Detection of Methylated DNA on a Power-Free Microfluidic Chip with Laminar Flow-Assisted Dendritic Amplification

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We report on a detection method for methylated DNA on a microfluidic chip, which needs no external power for fluid pumping. The methylated DNA was sandwiched by immobilized probe DNA and an anti-methylcytosine antibody. The fluorescence signal was amplified by our original amplification technology. The detection method was first optimized using a 22-mer DNA sequence and further validated using a 60-mer DNA sequence adapted from the SEPT9 gene. We were able to detect the methylated 60-mer DNA at 0.4 nM within 18 min.

**Keywords** DNA methylation, detection, immunoassay, power-free microfluidic chip, LFDA

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The methylation of cytosine in DNA involves the addition of a methyl group to the fifth position on the pyrimidine ring, resulting in 5-methylcytosine (5mC). In mammalian cells, DNA methylation is a major mechanism in the epigenetic regulation of gene expression, and plays a crucial role in cellular differentiation.1 Aberrant DNA methylation patterns have been associated with various diseases, including cancer.2-5 Thus, DNA methylation analysis is an important technology for biomedical research, such as epigenetics, developmental biology, and pathology. Moreover, aberrant methylation patterns in specific genes are expected to be new biomarkers for the early diagnosis or prognosis of cancer.6,4

Currently, various analysis methods for DNA methylation are employed. Most of them involve the combination of a discrimination method of 5mC from unmethylated cytosine and a downstream analysis method, such as PCR and microarray. The discrimination method can be a bisulfite treatment,9,10 immunoprecipitation,11 or methylation-sensitive enzymatic reaction.12 Among them, the bisulfite treatment, in which unmethylated cytosine is selectively converted into uracil, is the most accurate and versatile. However, the treatment takes about 16 h, and reportedly degrades 99% of the sample DNA.13 The other two discrimination methods are generally time-consuming and labor-intensive as well. To circumvent these lengthy reactions, sandwich-type assays have recently been reported.14,15 In these assays, specific target genes were first captured by probe DNA immobilized on solid surfaces. Then, 5mC recognition molecules, such as recombinant methyl-binding domain14 and anti-5mC antibody,15 were bound to 5mC on the target gene. These assays typically took 45-60 min.

We have been developing sandwich-type assays for biomolecules on power-free microfluidic chips, which are driven by degassed poly(dimethylsiloxane) (PDMS).16 The power-free microfluidic chip needs no external pump, and thus simplifies the whole detection setup. We have also invented a signal-amplification technology specialized for microfluidic chips: laminar flow-assisted dendritic amplification (LFDA).17 The combination of these two technologies, the power-free microfluidic chip and LFDA, has been proven to be effective for the detection of proteins,17,18 DNA,19 and microRNA (miRNA)20,21 with a typical assay time of 20 min.

In this paper, we report on the detection of 5mC in target single-stranded DNA on a power-free microfluidic chip for the first time. The target DNA was captured by probe DNA immobilized on a microchannel surface. At the same time, anti-5mC antibody was bound to the 5mC on the target DNA. The signal was amplified with LFDA. We first tried very short (22-mer) DNA for a proof-of-concept. After optimization of the immunoreaction using the short DNA, we have succeeded to detect much longer (60-mer) methylated DNA with a homologous sequence to the human SEPT9 gene, which is known to be a potential biomarker in blood for the diagnosis of colorectal cancer.9

**Experimental**

The microfluidic chip had the same design as that for miRNA detection20 (Fig. 1, upper left). Briefly, it consisted of a 30 × 25 × 2 mm PDMS (Sylgard 184, Dow Corning) part and a 76 × 26 × 1 mm glass substrate. A piece of adhesive tape was used for the power-free pumping technique.16 A straight line pattern (100 μm wide) of the capture probe (CP) DNA was made on the glass substrate, which was then covered with the PDMS part having microchannels (100 μm wide and 25 μm deep). Thus, the CP pattern and the microchannels became orthogonal to each other. A single assay was carried out with a pair of Y-shaped microchannels sharing one inlet (“center inlet” in Fig. 1). The left Y-channel was used for methylated DNA,
while the right one was for unmethylated DNA as a reference.

The PDMS part was fabricated as described elsewhere. The oligonucleotides listed in Table 1 were purchased from Gene Design (methylated DNAs) or Eurofins Genomics (others). CP-1 or CP-2 was immobilized onto the glass surface as described elsewhere. Briefly, 1% glutaraldehyde in carbonate buffer was reacted with an aminated glass slide (SD00011, Matsunami Glass) at 37°C for 2 h. A PDMS part having a straight microchannel was attached onto the glass surface. CP-1 or CP-2 (100 μM) in ultrapure water was injected into the microchannel and incubated at 37°C for 1 h. The PDMS part was detached from the glass surface in a stopping buffer (25 mM Tris, 0.05% Tween 20), and the glass substrate was washed by ultrasonication in the stopping buffer, followed by deionized water. The PDMS part having the Y-shaped microchannels was attached onto the glass surface. The PDMS-glass microfluidic chip was degassed in a vacuum chamber at 10 kPa for 1 h for power-free pumping.

The assay protocol is illustrated in Fig. 1 (lower left). It consisted of three steps. All of the solutions were injected with the power-free pumping technique. In each step, three aliquots were injected almost simultaneously (< 40 s). (Step 1) A blocking buffer (1% Roche Blocking Reagent, 0.02% SDS, 0.05% Tween 20, and 5 × SSC) was injected from all of the inlets. The injected volumes were 0.5 μL (left and right) and 1 μL (center). This blocking buffer was used for diluting all of the solutions described below. (Step 2) First, 0.5 μL of 0–1000 nM M-DNA1 or M-DNA2 (Table 1) was injected from the left inlet, while the same concentration of U-DNA1 or U-DNA2 (Table 1) was injected from the right inlet as a reference. Then, 1 μL of 1 μg/mL anti-5mC antibody (Active Motif cat# 39649, clone# 33D3) was injected from the center inlet. (Step 3) Subsequently, 3 μL of 5 or 10 μg/mL biotinylated anti-mouse IgG antibody (B-anti-IgG, KPL, cat# 16-18-06) was injected from the left and right inlets, while 6 μL of 2.5 or 5 μg/mL FITC-labeled streptavidin (F-SA) was injected from the center inlet. Finally, the fluorescence signal was captured by a charge-coupled device camera and analyzed using ImageJ software.

### Results and Discussion

The detection strategy for methylated DNA in this study is illustrated in Fig. 1 (right). The single-stranded methylated DNA is captured by the immobilized CP DNA. At the same time, the anti-5mC antibody is bound to the 5mC in the methylated DNA. In the next step, B-anti-IgG is bound to anti-5mC, thus triggering the initiation of LFDA. B-anti-IgG molecules also serve as cross-linkers between the F-SA molecules in the LFDA step, because each B-anti-IgG molecule generally has multiple biotin moieties. In the neighboring microchannel, unmethylated DNA is introduced as a reference. We first designed very short sequences (M-DNA1 and U-DNA1, Table 1) for proof-of-concept, so that we were able to use the same microfluidic chip, including the CP sequence, as that for miRNA detection. For this set of experiments, CP-1 (Table 1) was immobilized to the glass surface. M-DNA1 was

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### Table 1 Sequences of the oligonucleotides

| Symbol | Description         | Length | Sequencea,b |
|--------|---------------------|--------|-------------|
| M-DNA1 | Methylated DNA      | 22-mer | TAC GTT ATC AGA CTG ATG TTG A |
| U-DNA1 | Unmethylated DNA    | 22-mer | TAC GTT ATC AGA CTG ATG TTG A |
| M-DNA2 | Methylated DNA      | 60-mer | CGG ACC CCG TCA AGC CAG TGG ATG GGA TCA TTT CGG ACT TCG AAG GTG GGT GCT |
| U-DNA2 | Unmethylated DNA    | 60-mer | CGG ACC CCG TCA AGC CAG TGG ATG GGA TCA TTT CGG ACT TCG AAG GTG GGT GCT |
| CP-1   | Capture probe       | 26-mer | NH2-ATT TTT TTT TTT TTT TCA ACA TCA GT |
| CP-2   | Capture probe       | 35-mer | NH2-ATT TTT TTT TTT TTT TTA AGC ACC CAC CTT CTA AGT CC |

a. Written from 5′ to 3′. b. Underlined cytosine bases were 5mC.
injected from the left inlet, while the same concentration of U-DNA1 was injected from the right inlet. Using these sequences, we optimized the concentration of anti-5mC. The time course of the fluorescence with various concentrations of anti-5mC is shown in Fig. 2. These experiments were carried out with 10 μg/mL B-anti-IgG and 5 μg/mL F-SA. The fluorescence signal from M-DNA1 was increased with an elevated concentration of anti-5mC. However, at the same time, nonspecific binding to U-DNA1 was also aggravated. To evaluate the difference between the fluorescence intensities from M- and U-DNA1, we define the “signal-to-reference ratio” (SRR) as the fluorescence from M-DNA1 divided by the fluorescence from U-DNA1, immediately before nonspecific LFDA of U-DNA1 started. The nonspecific LFDA was judged by the rise in fluorescence from the baseline. The SRR values were 7.8, 16.8, and 13.8 for Figs. 2(a), 2(b), and 2(c), respectively (arrows in Fig. 2). For Fig. 2(d), SRR could not be determined. Therefore, we determined 1 μg/mL (Fig. 2(b)) as being the optimal anti-5mC concentration.

With the optimized anti-5mC concentration, we measured SRR for various M-DNA1 concentrations. The data were fitted with the four-parameter logistic function. As a result, we obtained the calibration curve shown in Fig. 3. The limit of detection (LOD) was determined by the intersection of the calibration curve and the horizontal “3σ” line, which was drawn at calculated zero-dose SRR plus 3-times the standard deviation of the blank measurements. The LOD of M-DNA1 was calculated to be 1.2 nM, which is much higher than the LOD of miRNA (0.5 pM for miR-21) on the same microfluidic chip.20 There are several possible explanations for this difference. Compared to miRNA detection (see Fig. S1(a) in Supporting Information (SI) for the detection strategy), two RNA-DNA binding reactions have been replaced by DNA-DNA binding and 5mC-antibody binding reactions, and biotin-streptavidin binding has been partially replaced by IgG Fc region-antibody binding. All of these three modifications may have weakened each binding reaction. The LOD might be improved by strengthening these reactions. For example, the IgG Fc region-antibody binding could be turned back to the biotin-streptavidin binding by the biotinylation of anti-5mC. Although the LOD has much room for improvement, the detection method presented here took less than 18 min, which is more rapid than any other existing methods for the detection of methylated DNA, to the best of our knowledge.13,14,15 In this study, for the first time, LFDA was induced by B-anti-IgG instead of biotinylated anti-streptavidin (B-anti-SA), which had long been used in our previous studies.17–21 As a preliminary experiment, we had confirmed that B-anti-IgG is as effective as B-anti-SA for induction of LFDA using the well-established miRNA detection system (Fig. S1 in Supporting Information). These results indicate that, 1) in LFDA, recognition of
streptavidin as an antigen is unimportant, compared to the biotin-streptavidin binding, and 2) the change of the antibody for LFDA in the proposed method is unlikely to have affected the LOD.

Next, for further validation of this detection method, we designed longer and more realistic sequences (M-DNA2 and U-DNA2, Table 1) adapted from human SEPT9 gene: a potential biomarker for colorectal cancer.8 We immobilized CP-2 (Table 1) onto the glass surface. The remainder of experiments was carried out in the same way as those for M- and U-DNA1, except that 5 µg/mL B-anti-IgG and 2.5 μg/mL F-SA were used. As a result, we obtained the calibration curve shown in Fig. 4. The LOD of M-DNA2 was calculated to be 0.4 nM. Although the reduction in the LOD from that of M-DNA1 (1.2 nM) has not been proven to be significant, there are two reasons for the possible reduction. First, the recognition sequence of CP-2 (20-mer) is longer than that of CP-1 (11-mer), making stronger binding to the target DNA. Second, M-DNA2 has five 5mC bases, compared to only one 5mC base in M-DNA1. In other words, M-DNA2 can bind more anti-5mC than M-DNA1. The effects of these factors will further be studied in our future work.

In conclusion, the detection of methylated DNA on a power-free microfluidic chip with LFDA has been demonstrated for the first time. With an assay time of less than 18 min and a sample volume of 0.5 µL, methylated DNA was successfully discriminated from its unmethylated counterpart. The LOD values obtained in this work were 0.4 and 1.2 nM. In our future work, the LOD should be improved by optimization of the reaction conditions, including the probe design and antibody biotinylation. Another direction of development should be the quantification of methylation, because the degree of methylation is of primary interest in realistic applications. The accuracy of the proposed method will be studied in our next step.

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Supporting Information

Comparison of LFDA using B-anti-SA and B-anti-IgG for miRNA detection. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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