

Ultrasensitive Optical DNA Biosensor Based on Surface Immobilization of Molecular Beacon by a Bridge Structure

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A novel biotinylated molecular beacon (MB) probe was developed to prepare a DNA biosensor using a bridge structure. MB was biotinylated at the quencher side of the stem and linked on a biotinylated glass cover slip through streptavidin, which acted as a bridge between MB and glass matrix. An efficient fluorescence microscope system was constructed to detect the fluorescence change caused by the conformation change of MB in the presence of complementary DNA target. The proposed biosensor was used to directly detect, in real-time, the target DNA molecules. The bridge immobilization method caused the proposed DNA biosensor to have a faster and more stable response. Under the optimal conditions, the newly developed DNA biosensor showed a linear response toward ssDNA in the range of 5 - 100 nM with a detection limit of 2 nM. It was interesting to note that the described biosensor was reproducible after being regenerated by urea.

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The development of DNA biosensors is motivated by their potential applications in diagnosis and genome mutation.¹ DNA hybridization biosensors offer considerable promise for obtaining sequence-specific information in a faster, simpler and less expensive manner compared to traditional hybridization assay.² In the past decade, a few DNA biosensors have been developed based on the hybridization of immobilization DNA and its target oligonucleotide at the surface of modified electrode,⁴ piezoelectric quartz crystal microbalance,⁵ surface plasmon resonance,^{6,7} optical fiber⁸ and planar waveguide.⁹ The customary fabrication procedures of these sensors usually include two steps: (1) the chemical modification of polymer or the activation of the matrix, and (2) the immobilization of single-stranded DNA on the sensor surface.⁸ Hence, the biosensor can determine its target in the sample. Because these sensors take advantage of the biospecific interactions, the selectivity is very high.

The optical technique has significantly enhanced the capability of DNA-based biosensors due to its high sensitivity.^{10,11} However, there are several critical limitations in optical biosensors, such as the need for labeling targets or an intercalation reagent. These limitations make it difficult to carry out real-time hybridization studies and to quantitatively monitor hybridization kinetics at an interface.

The molecular beacon (MB) was a new DNA fluorescence probe which is a kind of single-stranded oligonucleotide consisting of a probe sequence embedded within complementary sequences that form a hairpin stem.¹²⁻¹⁴ A fluorophore and a quencher are linked to the two ends of the stem, which keeps these two moieties in close proximity, causing the fluorescence of the fluorophore to be quenched by energy transfer. When the probe encounters a target DNA molecule, the MB undergoes a spontaneous conformational reorganization that forces the stem apart, leading to the

restoration of fluorescence. A series of optical DNA biosensors have been proposed, based on MB as the probe.¹⁵⁻¹⁹ For the development of DNA biosensor, MB was modified by biotin and immobilized on optical fiber core surfaces by absorbed streptavidin. However, the absorption method for streptavidin immobilization is not very efficient. Later, Klenerman described a new method to immobilize biotinylated DNA.²⁰ A glass cover slip was biotinylated and streptavidin-functionalized microspheres were deposited onto the biotinylated matrix. Then biotinylated DNA was linked to the microspheres and streptavidin-functionalized microspheres acted as the "bridge" of the glass and biotinylated DNA.

In this paper, an optical DNA biosensor based on streptavidin-biotin interaction was proposed by the bridge immobilization method, which obviously enhanced the freedom of the immobilized MB. The streptavidin was immobilized on the glass cover slip through the absorption of albumin bovine serum-biotin and connected to the biotinylated MB. The real-time response of the DNA biosensor was recorded by a PMT. The proposed sensor will be used to quantitatively detect its complementary DNA.

Experimental

Materials

MB (5'-TMR-CCT AGC TCT AAA TCG CTA TGG TCG CGC (biotin dT) AG G-DABCYL-3') and its complementary single-stranded DNA (5'-GCG ACC ATA GCG ATT TAG A-3') were synthesized by TriLink BioTechnologies, Inc. (San Diego, CA). The one base mismatch oligonucleotide (5'-GCG ACC ATA TCG ATT TAG A-3') and noncomplementary oligonucleotide (5'-GCG ATG GTG TGG ACA TTC A-3') were synthesized by Shanghai Shengong Biotechnology Co. (Shanghai, China). The structure is shown in Fig. 1. Tetramethylrhodamine (TMR) was labeled as the fluorophore and (4-dimethylaminophenylazo)benzoyl (DABCYL) was

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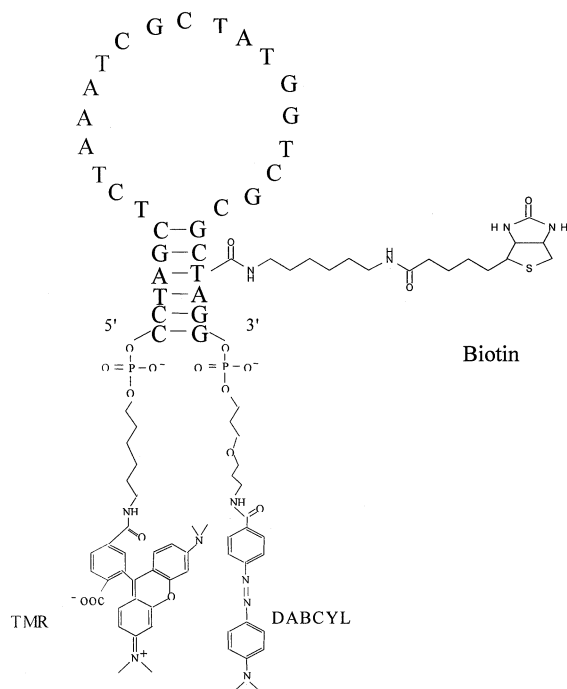


Fig. 1 The structure of biotinylated MB. 5'-TMR-CCT AGC TCT AAA TCG CTA TGG TCG CGC (biotin dT) AG G-DABCYL-3'.

added as the quencher. Biotin was linked to the quencher end of the stem of the MB. Albumin bovine serum-biotin (BSA-biotin) and affinity-purified streptavidin were purchased from Sigma (St. Louis, MO). A batch of silica optical fibers (ϕ 330 μ m) was obtained from Beijing Glass Institute (Beijing, China). Glass cover slip (22 \times 22 mm) was supplied by Fisher Scientific Co. (Pittsburgh, PA). All chemicals were analytical reagents and were used without further purification. Double distilled water was used throughout.

Immobilization of MB on glass cover slip

The immobilization of MB through a bridge structure is illustrated in Fig. 2(A). A piece of glass cover slip was rinsed in 1 mol/L aqueous ammonia and washed with water for several times. The activated glass was biotinylated by incubation for 12 h with 1 mg/mL BSA-biotin solution (in 20 mM phosphate buffer, pH = 7.0). The biotinylated glass was rinsed with water to remove the unabsorbed BSA-biotin. A drop of 1 mg/mL streptavidin in 20 mM phosphate buffer (pH = 7.0) was deposited on the glass cover slip, the system was incubated for 1 h, then washed with a lot of water to remove the unbound streptavidin. Then a drop of 50 μ L of 1 μ M MB solution buffered with 20 mM phosphate solution (pH = 7.0) was deposited on the streptavidin-functionalized glass overnight at 4°C to allow the biotinylated MB to be immobilized on the surface. The prepared sensor was stored in 10 mM phosphate buffer at 4°C for future use.

The direct immobilization of streptavidin on the glass was carried out according to reference¹⁸ and as shown in Fig. 2(B). A drop of 1 mg/mL streptavidin buffered with 20 mM phosphate was deposited on a glass cover slip for 12 h at 4°C. After washing with buffer several times, the streptavidin was stabilized by crosslinking with glutaraldehyde (1%) for 1 h at 4°C, followed by incubating in buffer for 3 h at 4°C to remove the unbound streptavidin. The following steps for the immobilization of biotinylated MB were a similar procedure.

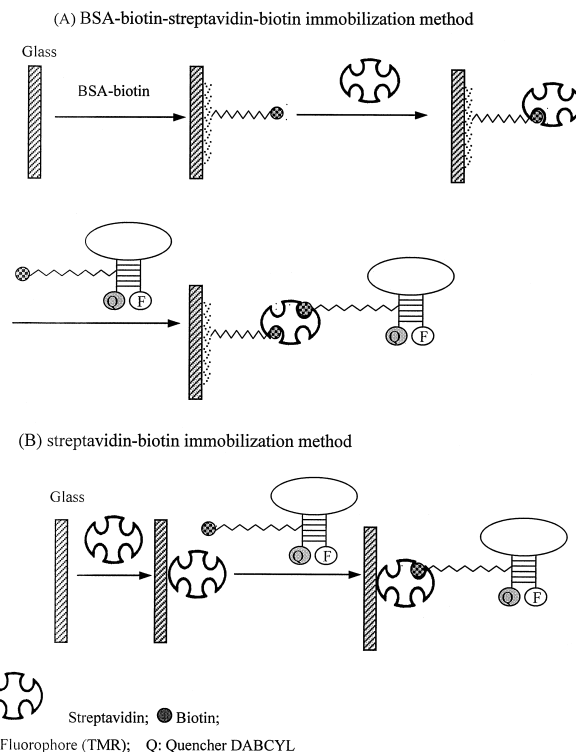


Fig. 2 Schematic diagram of the immobilization of biotinylated molecular beacon on glass cover slip.

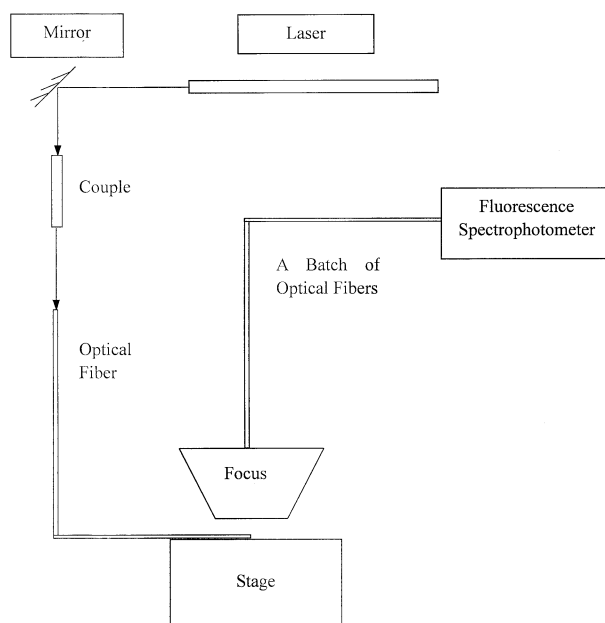


Fig. 3 The schematic diagram of the measurement system.

Optical instrumentation and procedure

To detect the fluorescence of the MB immobilized on the glass cover slip, an efficient optical system was built as shown in Fig. 3. The source for the excitation light was a 530 nm laser beam from a He-Ne laser (Melles Griot Laser Division, Carlsbad, CA). The laser beam was directed to an optical fiber through an optical fiber couple (Newport Corp, Irvine, CA). The other end of the optical fiber was directly pointed at the

glass cover slip on the stage of an E600 fluorescence microscope (Nikon, Japan). The emission light was collected via quartz microscope objectives with either 10× or 40× magnification and transmitted by a dichroic mirror. To specifically select the fluorescence signal, a 575 nm interference filter was put in front of the objective lens. One end of a batch of optical fibers was mounted on the top entrance port of the microscope. The other end of the fiber was directed to the PMT of an F2500 fluorescence spectrophotometer (Hitachi, Japan) to record the emission light. The modified glass cover slip was stabilized on the stage of the fluorescence microscope. After the sample was washed with hybridization buffer (20 mM Tris-HCl, pH 8.0, 100 mM MgCl₂), a 50 μL volume of a complementary oligonucleotide sample was added, and the sensor's response was monitored over time. In order to minimize photobleaching, a shuttered excitation source was applied during data acquisition. The initial reaction rates were used to characterize the sensor's activity, these rates were calculated using the linear portion of the hybridization dynamic curve over time. All of the measurements were carried out at 25°C with an air conditioner to maintain the hybridization temperature.

Results and Discussion

Immobilization of molecule beacon on glass cover slip

The affinity constant of the streptavidin-biotin system was $K = 10^{15} \text{ M}^{-1}$. Hence, the most important step for the fabrication of the sensor was how to immobilize the streptavidin on the glass cover slip. Streptavidin is a kind of protein. The conventional methods for the immobilization of a protein included adsorption, covalent bind and entrapment by polymer. In order to reduce the steric hindrances, a new bridge connection method was proposed to immobilize MB. BSA conjugating with biotin was absorbed on the glass cover slip. Then streptavidin was deposited on the biotin-modified glass surface and bound with biotin on BSA. It was known that one streptavidin molecule had four binding sites to biotin.²¹ So the immobilized streptavidin could use another binding site to interact with the biotin conjugated on MB. The streptavidin served as a bridge between the glass surface and the biotin-modified MB. The hybridization would complete in 10 min. However, the response time was 20 min for the direct immobilization of streptavidin on the glass cover slip. This phenomenon may be explained by a process in which the bridge structure reduced the steric hindrances associated with biotinylated MB. Then the target DNA could easily bind the immobilized MB after reaching the sensor surface. Moreover, the enhancement efficiency could reach 2.7 times for bridge immobilization and only 1.6 times for direct immobilization (Fig. 4).

The optimum of the hybridization condition

Since there are phosphate groups on the oligonucleotide, single-strand DNA will dissociate in the solution and process negative charge, which would hinder the formation of a duplex for the electrostatic repulsion. The presence of cations in solution could counteract the negative charge on the oligonucleotide and accelerate the hybridization reaction. So the ionic environment was carefully studied to find the optimum hybridization condition. The influence of different cations on the stem stability of the immobilized MB is shown in Table 1. The residual fluorescence on the MB was due to the incomplete quenching. The more stable the stem structure in a MB was, the

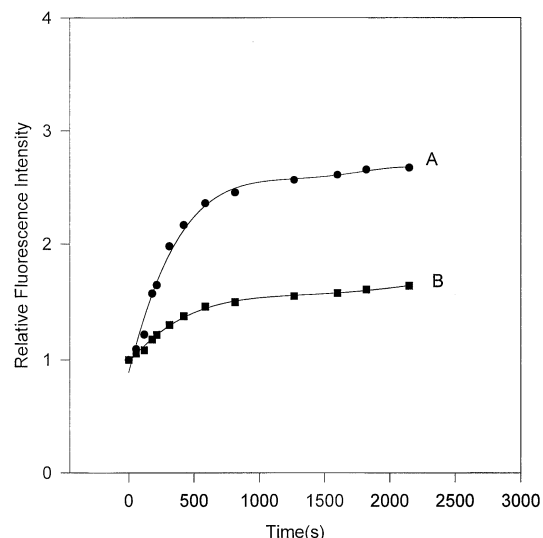


Fig. 4 The effect of different immobilization methods on the response: (A) BSA-biotin-streptavidin-biotin immobilization method; (B) streptavidin-biotin immobilization method. [cDNA] = 50 nM. All experiments were performed in hybridization buffer of 20 mM Tris-HCl, pH = 8.0.

Table 1 The fluorescence of immobilized MB on the condition of different cations, 20 mM Tris - HCl (pH = 8.0) as the buffer

	Ion concentration/mM	
	100	500
K ⁺	270	227
Na ⁺	260	250
Mg ²⁺	180	170
Ca ²⁺	168	145

Buffer with no cation: 420 mM.

less fluorescence yielded from MB. The experimental results showed that the divalent cations played a much more important role in forming stem structure than did the monovalent ions.

We further investigated the influence of Mg²⁺ concentrations on the hybridization of the immobilized MB with its target DNA. The results (Fig. 5) showed that the hybridization efficiency was greatly improved when the Mg²⁺ concentration was increased. All these would be simply explained if the presence of cations decreased the electrostatic repulsion between anionic chains of MBs and the target nucleic acid in the solution. But when the concentration of MgCl₂ was above 100 mM, there was little effect when a higher concentration of MgCl₂ was used. For example, the dynamic responses of the biosensor were analogous when 100 mM and 200 mM MgCl₂ were added in the hybridization solution.

The response of the MB biosensor to its target DNA molecules

The complementary DNA can directly hybridize with the immobilized MB on the glass cover slip at the optimum condition. Because the number of immobilized MB was fixed for the same-sized glass matrix, the formed duplex may be analogous for the different concentrations of cDNA. But the initial reaction rate was proportional to the concentration of complementary oligonucleotide. So initial reaction rates were used to characterize the response. The hybridization rate was

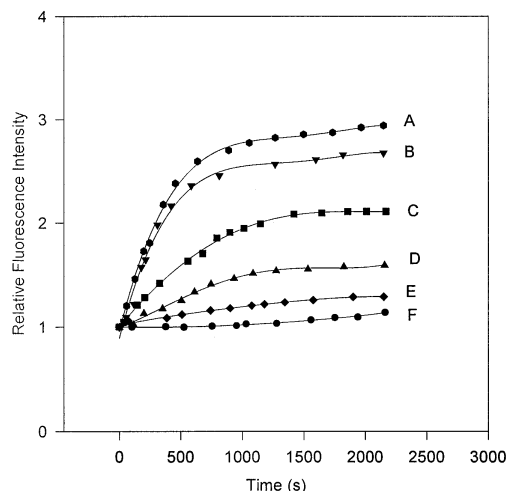


Fig. 5 The effect of ion concentration on the hybridization reaction, [cDNA] = 50 nM. All experiments were performed in hybridization buffer of 20 mM Tris-HCl, pH = 8.0. The concentrations of MgCl₂ were: (A) 500 mM; (B) 100 mM; (C) 10 mM; (D) 1 mM; (E) 0.1 mM; (F) no salts.

linearly increased with the increasing of target oligonucleotide concentration. A linear calibration response curve could be described by the following equation in the range 5 - 125 nM.

$$Y = 0.3525C + 1.890$$

Here Y was the initial reaction rate, C was the concentration of complementary DNA (nM), the linear relative coefficient $r = 0.9931$. The detection limit was approximately 2 nM.

Selectivity of the DNA biosensor

The ability to discern the single base mutation was an important characteristic for a DNA biosensor. The hybridization of the oligonucleotides to the MB immobilized on the glass cover slip was investigated and the real time response curve was recorded by a fluorescence spectrophotometer. Figure 6 shows the hybridization process of the DNA biosensor with complementary oligonucleotide (5'-GCG ACC ATA GCG ATT TAG A-3'), one base mismatch oligonucleotide (5'-GCG ACC ATA TCG ATT TAG A-3') and noncomplementary oligonucleotide (5'-GCG ATG GTG TGG ACA TTC A-3'). It was found that there was no significant fluorescence enhancement for noncomplementary DNA. When one base mismatch DNA was used, a weak fluorescence enhancement was obtained. This indicated that one base mismatched DNA also could hybridize with the immobilized MB, but the duplex was too weak to restore the fluorescence completely. The specificity of MB was higher than that for a linear probe, mainly due to the hairpin stem structure of the MB.

The hybridization reaction between DNA probe and target oligonucleotide could be expressed by the following equation:



The association constant (K_a) was defined as:

$$K_a = \frac{[\text{hybrid}]}{[\text{target}][\text{probe}]} = \frac{[v]}{[\text{target}][v_{\max} - v]}$$

Here v represented the initial reaction rate of the hybridization

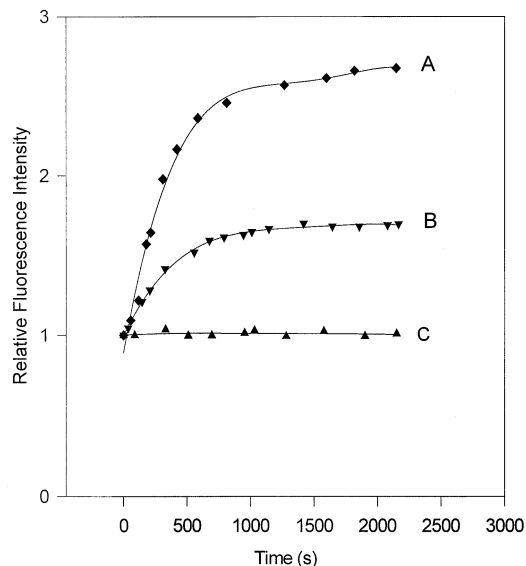


Fig. 6 The response of immobilized MB to (A) 50 nM complementary oligonucleotide; (B) 50 nM one base mismatch oligonucleotide; and (C) 50 nM noncomplementary oligonucleotide. All experiments were performed in hybridization buffer of 20 mM Tris-HCl, pH = 8.0.

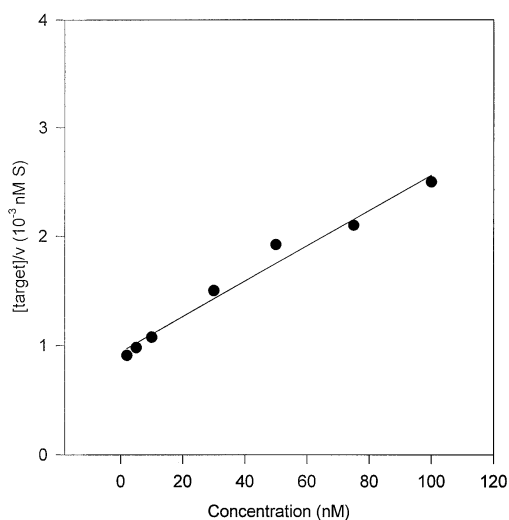


Fig. 7 Linear reciprocal plot of $[\text{target}]/v$ against $[\text{target}]$.

reaction between the immobilized MB and detected DNA and v_{\max} indicated the maximum initial reaction rate of the hybridization reaction. Because the amount of immobilized MB was very small, the hybridized DNA was also very small. Thus the equilibrium concentration could be considered as the initial concentration $[\text{target}]_0$. So the above equation could be written as:

$$\frac{[\text{target}]_0}{v} = \frac{[\text{target}]_0}{v_{\max}} + \frac{1}{v_{\max}K_a}$$

Reciprocal plots between $[\text{target}]/v$ and $[\text{target}]$ gave a simple straight line. The association constant and the maximum initial reaction rate were calculated from the slope and the linear correlation of Fig. 7, respectively. The results showed that K_a was equal to $1.725 \times 10^5 \text{ M}^{-1}$, which was smaller than the results

of DNA biosensor based on quartz crystal microbalance.² This may be caused by the loop-stem structure of MB.

Using a similar method, we also investigated the association constant (K_a) of the hybridization reaction between immobilized MB with one base mismatch oligonucleotide and noncomplementary oligonucleotide. The calculated K_a values were $3.8 \times 10^4 \text{ M}^{-1}$ and $8.6 \times 10^3 \text{ M}^{-1}$, respectively. This indicated that the proposed MB biosensors have 5 and 20 times selectivities toward one base mismatch oligonucleotide and noncomplementary oligonucleotide.

The regeneration and stability of MB based biosensor

A reusable biosensor is very important for practical applications. Thermal and chemical regeneration schemes involving prolonged incubation in hot water or 50% urea, are commonly used for the regeneration of different DNA biosensor formats. However, such a thermal method may cause the denaturation of BSA and streptavidin. So a chemical method using 50% urea solution was applied to regenerate the MB biosensor. After regeneration, the MB sensor returned to its original state in 5 min, which indicated that the stem of the MB was closed again. The regenerated biosensor had a similar fluorescence increase when it was hybridized to its target DNA. It was postulated that the major reason was due to the incomplete restoration to original hairpin structure after use.

Conclusion

A biotinylated MB was immobilized for the development of DNA biosensor through streptavidin-biotin binding on silica gel, which was formed by the sol-gel method. The MB based DNA biosensor can detect nonlabeled DNA target in real time with high sensitivity and one-base-mismatch selectivity. Compared with the previous results, the sol-gel based DNA biosensor processes three advantages: (1) The response time was significantly shortened to 10 min when the detected DNA was over 10 nM. The previously reported biosensor needed 20 min to complete the hybridization. (2) The silica gel prolonged the lifetime of the MB-based biosensor. The immobilized MB could keep its activity for three weeks if stored in the buffer, the lifetime of previously reported biosensor was only one week. (3) The gel formed by sol-gel method processed a larger surface size than the common glass cover at the same scope. The number of binding sites for biotinylation increased significantly. So the sensitivity also increased to sub-nmol/L. Moreover, the sol-gel matrix provided a solid support for the immobilized MB, which can be doped on the surface of many materials. This will widen the applicability of MB in both clinical and biotech fields.

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