

Rapid DNA Hybridization Analysis Using a PDMS Microfluidic Sensor and a Molecular Beacon

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A rapid DNA analysis has been developed based on a fluorescence intensity change of a molecular beacon in a PDMS microfluidic channel. Recently, we reported a new analytical method of DNA hybridization involving a PDMS microfluidic sensor using fluorescence energy transfer (FRET). However, there are some limitations in its application to real DNA samples because the target DNA must be labelled with a suitable fluorescent dye. To resolve this problem, we have developed a new DNA microfluidic sensor using a molecular beacon. By monitoring the change in the restored fluorescence intensity along the channel length, it is possible to rapidly detect any hybridization of the molecular beacon to the target DNA. In this case, the target DNA does not need to be labelled. Our experimental results demonstrate that this microfluidic sensor using a molecular beacon is a promising diagnostic tool for rapid DNA hybridization analysis.

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Introduction

It is very important to develop a quick and accurate way of analyzing DNA hybridization, because it plays an important role in profiling genetic diseases and gene expression. One of the most popular approaches to DNA hybridization analysis is the use of a microarray chip.¹ However, with the microarray technique, a long hybridization time (about 1–2 h) is required for hybridization when probe DNA sequences immobilized on a glass slide are incubated with a mixture of an unknown target DNA. It takes a long time for hybridization of the probe and target DNA oligomers because of the diffusion-limited hybridization kinetics on a solid phase.^{2,3} The microfluidic sensor overcomes this problem of slow hybridization on a microarray chip because hybridization occurs in a solution phase.^{4–10} However, a new detection method for use in the microfluidic device is required to identify any changes in fluorescence during hybridization, because non-hybridizing fluorescent oligomers cannot be washed from the channel.

To resolve this problem, we recently developed a new analytical method for DNA hybridization using a microfluidic channel device that involves fluorescence energy transfer (FRET).¹¹ For this purpose, probe and target DNA oligomers that share complementary base sequences were prepared. Each DNA oligomer was labelled with a different fluorescent dye at the 5' and 3' termini. Here, one oligomer is the fluorescence donor and the other is the acceptor. When the two fluorescent oligomers hybridize to form adjacent sequences in the microfluidic channel, the distance between the two fluorophores on the new hybrid becomes small enough for FRET to occur.

The microfluidic technique that we previously reported, which uses FRET for DNA hybridization analysis, is inconvenient for the analysis of real samples, such as chromosomal DNA, because both the probe and the target DNA strands must be labelled with a suitable fluorescent dye.¹¹ To resolve this problem, a new hybridization analysis technique using a molecular beacon (MB) was developed in this study. The MB is a single-stranded oligonucleotide that forms a stem-loop structure. In the MB, a fluorescent moiety is attached to the end of one arm, and a non-fluorescent quencher is attached to the end of the other arm.^{12–16} Binding this MB probe to its target DNA in a microfluidic channel creates energetically stable probe-target hybridization, and the fluorescence of the donor is restored. In this case, the target DNA need not be labelled. This dual-labelled fluorescent MB is expected to be used as a superior probe for the quantitative evaluation of target DNA. Our technique may satisfy the continuing need for rapid and inexpensive methods for hybridization-based investigations of single-nucleotide polymorphisms (SNPs).

Experimental

Preparation of the molecular beacon

All of the molecular beacon probe (MB-probe) and target DNA oligonucleotides were purchased from Bio Basic Inc. (Canada), and used without further purification. In this work, a molecular beacon, labelled with a fluorophore (FAM) and a quencher (DABCYL) attached to opposite ends of the stem, was used as the probe DNA oligonucleotide. The base sequence of the MB was (FAM)-5'-TACGAGGTTAAAGGCTCTCTCCCTGTCGTA-3'-(DABCYL). The base sequence of the target DNA oligonucleotide was 3'-CCATTTTCCGAGAGAGGGAC-5'. This DNA oligonucleotide is known to be closely related to azoospermia.¹⁷

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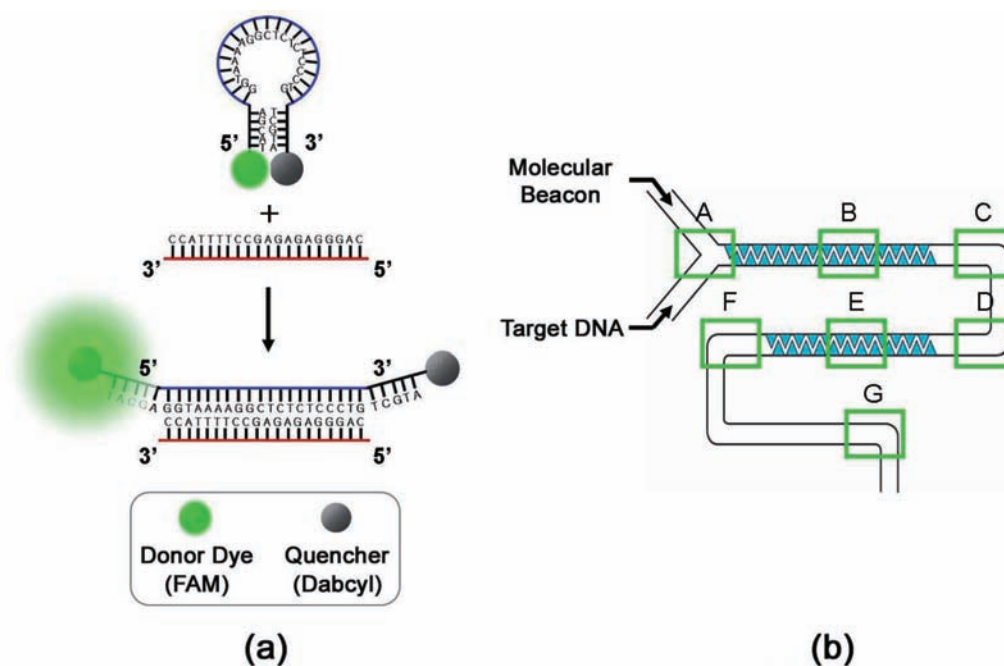


Fig. 1 (a) Schematic representation of the molecular beacon and its operating principle. Target hybridization leads to the separation of the fluorophore (FAM) and quencher (DABCYL) and a consequent fluorescent signal. (b) Schematic representation of an alligator-teeth-shaped PDMS microfluidic channel. The seven boxes denote the fluorescence measurement areas. The MB and the target DNA are injected into the channel using a microsyringe pump.

A significant percentage of patients with non-obstructive azoospermia test positive for small deletions in the DNA of the Y chromosome, at the locus *DYS 209*; those gene sequences are closely associated with the deleted segments. For comparison purposes, hybridization of a non-complementary target DNA (mismatched base sequence: 3'-CTCTAGACTTAGTCTCGCAG-5') with the MB was also investigated. All samples were dissolved in a 0.01 M PBS buffer solution (NaCl 0.138 M, KCl 0.0027 M, and pH 7.4) and stored at 4°C. All hybridization experiments were performed at room temperature.

Fabrication of a PDMS microfluidic channel

The procedure for fabricating alligator-teeth-shaped poly(dimethylsiloxane) (PDMS) microfluidic channels has been reported elsewhere.^{18,19} A microfluidic channel was fabricated by stacking two PDMS layers that had upper and lower teeth patterns. These layers were produced by the pattern replication of mould masters. Two epoxy-based photoresist (EPON) mould masters, including upper and lower teeth patterns, were fabricated. A thick patterned layer with the lower pattern was constructed by pouring a mixture of PDMS prepolymer and a curing agent, in a 10:1 ratio, onto the lower mould master and curing it for 2 h on a hot-plate at 80°C. The layer with the upper teeth pattern was fabricated by the compression of micromoulding made of PDMS elastomer. The PDMS prepolymer was poured into the mould master and compressed with transparent film and an aluminium disk. It was then cured for 2 h. A thin upper layer was produced by separating it from the mould master. To bond the upper and lower layers, the surfaces of both layers were activated in an oxygen plasma. Both layers were then aligned using our homemade aligner. Methanol was used as a surfactant between the two layers. Finally, a cover glass was stacked onto the upper layer.

In the microfluidic channel used, triangular structures (base,

300 µm; height, 300 µm; thickness, 150 µm) were located on the lower and upper surfaces of the channel in a zigzag manner within a rectangular duct (width, 300 µm; height, 300 µm). The total length of the microfluidic channel was 30 mm. The distances between detection points A to F were 4 mm, respectively. The distance from F to G was 6 mm. While confluent streams of MB and target DNA traveled along the microfluidic channel, the transverse and vertical dispersions of the fluid occurred simultaneously through the upper and lower teeth.

Fluorescence detection

The fluorescence emission spectra were measured using a Leica TCS SP confocal fluorescence microscope. The confluent mixing streams of the molecular beacon and the target DNA were analyzed using two-dimensional confocal fluorescence images in the *x-y* plane located perpendicular to the optical axis using a 10× water-immersion objective lens, the resolution of which was estimated to be 1 µm. Laser excitation of FAM occurred at $\lambda = 488$ nm, and the emitted fluorescent light was detected between $\lambda = 500$ and 640 nm. The image size was 512 × 512 pixels, and the width of each pixel was 0.49 µm. The fluorescence spectra were also measured using the λ -scanning mode of the confocal laser scanning microscope to quantitate any change in the fluorescence intensity during hybridization of the MB and the target DNA.

Results and Discussion

MB is a DNA oligonucleotide labelled with a fluorophore at the 5' end and a quencher at the 3' end. As shown in Fig. 1(a), MB has a hairpin structure, in which the fluorophore, FAM, and the quencher, DABCYL, are in close proximity, so that energy from

the fluorophore is transferred to the quencher molecule. As a result, the energy is absorbed by DABCYL, and no fluorescent signal is observed. In the presence of target DNA, the loop section of the MB hybridizes with the complementary target DNA. Because hybridization between the loop and the target is stronger than that within the stem, the MB undergoes a conformational change and the fluorophore and quencher molecules are separated from each other. Consequently, the fluorescent signal is restored. By monitoring the change in the restored fluorescence intensity in a microfluidic channel, it is possible to quantitatively evaluate the unlabelled target DNA.

Figure 1(b) shows a schematic drawing of the alligator-teeth-shaped PDMS micromixer. The triangular structures are located on the lower and upper surfaces of the channel, within a rectangular duct, in a zigzag manner. The mixing of the MB

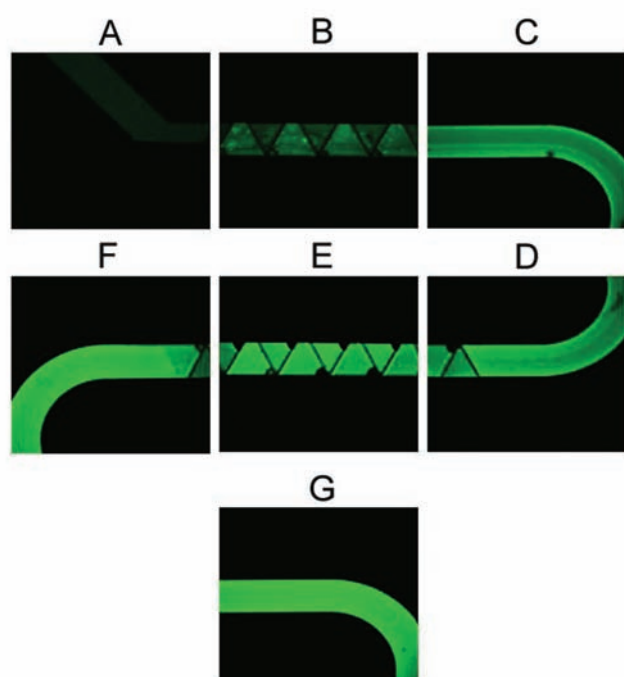


Fig. 2 Confocal fluorescence images of lateral confluent streams along the channel. The green light becomes brighter as the distance along the channel increases.

and the target DNA proceeds in the alligator-teeth-shaped PDMS microfluidic channel. The MB and specific target DNA are introduced into the channel using microsyringes connected by tubes to inlet pipettes. The flow rate is simultaneously controlled using a microsyringe pump. While the confluent liquid streams travel along the microfluidic channel, the transverse and vertical dispersion of the fluid occurs simultaneously through the upper and lower teeth. The seven rectangles denote the fluorescence measurement areas. Our previous work^{20,21} demonstrated that this three-dimensional PDMS channel is very effective in mixing two laminar-flow streams when compared with a serpentine or twisted chaotic channel, because stronger advection is developed by the simultaneous vertical and transverse dispersion of the confluent streams. Using this saw channel, together with a fluorescence/Raman optical detection technique, we successfully performed quantitative analyses of duplex DNA oligonucleotides,²⁰ cyanide pollutants in water,¹⁸ glucose-catalyzed reactions,²¹ and DNA oligomers.¹¹ To determine the optimal flow rate for fluorescence measurements, the flow rate was changed from 1 to 20 $\mu\text{L}/\text{min}$ by regulating the microsyringe pump. At a slow flow rate of 1–5 $\mu\text{L}/\text{min}$, the two confluent streams of the MB-probe and the target DNA were not completely mixed. Conversely, at a fast flow rate of 15–20 $\mu\text{L}/\text{min}$, the mixing efficiency was greatly enhanced. However, at that rate, the reaction time between the MB-probe and the target DNA was insufficient for complete hybridization. Therefore, a flow rate of 10 $\mu\text{L}/\text{min}$ was chosen as being optimal flow rate.

Figures 2 and 3 show confocal fluorescence images at seven different channel distances and their corresponding fluorescence emission spectra. Here, the flow rate was maintained at 10 $\mu\text{L}/\text{min}$. Solutions of the MB (1×10^{-5} M) and target DNA (1×10^{-5} M) in a 1:1 molar ratio were introduced into the channel, and complete mixing of both solutions was achieved after they passed an alligator-shaped saw channel. The small black circles of channel positions B and E in Fig. 2 are air bubbles. The flowing conditions in a microfluidic channel were very carefully controlled to remove the bubbles. However, it was not so easy to completely remove small bubbles, especially those caught in between triangular teeth. Nonetheless, the fluorescence detection for the quantitative evaluation of DNA hybridizations was not seriously interfered with those air bubbles.

Figure 3 shows the average fluorescence emission spectra

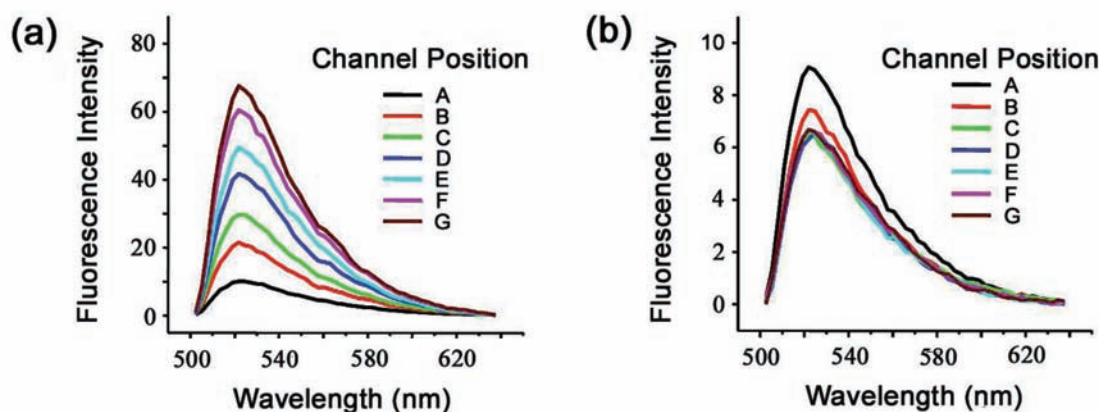


Fig. 3 Changes in the fluorescence emission intensity corresponding to the measurement areas denoted in Fig. 1. (a) Fluorescence emission spectra for the hybridization of the MB and the complementary target DNA. (b) Fluorescence emission spectra for the hybridization of the MB and a non-complementary target DNA.

corresponding to the measurement area in Fig. 1. Here, the fluorescence emission intensity was determined by averaging all of the pixel points in a given area. They were calculated using the software installed in the Leica confocal laser scanning microscope. As shown in Fig. 3(a), the relative fluorescence intensity of the complementary target DNA gradually increased with increasing distance along the channel. This means that the fluorescence intensity of FAB was restored by hybridization between the MB and the target DNA. In other words, the stem-loop configuration of the MB was disrupted by the hybridization and the fluorescence intensity was gradually restored with increasing distance along the channel. For comparison purposes, the fluorescence emission spectra for the hybridization between the MB and a non-complementary target DNA were also measured. In this case, no change in the fluorescence intensity was observed along the channel length. Only a small change in intensity from channel positions A to C was observed due to the dilution effect of the phosphate-buffered saline solution. A continual increase of the fluorescence intensity from A to G in Fig. 3(a) indicated that the hybridization reaction between the MB and complementary target DNA took a much longer time than simple mixing between the MB and non-complementary target DNA. As a result, the fluorescence intensity might have increased after

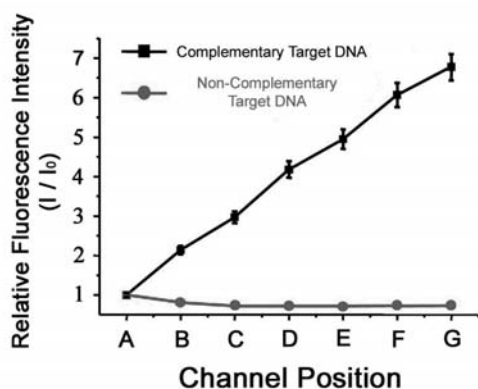


Fig. 4 Changes in the relative fluorescence intensity for the hybridization of complementary and non-complementary DNA oligomers along the channel length. Each channel position corresponds to a fluorescence measurement area denoted in Fig. 1. The molar ratio of the MB and target DNA is 1:1.

position G for complementary target DNA at a flow rate of 10 $\mu\text{L}/\text{min}$. However, it was impossible to monitor the change in the fluorescence intensity over position G for the PDMS microfluidic channel used in this study. In order to find the equilibrium point for complete DNA hybridization, the hybridization reaction between MB and complementary target DNA was performed in a microtube at room temperature. The reaction was performed for 60 min under a vigorous shaking condition. Then, its fluorescence intensity was compared with the value at channel point G. According to our experimental data, it is believed that over 90% of the hybridization reaction is completed at channel position G. The changes in the relative fluorescence intensity along the channel length for the complementary and non-complementary target DNAs are plotted in Fig. 4.

The fluorescence intensity changes with different molar ratios of the MB and target DNA were also investigated. The results are presented in Fig. 5(a). With an increase in the molar ratio from 0 to 1:1, the relative fluorescence intensity for the complementary target DNA gradually increased. However, there was no intensity change with the non-complementary target DNA. The fluorescence intensities during the hybridization of non-complementary and complementary target DNA oligomers, compared with that of the MB alone, are shown in Fig. 5(b). Here, all of the fluorescence intensity data correspond to the values measured at position G. The fluorescence intensity for the complementary DNA was about 10-times stronger than that of the non-complementary DNA. This great difference is expected to allow the development of a superior probe for a quantitative evaluation of target DNA.

Conclusions

Recently, we reported a microfluidic technique that uses FRET for DNA hybridization analysis.¹¹ However, this technique is inconvenient for the analysis of unknown DNA samples, because both the probe and the target DNA strands must be labelled with a suitable fluorescent dye. In the present study, a new hybridization analysis technique using a molecular beacon (MB) and the PDMS microfluidic channel was developed. The MB is a single-stranded oligonucleotide that forms a stem-loop structure. In the MB, a fluorescent moiety is attached to the end of one arm and a non-fluorescent quencher is attached to the end of the other arm. By monitoring the change in the intensity of the restored fluorescence along the channel length, it is

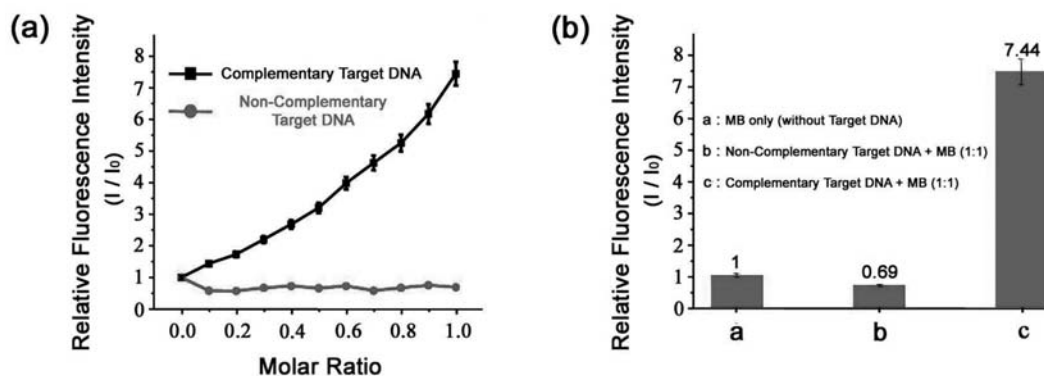


Fig. 5 (a) Changes in the relative fluorescence intensity for different molar ratios of the MB and the target DNA. (b) Comparison of the relative fluorescence intensities for the MB only, a non-complementary target DNA with the MB, and the complementary target DNA with the MB.

possible to rapidly detect the hybridization of MB with the target DNA. In this technique, the target DNA need not be labelled. When the detection of DNA hybridization was performed at a flow rate of 10.0 $\mu\text{L}/\text{min}$, it took less than 20 s for total analysis. Our experimental results demonstrated that the MB-based DNA analysis technique, combined with the continuous flow of the microfluidic device, is a promising diagnostic tool that can be applied to high-throughput bioanalyses.

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