepare biotinylated MBs for future use. A few cover slips (18 × 18 mm, Fisherfinest, Fisher, GA; Cat. No. 12548A) were cleaned with 10 M NaOH solution and thoroughly rinsed with deionized water. The treated cover slips were dried with compressed nitrogen and incubated with 1 mg/ml avidin solution in pH 7.1, 10 mM phosphate buffer for 12 h at 4 °C. After the cover slips were washed three times with deionized water and dried with nitrogen, 0.4-μl 100 nM biotinylated MB solutions were put on the avidin-immobilized cover slips and incubated at room temperature in a humid environment for 1 h. Four different MB probes were spotted on the cover slips in a 4 × 4 format. From top to bottom, the probes were MB1 (MB–linker 1 duplex), MB2 (MB–linker 2 duplex), MB3 (MB–linker 3 duplex), and MB4 (MB–linker 4 duplex). The unbound MBs were then washed away with deionized water and the cover slips were dried with nitrogen.

Table 1
Molecular beacon and oligonucletides

MB	5′-*TGGTGTGGTTGG*TTT(TEMRA-dC) CGAGCTGGATTAAGTATGCTGCTCGG-BHQ-3′
cDNA	3′-ACCTAATTCATACGACGATTT-5′
ncDNA	3′-AAAACATGACTATAGCTAGAA-5′
Linker 1	Biotin-3′-*ACCACACCAACC*-5′
Linker 2	Biotin-3′-$(T)_{10}$*ACCACACCAACC*-5′
Linker 3	Biotin-3′-$(T)_{20}$*ACCACACCAACC*-5′
Linker 4	Biotin-3′-$(T)_{30}$*ACCACACCAACC*-5′

Hybridization of molecular beacon array

After MBs were immobilized on the cover slip, 4 μl 100 nM DNA (or the corresponding buffer solution) in desired pH and ionic strength were placed on the MB spots and hybridized at room temperature in a humid environment for 2 h. The cover slips were washed and dried prior to fluorescent detection.

Imaging of molecular beacon array

The samples were imaged with a home-built fluorescence microscope setup, shown in Fig. 1. A 1.00-W 514-nm laser beam was produced from an Innova I307C argon ion laser (Coherent, Santa Clara, CA). After passing through a 60° light-shaping diffuser (Edmund Industrial Optics, Barrington, NJ), a 10× beam expander, and a focus lens, the laser beam was refocused onto the input end of a Fostec optical fiber light bundle and directed onto the sample stage. The emission signal was collected by a close-focus zoom lens (6 × CCD C-Mount Lens; Edmund Industrial Optics; Cat. No. A52-274) mounted on an ICCD camera (PentaMAX EEV 512 × 512 FT; Princeton Instrument, Trenton, NJ). A 580DF20 bandpass filter (Omega Optical, Brattleboro, VT) was used to select the desired fluorescence signal.

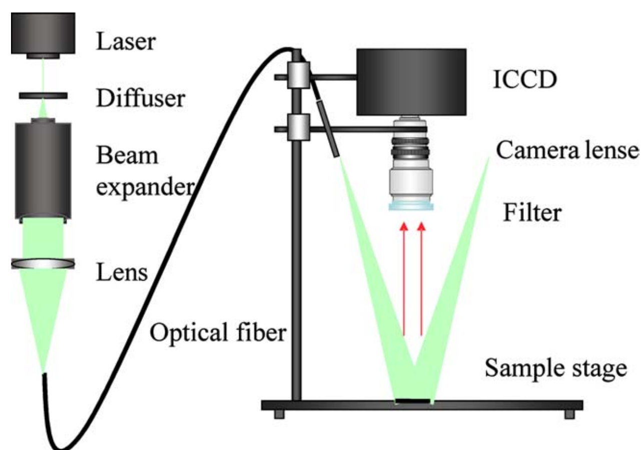

Fig. 1. Optical imaging setup for the molecular beacon DNA array.

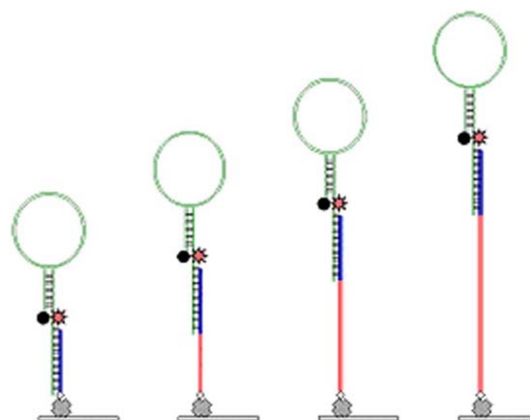

Fig. 2. Immobilized MBs with different linkers on the cover slips through biotin–avidin interaction.

The lens was 18 cm away from the sample stage for large-area optical imaging. The temperature control for the ICCD was set at −20 °C and the MCP gain on the ICCD was set at 80. The ICCD was controlled by WinView software (Roper Scientific, NJ) with an integration time of 500 ms. Images were analyzed by ImageJ software (NIH, http://rsb.info.nih.gov/ij/).

Results and discussion

Design of MBs for surface immobilization with different separation distances

When the MB is attached on the substrate by avidin–biotin, its hairpin structure can rotate randomly until being absorbed on the substrate through a nonspecific surface interaction. This adsorption will destabilize the MB hairpin structure and cause an increase of fluorescence intensity (background signal). To minimize the substrate surface effects on MB hybridization by a glass substrate surface, we need to prevent the MB from being absorbed on the surface and to have the MBs spaced adequately away from the surface. It has been reported that a linker arm between the glass surface and the terminus of the primer will enhance the efficiency of hybridization between immobilized DNA sequences and mobile DNA templates [20]. We have thus designed MBs with various linker lengths as shown in Fig. 2. The rationale is to use poly-T as a means to realize various separation distances between the MB probes and the surface. It is believed that poly-T is quite rigid [21] and the linker length is thus increased with the number of Ts in the strand. As shown in Table 1, the MB was designed with 15 bases in the loop and 6 bp in the stem. Tetramethylrhodamine was labeled at the 5′ terminal as the fluorophore and a black hole quencher was labeled at the 3′ terminal as the quencher. A 15-base sequence was extended from the 5′ end of the MB as a tail for hybridizing with the complementary sequence at the 5′ end of the linkers. Four different linkers were chosen in this study. Each linker had the same sequence at the 5′ end but a different length of poly-thymine at the other end (3′). Each linker was labeled with a biotin molecule at the end of the 3′ terminal. After the MBs were mixed with the biotinylated linkers, we were able to obtain biotinylated MBs with different linker lengths. Each biotinylated MB had a tail with a biotin molecule at the 3′ terminal. These MBs were immobilized on the avidin film on the cover slips through biotin–avidin interaction as shown in Fig. 2. Using these modified MBs, we prepared an MB-based DNA array. As shown in Fig. 3, there is an increased fluorescence intensity of the immobilized MB when it hybridizes with the target DNA. Before discussing the results of different factors on hybridization, we first define the fluorescence enhancement factor as the ratio of the net fluorescence intensity (background subtracted) of the hybrids (MB–cDNA, after hybridization) to that (background subtracted) of the MB alone (before hybridization). This definition is consistent with previous MB literature [11,12]. Two factors may contribute to improving this ratio for DNA analysis: more stable MBs on the surface, i.e., lower MB

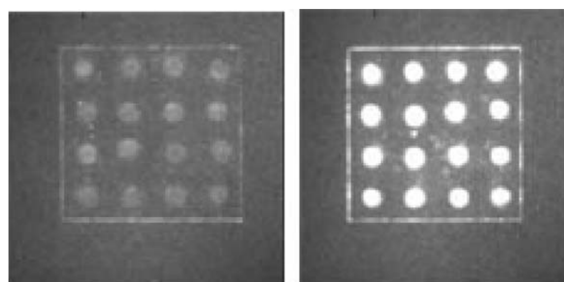

Fig. 3. Fluorescence images of the MB array before (left) and after (right) hybridization with the cDNA target. The images are obtained by imaging a 4 × 4 array area. The array was prepared by immobilizing MB2 on a glass surface.

background fluorescence intensity before hybridization, and higher activity of the immobilized MB, i.e., higher fluorescence intensity of the duplex after hybridization.

Effect of pH on the molecular beacon array

pH is one of the most important factors that can affect the performance of DNA arrays. In most published experiments, a pH around 8 is chosen for the hybridization buffer solution [2,7,12,14,22]. Here we investigated the effect of pH on the fluorescence intensity of immobilized MB and MB–cDNA duplex by performing the hybridization in 20 mM Tris–HCl, 100 mM $MgCl_2$ solution with different pH levels (pH at 6.0, 7.2, 8.2, 9.1, and 9.9). As shown in Fig. 4, the fluorescence intensity of the immobilized MB decreases with increasing pH for all four linker lengths. In contrast, the fluorescence intensity of the MB–cDNA hybrids increases slightly under the same conditions. It should be mentioned that all the fluorescence intensity data shown in the Figures of this paper are unmodified original data, which included the fluorescence of each MB and the substrate. In each Figure, all the data points came from the same substrate and had the same background, so that the effect on the (unmodified) fluorescence intensity is the same as that on the net fluorescence intensity. Fig. 4 also shows that pH plays an important role in the fluorescence enhancement factor, which by definition is the ratio of the net fluorescence intensity of the hybrid to that of the MB. As the pH was increased from 6.0 to 9.9, the fluorescence enhancement factor increased from 2 to 5.5, suggesting that an appropriate selection of pH can increase the sensitivity of the MB array.

Like conventional MBs in bulk solution, immobilized MBs are supposed to be in a closed form and to have relatively weak fluorescence. However, the surface property of avidin-coated glass is quite complicated and, in fact, can interact nonspecifically with the backbone of the immobilized MB and hence destabilize the MB hairpin structure. By changing the pH of the solution, the surface property will be changed. Avidin is a highly cationic glycoprotein (66 kDa) with an isoelectric point of about 10.5 [23]. Its positively charged residues and its oligosaccharide component can interact nonspecifically with the negatively charged MB DNA probes. As the pH of the hybridization buffer increases, the net positive charge of the avidin will decrease, thereby weakening the electrostatic interactions between the MB and the avidin surface. Using a higher pH, therefore, has two distinct advantages for immobilized MB arrays. First, the fluorescence intensity before hybridization results solely from the immobilized MB. Less surface adsorption,

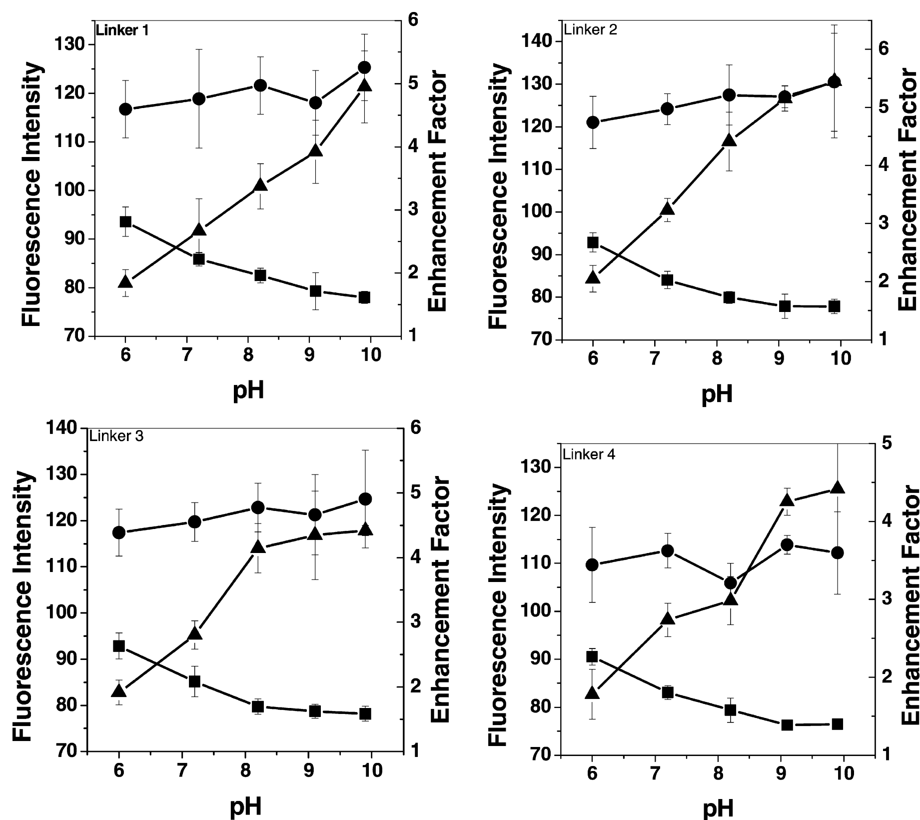

Fig. 4. Effect of pH on the MB array with different linker lengths (■, fluorescence intensity of MB; ●, fluorescence intensity of MB–cDNA; ▲, enhancement factor).

resulting from a higher pH, enables easier maintainance of the MB closed structure. This will result in lower background fluorescence (Fig. 4). Second, the lower adsorption will also increase the efficiency of hybridization. The less an MB is adsorbed on the substrate, the easier it can hybridize with target DNA, resulting in an increase of the fluorescence intensity of the hybridized MB. Because each MB array was made at the same working condition, the total numbers of immobilized MBs were the same at different pH conditions. The fluorescence intensity of those hybridized MBs will compensate the decreased fluorescence intensity due to less adsorption (fewer denatured MBs), which presents a slight increase in the total fluorescence intensity after hybridization (Fig. 4). With an increase in the fluorescence intensity after hybridization and a decrease in the background fluorescence intensity, the fluorescence enhancement factor will thus be considerably higher as the pH increases.

Effect of ionic strength on the molecular beacon array

It has been reported that ionic strength, especially the concentration of divalent cations, will affect the hybridization of an MB with cDNA [11,14,15]. Here we studied the effect of $MgCl_2$ concentration on the immobilized MB array. Hybridization was performed at pH 9.9, in a 20 mM Tris–HCl solution with different $MgCl_2$ concentrations (0, 3, 50, 100, and 200 mM). It can be seen from Fig. 5 that no fluorescence change could be observed in the absence of $MgCl_2$, suggesting that the hybridization of the MB with its target DNA was greatly hindered without $MgCl_2$. The fluorescence intensity of an immobilized MB was found to decrease with the increase of $MgCl_2$ concentration from 3 to 200 mM, while that of the MB–cDNA was enhanced when the $MgCl_2$ concentration was increased from 0 to 100 mM. When the $MgCl_2$ concentration was further increased to 200 mM, the fluorescence intensity of MB–cDNA was decreased instead. As shown in Fig. 5, the fluorescence enhancement factor was increased from 1 (implying no hybridization) to 5.5 upon increasing the $MgCl_2$ concentration from 10 to 100 mM, and the enhancement factor was decreased to 4 upon further increasing the $MgCl_2$ concentration to 200 mM. This result suggested that ionic strength was also important in the performance of the MB DNA array.

We have kept $MgCl_2$ concentration at 100 mM and further studied the ionic effect by increasing KCl concentration. Hybridization was performed at pH 9.9, in a

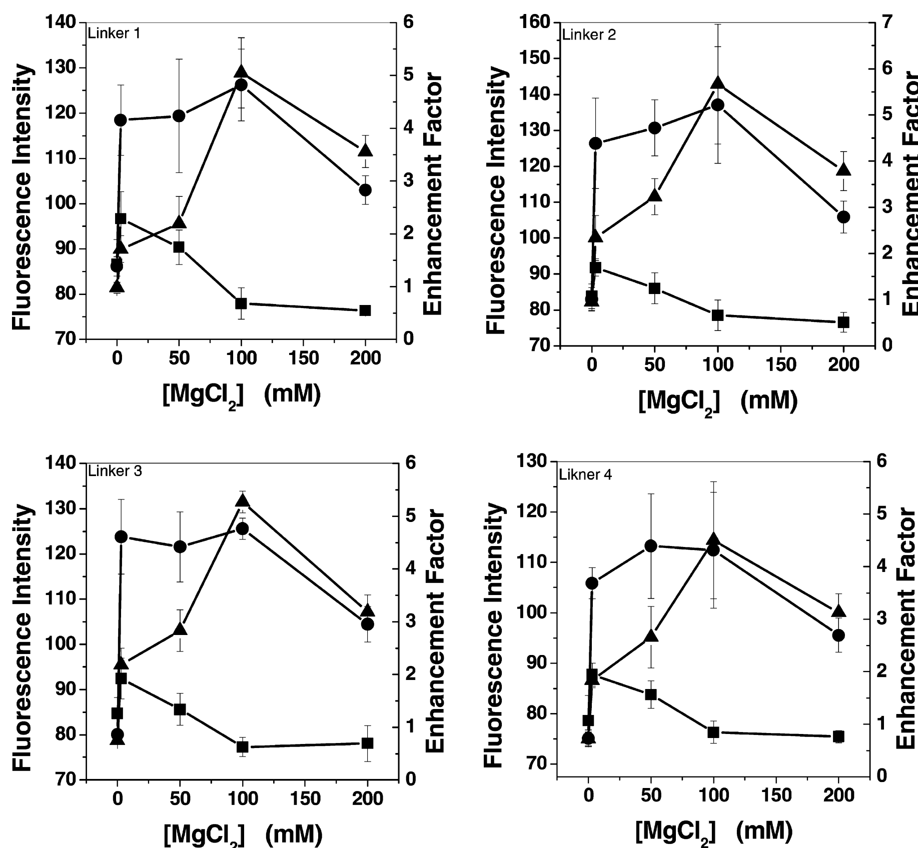

Fig. 5. Effect of $MgCl_2$ on the MB array with different linker lengths (■, fluorescence intensity of MB; ●, fluorescence intensity of MB–cDNA; ▲, enhancement factor).

20 mM Tris–HCl, 100 mM $MgCl_2$ solution with different KCl concentrations (0, 100, 200, 300, and 400 mM). We found that the fluorescence images of an immobilized MB became blurry and uneven with KCl concentration above 200 mM (data not shown). Also, the fluorescence intensity of MB–cDNA decreased with increase of KCl concentration.

The effect of ionic strength on the MB array can be explained based on charges. By increasing the salt concentrations, the metal cations can stabilize the negatively charged MB backbones, hence helping maintain the MB stem-and-loop structure. The stable stem structure contributes to the lower background fluorescence intensity of the MB array. Meanwhile, those metal ions can decrease the electrostatic repulsion between the negatively charged MB chain and the negatively charged target DNA chain, consequently benefiting their hybridization. However, further increasing ionic strength can cause cations to be enriched on the MB and target DNA. The positively charged chains will again repulse each other and retard the hybridization process. In summary, the stabilization of the MB and MB–cDNA duplex is optimized at a certain ionic strength, beyond which MB stability hinders the hybridization with cDNA.

Effect of linker length on the molecular beacon array

The effect of linker length can be viewed from two different angles. The first angle is whether a linker is necessary for an MB intended for surface immobilization, and the second angle is what constitutes the best linker length. As discussed before, the best enhancement factor for surface immobilization of previous MBs was around 2. As shown in Figs. 4 and 5, the enhancement factor could be as high as 5.5 under optimal conditions and mostly higher than 2 under a variety of experimental conditions. This clearly shows that the added linker in MBs used in this study does improve MB performance in detecting targets. However, the linker length cannot be too long. As shown in Figs. 4 and 5, under all working conditions that we tested, the fluorescence intensity of an immobilized MB decreased slightly with linker length (15 bases for MB1, 25 bases for MB2, 35 bases for MB3, and 45 bases for MB4). On the other hand, the fluorescence intensity of MB–cDNA hybrids increased with linker length from MB1 to MB2 and then decreased when linker length was further increased from MB2 to MB4. The highest MB array sensitivity can be obtained with a linker length of 25 bases for all tested samples and under different experimental conditions.

It has been reported that the length (or the linker length) of the immobilized linear DNA plays an important role in hybridization [3,24]. The observation of the length effect on the hybridization of an immobilized MB with its complementary DNA was not surprising. A long linker helps the MB reduce its adsorption onto the surface. This will also make it easier for an MB to react with its target DNA. However, when poly-T was used as the linker in our experiments, a higher negative charge close to the surface area was built up when the linker length was increased, which could repel the negatively charged target DNA and reduce the formation of MB–cDNA duplex. Both effects have contributed to the MB-based assay and resulted in an optimal linker length in our current experiments. A neutral straptavidin would reduce this potential problem in the immobilization.

Sensitivity and selectivity of the molecular beacon array

Based on the results obtained from all the abovementioned experiments, MB2 was chosen to prepare the array and a pH 9.9, 20 mM Tris–HCl, 100 mM $MgCl_2$ solution was used as the buffer for MB hybridization. To investigate the sensitivity and selectivity of our MB array, complementary and random DNAs were used. A clear difference between MB–cDNA (Fig. 6A, row 2) and MB (Fig. 6A, row 1) can be observed at a cDNA concentration of 1 nM. Little difference can be found between MB and MB/noncomplementary DNA (Fig. 6B, row 3). As shown in Fig. 6A, the fluorescence increase resulting from the addition of 1 nM complementary DNA was 56 times higher than that by addition 1 nM noncomplementary DNA. The use of 5 nM cDNA will enable easier differentiation of fluorescence intensities on the array. The high sensitivity makes our MB array an excellent technique for label-free, simultaneous multiple-target analysis.

It is worth noting that the error bars in Figs. 4 and 5 are relatively large. There are three potential reasons for this. First, the automation of our array is minimal. We handled all samples manually, and there was a reproducibility problem. Second, the immobilization of the MBs on the array surface could produce irreproducibility between different arrays. Third, our optical measurement could be a source of error as it was a homemade setup. It is highly sensitive but may not be rigid enough to give reproducible measurements with low concentrations such as those used in this study. These problems will be addressed in future research to improve the reproducibility of the arrays.

This study demonstrates a new method to optimize the preparation of MB arrays with a significantly increased enhancement factor. However, we realize that this enhancement needs to be even larger for situations where only a few copies of the target DNA/RNA exist. The enhancement factor will be further improved with better monitoring techniques such as those used in our single immobilized MB detection [25] or with a gold surface used for both immobilization and quenching [26]. Our study of the linker length effect on MB immobilization should be useful in improving the fabrication and application of MB arrays.

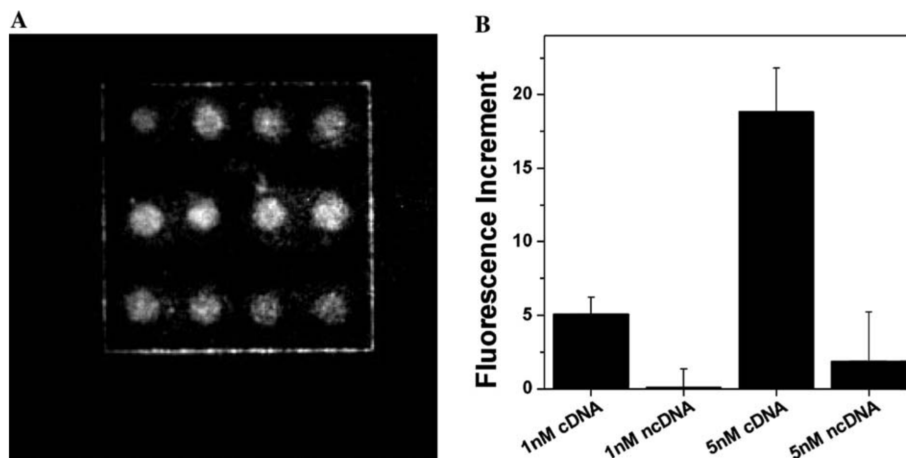

Fig. 6. Sensitivity and selectivity of the MB array. (A) Fluorescence image of the MB2 array after hybridization in a pH 9.9, 20 mM Tris–HCl, 100 mM $MgCl_2$ buffer with 1 nM cDNA (row 1, MB; row 2, MB–cDNA; row 3, MB–noncomplementary DNA). (B) Fluorescence enhancement of cDNA and noncomplementary DNA (vertical sticks represent the standard deviation).

Conclusion

Its unique properties have made the molecular beacon an excellent probe for label-free gene detection in biochip and biosensor applications. We have developed a modified surface-immobilization strategy for the preparation of molecular-beacon-based DNA arrays. To minimize surface effects, we used different length linkers for MB immobilization. The biotin-labeled molecular beacon probes are immobilized on an avidin-coated glass surface for DNA array development. The molecular-beacon-based array has been studied by characterizing the effects of pH, ionic strength, and linker length. It is clear that the new molecular beacons have much higher enhancement after hybridization than previous molecular beacons (from about 2 to 5.5). A 25-base linker is the optimal length for MB hybridization under our experimental conditions. If the linker length is shorter than 25 bases, the MB array sensitivity increases with linker length. Further increasing the linker length results in a decrease of the sensitivity. We have demonstrated that the newly developed MB array can be used for DNA detection and with multiple DNA targets.

Acknowledgments

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