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**Multiplex MicroRNA Detection on a Surface-Functionalized Power-Free Microfluidic Chip**

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Abstract

Circulating microRNAs (miRNAs) have emerged as promising cancer biomarkers, because their concentration profiles in body fluids are associated with the type and clinical stage of cancer. For multiplex miRNA detection, a novel surface-functionalized power-free microfluidic chip (SF-PF microchip) was developed. The inner surface of the microchannels of the SF-PF microchip was functionalized via electron beam-induced graft polymerization and immobilization of capture probe DNAs. Simultaneous and specific duplex miRNA detection was achieved on the line-type SF-PF microchip, and the detection limits of this microchip were 19.1 and 47.6 nmol·L⁻¹ for miR-16 and miR-500, respectively. Moreover, simultaneous, and specific triplex miRNA detection was achieved on the stripe-type SF-PF microchip. The sample volume required for this microchip was 0.5 μL, and the time required for detection was 17 min. These results indicate that up to six types of miRNAs could be detected without compromising the advantages of the previous SF-PF microchips for cancer point-of-care testing.
Introduction

Early detection of cancer is important from the viewpoint of effective treatment, and many biomarker-based diagnostic methods are reported. Circulating microRNAs (miRNAs) in the body fluids serve as promising biomarkers for the early detection of cancer. Changes in intracellular and blood miRNA concentrations have been reported to be associated with the type and clinical stage of cancer. Therefore, multiplex miRNA detection is essential for cancer diagnosis because changes in the miRNA concentration profile can aid the identification of cancer type and stage.

For practical application of miRNA-based cancer point-of-care testing (POCT), superior multiplex miRNA detection methods are required, because current well-established miRNA analytical methods, such as microarray, deep sequencing, and qPCR, require a relatively large equipment, are expensive, and take a long time to generate results. In contrast, cancer POCT requires a relatively small equipment, is inexpensive, quicker, and involves simple operation. Microfluidic chips (microchips) are a promising candidate as a device substrate. Although miRNA detection methods using microchips have been reported, there are challenges in cancer POCT, such as the need for a pump to transport the liquid.

Our research group has developed a surface-functionalized power-free microchip (SF-PF microchip). The substrate of the SF-PF microchip is made of polydimethylsiloxane (PDMS). As the SF-PF microchip uses power-free technology based on the specific property, high gas solubility, of PDMS, it does not require an external power source, such as a pump, to transport liquid and can be manufactured at a relatively low cost. In addition, for miRNA detection, the inner surface of microchannels can be easily functionalized with a polymer via simultaneous irradiation.
method of electron beam-induced graft polymerization and immobilization of capture probe DNA (CP DNA), presenting the half complementary sequence of the target miRNA, on the grafted polymer. By capturing miRNAs with CP DNA and using fluorescent-labeled detection probe DNA (DP DNA), the complementary sequence of the other half of the target miRNAs can be detected based on fluorescent signals. In a previous study, only one type of miRNA could be detected\textsuperscript{13}; thus, multiplex miRNA detection using the SF-PF microchip is imperative for cancer POCT.

Here, we achieved multiplex miRNA detection on the SF-PF microchips by the following two approaches: (1) simultaneous and specific duplex miRNA detection using two differently colored fluorescent dyes on a line-type SF-PF microchip and (2) simultaneous and specific triplex miRNA detection based on different detection positions on a stripe-type SF-PF microchip. As target cancer biomarkers, hsa-miR-16\textsuperscript{5}, hsa-miR-21-5p\textsuperscript{16}, and miR-500a-3p\textsuperscript{17} were employed.

**Experimental**

*Reagents and chemicals*

The epoxy-group-containing vinyl monomer glycidyl methacrylate (GMA) was purchased from Sigma-Aldrich (St Louis, MO, USA) and used without further purification. \textit{N,N}-Dimethylformamide (DMF) was purchased from Fujifilm Wako Pure Chemicals Corp. (Osaka, Japan). PDMS (Sylgard 184, Dow Corning, Midland, MI) was purchased from Dow Corning Co. (Midland, MI, USA). Oligo-DNAs were purchased from Eurofins Genomics Co. (Ebersberg, Germany). MicroRNAs were purchased from Fasmac Co., Ltd. (Kanagawa, Japan). Other reagents were of analytical grade or higher.
Preparation of a surface-functionalized power-free PDMS microfluidic chip (SF-PF microchip)

The SF-PF microchip for multiplex miRNA detection was prepared as follows. First, a PDMS microchip with a recessed pattern of line-shaped microchannels (line-type, Fig. 1 (a)) or three parallel line-shaped microchannels (stripe-type, Fig. 1 (a)) was fabricated as described elsewhere\textsuperscript{18}. The stripe-type microchannel was designed to align the length to the detection parts of the three microchannels and to observe the detection parts at the same time in a microscopic field. The width and height of the microchannels were 100 and 25 μm, respectively. Next, these microchannels were filled with 10 μL of a solution containing GMA and DMF (Fig. 1 (b)); GMA concentration was 2.0 mol·L\textsuperscript{-1}. Thereafter, the inlet and outlet of the microchannel were sealed with Teflon tape and packed in a gas barrier bag (Lamizip, AS ONE Corp., Tokyo, Japan) containing an oxygen scavenger (Ageless\textsuperscript{®} from Mitsubishi Gas Chemical Co., Japan). Furthermore, the microchip was irradiated with a 20 kGy electron beam at an energy of 2.0 MeV and a current of 20 mA using an accelerator (Dynamitron; Radiation Dynamics, Ltd.). Finally, the irradiated PDMS microchip was washed with methanol via sonication three times and dried under reduced pressure. Thus, the polyGMA(PGMA)-grafted PDMS microchip was obtained. Next, amino-group-modified probe DNAs (CP DNAs) for miRNA capturing were three-dimensionally immobilized onto PGMA via an epoxy-ring-opening reaction (Fig. 1 (b)). The sequences and abbreviations of the miRNAs, CP DNAs, and DP DNAs used in this study are listed in Table 1. The grafted PDMS part was filled with 2.0 μL of 50 μmol·L\textsuperscript{-1} CP DNA aqueous solution and incubated at 37°C for 2 h. For duplex miRNA detection, the line-type microchip was functionalized with CP16 and CP500. For triplex miRNA detection, the stripe-type microchip was functionalized with CP16 (left
channel), CP21 (center channel), and CP500 (right channel). For evaluating the amount of CP DNA immobilized, two types of fluorescent-labeled CP DNAs (CP 21-TAMRA and CP 500-6Fam) were immobilized on the microchannel inner surface of the line-type microchip. Images of the channel were obtained under a fluorescence microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan), and the fluorescence intensity was analyzed using image analysis software (ImageJ 1.45s, National Institute of Health, Bethesda, MD, USA). Finally, the CP-DNA-immobilized PDMS microchip was set at 10 kPa for 1 h and degassed to obtain the SF-PF microchip.

Procedure of duplex miRNA detection on the line-type SF-PF microchip

The procedure of duplex miRNA detection is shown in Fig. 2. First, the fluorescent-labeled DP DNAs (DP16-Fam and DP500-TAMRA; both 1.0 μmol·L⁻¹) were pre-hybridized with the target miRNAs (miR-16 and miR500a-3p; both 1.0 μmol·L⁻¹) for 5 min in 10 μL of hybridization buffer [1% blocking reagent, 0.02% SDS (w/v), 5× SSC, and 0.05% Tween 20]. After 2 min, 0.5 μL of hybridization buffer was passed through the line-type SF-PF microchip for 3 min for blocking. The pre-hybridization and blocking steps were conducted simultaneously. Next, 0.5 μL of pre-hybridized solution was injected into the microchip for 5 min. Finally, hybridization buffer was injected into the SF-PF microchip for 7 min for washing the microchannel. After washing, images of the channel were obtained under a fluorescence microscope (Biozero BZ-8100, Keyence, Osaka, Japan), and the fluorescence intensity was analyzed using the ImageJ. The total time required for detection was 17 min and all injections were conducted by power-free pumping.
Procedure of triplex miRNA detection on the SF-PF microchip

The principal of triplex miRNA detection is shown in Fig. 2. Three types of CP DNAs were immobilized onto each channel of the stripe-type microchip. Same as that in the duplex miRNA detection, the fluorescent-labeled DP DNAs and target miRNAs were pre-hybridized in a microtube for 5 min. Hybridization buffer was injected into the stripe-type SF-PF microchip for 3 min for blocking. Thereafter, pre-hybridized solution was injected into the microchip for 5 min. After washing the microchannel with hybridization buffer for 7 min, images were obtained under a fluorescence microscope, and the fluorescence intensity was evaluated using the ImageJ. The total time required for detection was 17 min and all injections were conducted by power-free pumping.

Results and Discussion

Amount of capture probe DNAs immobilized on the microchannel

The amount of CP DNAs immobilized on the microchannel inner surface is presented in Fig. 3. For evaluation, two types of fluorescent-labeled CP DNAs (CP 21-TAMRA and CP 500-6Fam) were immobilized onto the microchannel inner surface of the line-type microchip, and the fluorescence intensity was converted to amount using a calibration curve of surface density versus fluorescence intensity. The amount of double CP DNAs immobilized on the microchannel surface was same irrespective of the DNA sequences and was lower than that of single CP DNAs. This is because the epoxy rings used for immobilization compete in these two types of CP DNAs. The amount of CP DNA immobilized was higher than that of single CP DNAs on the glass surface and was corresponding to the previous study. Immobilization of both types of CP DNAs on the
Duplex miRNA detection on the line-type SF-PF microchip

Duplex hsa-miR-16 (miR-16) and hsa-miR-500a-3p (miR-500) detection using the line-type SF-PF microchip is presented in Fig. 4. Both green and red fluorescence signals were observed for solutions containing these two types of miRNAs. Green and red fluorescence signals were observed from the solutions containing miR-16 and miR-500, respectively. The fluorescence intensities of the signals derived from the target miRNAs were higher than those of the signals derived from controls at 5% level of significance using one-tailed t-test (n = 3). CP DNAs immobilized to the polymer grafted onto the inner microchannel surface recognized the complementary miRNA. Thus, the specificity of the CP DNAs to the target miRNAs was confirmed. The limits of detection (LODs) of the SF-PF microchip were calculated from the calibration curves (Fig. S1) according to the 3σ criterion. The LODs were 19.1 and 47.6 nmol·L⁻¹ for miR-16 and miR-500, respectively. These LODs were two orders of magnitude higher than the previously reported values¹³. This is because the present study used two types of CP DNAs immobilized to the same microchannel inner surface and reduced amount of immobilized CP DNAs compared with the previous studies in which only one type of CP DNA was used. In addition, simultaneous irradiation method of electron beam-induced grafting provides variation to the obtained materials compared with that of the UV light induced grafting¹⁴. The variation may also affect to the LODs. Some parameters affect to LODs were reported in previous study²⁰ but still it remains unclear.

In body fluid, the miRNA exist sub-femtomolar to sub-picomolar levels²¹ and the thresholds to diagnose various diseases are different. Therefore, the dominant parameters to determine the LODs must be clarified and the LOD must be improved.
and controlled in the future study. The sample volume required for detection was 0.5 μL, and the time required for detection was 17 min. Therefore, duplex miRNA detection on the line-type SF-PF microchip could be achieved.

*Triplex miRNA detection on the stripe-type SF-PF microchip*

Triplex miR-16, miR-21, and miR-500 detection is presented in Fig. 5. Fluorescence signals were observed only in microchannels in which CP DNAs complementary to the target miRNAs were immobilized. Fluorescence intensities of the signals derived from the target miRNAs were higher than those of the signals derived from controls at 5% level of significance using one-tailed t-test (n = 3). The specificity of CP DNAs to the target miRNAs was confirmed, and triplex miRNA detection on the stripe-type SF-PF microchip was achieved. The difference and variation of the signal intensities between triple, double, and single miRNA detections probably come from the variation of the grafted materials by the simultaneous irradiation method of electron beam-induced grafting as described in elsewhere. The sample volume required for detection was 0.5 μL, and the time required for detection was 17 min—values same as that for duplex miRNA detection using the line-type SF-PF microchip. Taken together, up to six types of miRNAs could be detected by immobilizing two types of CP DNAs in each channel of this SF-PF microchip.

**Conclusions**

For detecting biomarker miRNAs, a novel SF-PF microchip was prepared in which several types of CP DNAs are immobilized. On the line-type SF-PF microchip, simultaneous and specific duplex miRNA detection was demonstrated via two differently colored fluorescent dyes. On the stripe-type SF-PF microchip, simultaneous
and specific triplex miRNA detection was demonstrated via different detection positions. Overall, up to six types of miRNAs could be detected without compromising the advantages of the previous SF-PF microchips, such as device portability, small sample volume, and short detection time. By designing a new stripe-type microchip, the number of detectable miRNAs could increase and that may help to provide a reliable diagnosis. For establishing miRNA profile-based cancer POCT methods using the SF-PF microchip, detection sensitivity must be improved. In this light, development of a new laminar flow-assisted dendritic amplification except avidin–biotin complex formation is a possible solution. In addition, miRNA signal detection by visual inspection would be preferable for practical use. Using gold nanoparticles instead of fluorescent dyes, the phenomenon of color change due to aggregation and dispersion of the nanoparticles may be utilized for miRNA detection. Owing to the suitable advantages of the SF-PF microchip for POCT, further research might contribute to the establishment of a new POCT method for various diseases and improvement of healthcare even in developing countries.

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| Nucleic Acids                          | Abbreviation | Sequences (from 5’ to 3’)                      |
|---------------------------------------|--------------|------------------------------------------------|
| target miRNA (hsa-miR-16)             | miR-16       | UAG CAG CAC GUA AAU AUU GGC G                   |
| capture probe DNA for miR-16          | CP16         | NH2-C6- TTT TTT TTT TTT CAA TAT TTA C           |
| detection probe DNA for miR-16        | DP16-6Fam    | GTG CTG CTA-6Fam                                |
| target miRNA (hsa-miR-21-5p)          | miR-21       | UAG CUU AUC AGA CUG AUG UUG A                   |
| capture probe DNA for miR-21          | CP21         | NH2-C6- TTT TTT TTT TTT TCA ACA TCA GT          |
| detection probe DNA for miR-21        | DP21-6Fam    | CTG ATA AGC TA-6Fam                             |
| target miRNA (hsa-miR-500a-3p)        | miR-500      | AUG CAC CUG GGC AAG GAU UCU G                   |
| capture probe DNA for miR-500         | CP500        | NH2-C6- TTT TTT TTT TTT CAG AAT CCT TGC         |
| detection probe DNA 1 for miR-500     | DP500-6Fam   | CCA GGT GCA T-6Fam                              |
| detection probe DNA 2 for miR-500     | DP500-TAMRA  | CCA GGT GCA T-TAMRA                            |
Figure Captions

**Fig. 1** Microchip types and surface functionalization scheme for the surface-functionalized power-free (SF-PF) microchips. (a) Types of polydimethylsiloxane (PDMS) microchips as a substrate. (b) Scheme of surface functionalization utilizing electron beam-induced graft polymerization. Glycidyl methacrylate (GMA) was used as a monomer for grafting. Two and three different capture probe DNAs were immobilized on the line-type and stripe-type microchips, respectively.

**Fig. 2** Procedure of multiplex detection of miRNAs on the SF-PF microchips. (left: duplex miRNA detection on the line-type microchip, right: triplex miRNA detection on the stripe-type microchip).

**Fig. 3** Amount of capture probe DNAs immobilized on the inner surface of the SF-PF microchip. Error bars indicate ±SD.

**Fig. 4** Duplex miRNA detection on the line-type SF-PF microchip. Green and red signals indicate miR-16 and miR-500, respectively. Error bars indicate ±SD, *p < 0.05, **not significant.

**Fig. 5** Triplex miRNA detection on the stripe-type SF-PF microchip. Error bars indicate ±SD.
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Fig. 5 Triplex miRNA detection on the stripe-type SF-PF microchip. Error bars indicate ±SD.
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  - miRNAs

- **Stripe type**
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