Multiplex MicroRNA Detection on a Power-free Microfluidic Chip with Laminar Flow-assisted Dendritic Amplification

Ryo Ishihara, Kazuki Hasegawa, Kazuo Hosokawa,† and Mizuo Maeda

Bioengineering Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

MicroRNA (miRNA) profile-based point-of-care (POC) diagnostic methods have attracted considerable attention. In our laboratory, singleplex miRNA detection on a power-free poly(dimethylsiloxane) (PDMS) microfluidic chip with laminar flow-assisted dendritic amplification (LFDA) has been developed. In this study, to obtain the miRNA profile and to improve the reliability of the diagnosis, multiplex miRNA detection on the same system is demonstrated without compromising any advantages of the singleplex miRNA detection. The limit of detection (LOD) was at the femto- to picomolar level and the assay time was 20 min. The sensitivity, rapidity, and portability of the microfluidic chip are adequate for POC diagnosis.

Keywords MicroRNA, multiplex, point-of-care diagnosis, power-free microfluidic device, LFDA

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Early diagnosis is a key for the effective treatment of any disease. As biomarkers for the early detection of Alzheimer’s disease or various cancer types, microRNAs (miRNAs) have shown great promise.1–4 They are non-protein-coding single-stranded RNAs having approximately 18–24 bases and play important roles in physiological and pathological processes, such as development, differentiation, apoptosis, and stress responses.5 Over 2500 human miRNAs are known to date. In 2008, miRNAs that circulate in human body fluid at very low concentrations, i.e., at femto- to picomolar levels, were discovered.3,6 These miRNAs are referred to as circulating miRNAs. Subsequently, many studies have claimed that circulating miRNA expression profiles can be used for cancer detection, classification, and progression.3,4,7,8 Circulating miRNAs are expected to be novel, minimally invasive biomarkers.

Effective screening is essential to achieve a high early cancer detection rate. To that end, point-of-care (POC) diagnosis, as represented by pregnancy tests, has attracted attention. POC diagnosis requires short detection times, small sample volumes, and the portability of devices. Some miRNA detection methods have already been established, such as qPCR,9,10 microarrays,11,12 and deep sequencing.13 However, these methods do not meet the requirements for POC diagnosis because they take more than a few hours, and require at least 100 μL of sample solution and large equipment. Among various miRNA detection methods, microfluidic chip-based methods meet the requirements other than portability. The chip itself is small and enables a short detection time and a small required sample volume due to its microscale channel structure;14 however, relatively large external power sources for fluid pumping are needed and they prevent portability of the microfluidic chip. Hence, we utilized our power-free microfluidic chip driven by energy that is stored in degassed poly(dimethylsiloxane) (PDMS) to overcome this drawback because it eliminates the need for external power sources for fluid pumping.15 The power-free pumping method is essential for microfluidic chip-based POC diagnosis.

In our laboratory, toward POC diagnosis singleplex biomarker miRNA or protein detection on a power-free microfluidic chip with laminar flow-assisted dendritic amplification (LFDA) has been developed.16–19 LFDA is an amplification technique that was recently invented by our group.18,20 In this study, to obtain the miRNA profile and to improve the reliability of the diagnosis, multiplex miRNA detection with LFDA on a power-free microfluidic chip was demonstrated without compromising advantages of the singleplex. This is the first report of multiplex biomarker detection on this power-free microfluidic chip and an LFDA combined platform. Especially, the specificity and validity of the assay for target miRNAs was confirmed. MiR-16, miR-21-5p, and miR-500a-3p were used as target miRNAs because they are known as cancer biomarkers.21 Sensitive multiplex detection of miRNAs on power-free microfluidic chips will contribute to miRNA profile-based POC cancer diagnosis.

Experimental

Two types of PDMS (Sylgard 184) chips were fabricated for this experiment. One was a PDMS chip for the immobilization of capturing probe DNAs with a recessed pattern of three parallel-line-shaped microchannels that were 30 μm in width and 25 μm in height. The other was a PDMS chip for the detection of miRNAs with a recessed pattern of a pair of Y-shaped microchannels (Fig. 1) that were 100 μm in width and 25 μm in height. The fabrication protocol for the PDMS chips was described in detail elsewhere.15 Oligonucleotide sequences used in this study are summarized in Table 1. Capture and detection probe DNAs corresponding to miR-X are referred to as CP-X

†To whom correspondence should be addressed.
E-mail: k-hoso@riken.jp
Fluorescence signal amplification by LFDA. Fluorescein SDS, 5 (Wako, Osaka, Japan) in carbonate buffer (100 mM, pH 9.4) was incubated at 37°C for 1 h using the PDMS chip described above, so that the probe DNAs that were three parallel line shape and 30 μm in width were patterned onto the glass surface via the glutaraldehyde. Second, a PDMS chip with a pair of Y-shaped microchannels (described above) was attached to the glass plate with immobilized CP-DNAs to form a microfluidic device used for a blank reference.

Typical fluorescence images obtained by injecting a high concentration of miRNA non-target miRNAs and those of 0 M sample were not statistically significant (p = 0.07 – 0.58 with two-tailed t-test). These data suggest that LFDA successfully occurred in a target sequence-dependent manner without any significant cross reaction with unrelated CP DNAs.

Results and Discussion

Typical fluorescence images obtained by injecting a high concentration of miRNA to evaluate the specificity of the assay for target miRNA are shown in Fig. 2(a). Bright lines appeared at the expected positions; they appeared only in those areas with the complementary probe DNA in which the target miRNAs were supplied. The signal-to-blank ratios (SBRs) calculated from the images, which were estimated by dividing the fluorescence intensity of the left microchannel by the fluorescence intensity of the right microchannel, are shown in Fig. 2(b). Solutions containing miRNA and blank reference were injected into the left and right-hand microchannels, respectively. The differences between the SBRs of 1000 pM non-target miRNAs and those of 0 M sample were not statistically significant (p = 0.07 – 0.58 with two-tailed t-test). These data suggest that LFDA successfully occurred in a target sequence-dependent manner without any significant cross reaction with unrelated CP DNAs.

By using various concentrations of target miRNAs, the calibration curve for multiplex miRNA detection was obtained, and is shown in Fig. 3 (solid circles). The data for the calibration curve fitted with the four-parameter logistic function, and the LODs were calculated by the 3σ criterion. LODs for miR-16, miR-21-5p, and miR-500a-3p were 48 fM, 1.4 pM, and 24 zeptomoles when correcting for the sample volume required (0.5 μL of sample solution in this assay). The 3σ criterion. LODs for each target miRNA were fitted with the four-parameter logistic function, and the LODs were calculated by the 3σ criterion. LODs for miR-16, miR-21-5p, and miR-500a-3p were 48 fM, 1.4 pM, and 140 fM, respectively. The LOD for miR-21-5p was consistent with our previous study17 and the LOD of 48 fM corresponded to 24 zeptomoles when correcting for the sample volume required (0.5 μL of sample solution in this assay). Three to 30-times differences in the LOD were observed between miRNAs of different sequences. These data indicate that control of the affinity between target miRNA and corresponding CP-DNAs is effective for controlling the LOD. Multiplex detection was verified by comparing the calibration curve for

Table 1  Sequences of the oligonucleotides

| Abbreviation | Sequence (from 5' to 3') |
|--------------|-------------------------|
| MiR-500a-3p  | AUG CAC CUG GGC AAG GAU UCU G |
| MiR-21-5p   | UAG C1U AUC AGA CUG AUG UUG A |
| MiR-16      | UAG CAC CAC G UAA AUA UUG GCG |
| CP-500      | NH2-TTT TTT TTT TTT TTT CAG AAT CCT TGC |
| CP-21      | NH2-TTT TTT TTT TTT TTT TCA ACA TCA GT |
| CP-16      | NH2-TTT TTT TTT TTT TTT CGC CAA TAT TTA C |
| DP-500      | CCA GGT GCA T - Biotin |
| DP-21      | CTG ATA AGC TA - Biotin |
| DP-16      | GTG CTG CTA - Biotin |

and DP-X, respectively.

The power-free microfluidic device for multiplex miRNA detection was prepared as follows. First, 1% of glutaraldehyde (Wako, Osaka, Japan) in carbonate buffer (100 mM, pH 9.4) was incubated at 37°C for 2 h and immobilized on an aminated glass entire surface (SD00011, 25.4 mm × 76.2 mm, Matsunami Glass Ind., Ltd., Kishiwada, Japan) and biotinylated probe DNAs, DP-16, DP-21, and DP-500, were supplied. The signal-to-blank ratios (SBRs) calculated from the images, which were estimated by dividing the fluorescence intensity of the left microchannel by the fluorescence intensity of the right microchannel, are shown in Fig. 2(b). Solutions containing miRNA and blank reference were injected into the left and right-hand microchannels, respectively. The differences between the SBRs of 1000 pM non-target miRNAs and those of 0 M sample were not statistically significant (p = 0.07 – 0.58 with two-tailed t-test). These data suggest that LFDA successfully occurred in a target sequence-dependent manner without any significant cross reaction with unrelated CP DNAs.

Fig. 1 Schematics of multiplex microRNA detection and typical fluorescence images. (a) Blank reference. (b) 1000 pM of miR-500. (c) 1000 pM of miR-16 and -500. (d) 1000 pM of miR-16, -21, and -500.
singleplex data shown in Fig. 3 (solid triangles) with that for multiplex data. The calibration curves for multiplex miRNA detection corresponded to that for singleplex miRNA detection. The required sample volume was 0.5 μL and assay time was 20 min.

In conclusion, rapid and sensitive multiplex miRNA detection utilizing LFDA on the power-free microfluidic chip, which enables the use of a portable microfluidic device, was demonstrated. Because each cancer or disease is thought to have a small number of corresponding marker miRNAs, the multiplex miRNA detection method brings us one step closer to the POC diagnosis of not only cancers, but also other diseases. For practical applications, some technical challenges remain. For example, LODs are needed to be improved or controlled due to respective miRNA cut-off values. Methods for the extraction and purification of miRNAs from body fluids are needed, because miRNAs exist in body fluids in various forms, such as in complexes with Ago2 proteins, encapsulated in extracellular vesicles, and encapsulated in high-density lipoprotein particles.23 Easier miRNA detection methods are preferable, such as methods that enable detection with the naked eye. For POC diagnosis and improved healthcare environments, especially in resource-poor countries, further studies are in urgent need.

Fig. 2  Evaluation of specificity for respective target microRNAs. (a) Typical fluorescence pictures. (b) Signal-to-blank ratio for respective capturing probe DNAs. The error bars indicate ±1 SD (n = 3).

Fig. 3  Calibration curves for multiplex microRNA detection. The error bars indicate ±1 SD (n = 3).
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References

1. V. Dorval, P. T. Nelson, and S. S. Hebert, Front. Mol. Neurosci., 2013, 6, 24.
2. P. Kumar, Z. Dezso, C. MacKenzie, J. Oestreicher, S. Agoulnik, M. Byrne, F. Bernier, M. Yanagimachi, K. Aoshima, and Y. Oda, PLoS One, 2013, 8, e69807.
3. P. S. Mitchell, R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin, and M. Tewari, Proc. Natl. Acad. Sci. U. S. A., 2008, 105, 10513.
4. N. Kosaka, H. Iguchi, and T. Ochiya, Cancer Sci., 2010, 101, 2087.
5. D. P. Bartel, Cell, 2004, 116, 281.
6. C. H. Lawrie, S. Gal, H. M. Dunlop, B. Pushkaran, A. P. Liggins, K. Pulford, A. H. Banham, F. Pezzella, J. Boultwood, J. S. Wainscoat, C. S. Hatton, and A. L. Harris, Br. J. Haematol., 2008, 141, 672.
7. Y. Toiyama, M. Takahashi, K. Hur, T. Nagasaka, K. Tanaka, Y. Inoue, M. Kusunoki, C. R. Boland, and A. Goel, J. Natl. Cancer Inst., 2013, 105, 849.
8. Y. Yamamoto, N. Kosaka, M. Tanaka, F. Koizumi, Y. Kanai, T. Mizutani, Y. Murakami, M. Kuroda, A. Miyajima, T. Kato, and T. Ochiya, Biomarkers, 2009, 14, 529.
9. C. Chen, D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisins, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, K. Q. Lao, K. J. Livak, and K. J. Guegler, Nucleic Acids Res., 2005, 33, e179.
10. P. Mestdagh, T. Feys, N. Bernard, S. Guenther, C. Chen, F. Speleman, and J. Vandesompele, Nucleic Acids Res., 2008, 36, e143.
11. P. T. Nelson, D. A. Baldwin, L. M. Scearce, J. C. Oberholtzer, J. W. Tobias, and Z. Mourelatos, Nat. Methods, 2004, 1, 155.
12. T. Ueno and T. Funatsu, PLoS One, 2014, 9, e90920.
13. M. Baker, Nat. Methods, 2010, 7, 687.
14. N. Lion, F. Reymond, H. H. Girault, and J. S. Rossier, Curr. Opin. Biotechnol., 2004, 15, 31.
15. K. Hosokawa, K. Sato, N. Ichikawa, and M. Maeda, Lab Chip, 2004, 4, 181.
16. H. Arata, H. Komatsu, A. Han, K. Hosokawa, and M. Maeda, Analyst, 2012, 137, 3234.
17. H. Arata, H. Komatsu, K. Hosokawa, and M. Maeda, PLoS One, 2012, 7, e48329.
18. K. Hosokawa, M. Omata, and M. Maeda, Anal. Chem., 2007, 79, 6000.
19. H. Okada, K. Hosokawa, and M. Maeda, Anal. Sci., 2011, 27, 237.
20. K. Hosokawa and M. Maeda, Lab Chip, 2009, 9, 464.
21. M. J. Lodes, M. Caraballo, D. Suciu, S. Munro, A. Kumar, and B. Anderson, PLoS One, 2009, 4, e6229.
22. K. Hosokawa, M. Omata, K. Sato, and M. Maeda, Lab Chip, 2006, 6, 236.
23. N. P. Hessvik, K. Sandvig, and A. Llorente, Front. Genet., 2013, 4, 36.