Spatial distribution of laminar flow-assisted dendritic amplification†

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In this paper, we report spatial distribution of laminar flow-assisted dendritic amplification (LFDA) product. LFDA is a recently invented signal amplification method dedicated to biomolecular binding events on microchannel walls. Onto the bound biomolecule, a dendritic structure is constructed by supplying two building blocks from laminar streams produced by a Y-shaped microchannel. In view of the extension of LFDA to simultaneous amplification of multiple binding spots, we have investigated the distribution of the LFDA product across and along the microchannel with the course of time. We fabricated a Y-shaped microchannel with a cross section of 110 μm × 22 μm using poly(dimethylsiloxane). As the LFDA building blocks, FITC-labeled streptavidin and biotinylated anti-streptavidin were injected from the two inlets of the microchannel at a mean flow velocity of 6.2 mm s⁻¹ (after the confluence). Nonspecific adsorption of the building blocks formed the seed layer of LFDA. The progress of LFDA was monitored with a fluorescence microscope up to 10.1 mm of microchannel length. After 5 min or later, the fluorescence intensity profile across the microchannel showed a peak at the center of the channel. With the course of time, the peak height grew exponentially except for slight saturation, but the peak width was almost constant. Along the microchannel, the peak height decreased almost linearly with the increasing logarithm of the distance, and the peak width was broadened in accordance with the 1/3 power law.

Introduction

A major application field of microchannels is detection of specific binding between biomolecules such as antigen–antibody binding and DNA–DNA binding. For easy bound/free separation, the microchannel walls have frequently been used as reaction sites. This strategy has successfully been demonstrated in immunoassays⁴–¹⁰ as well as in DNA assays.¹¹–¹⁷ This strategy has at least three advantages. The first two are common to most of other micro-total analysis systems: (1) small sample volume, which is self-evident; and (2) short analysis time, which is due to shortened distance that the analyte molecules must travel by diffusion.¹⁴ The third advantage is rather unique: (3) straightforward extension toward multi-target analysis in a single microchannel. In this case, one of the four walls of the microchannel is made of a microarray-like substrate having multiple binding spots or stripes.⁸–¹⁷

In conventional, macroscale solid-phase assays, the signal of the molecular binding event on the solid surface is often amplified through chemical means to achieve high sensitivity. As seen in enzyme-linked immunosorbent assays, the enzymatic amplification methods are the most popular. However, in our experience, the enzymatic methods are not always easy to transfer to microfluidic formats. The main difficulty was caused by the mobility of the enzymatic reaction product; the product freely came to and went from the binding site, thus yielding a signal only weakly related to the amount of the bound analyte.

Alternatively, there have been several reports on dendritic amplification (DA) methods, in which signaling products are strictly localized at the binding sites, for macroscale assays.¹⁹–²⁴ The DA methods employ a pair of multivalent building blocks (molecules or particles) which can bind to each other. One of the building blocks also binds, directly or indirectly, to the surface-bound analyte molecule. In the conventional DA methods, the amplification is performed by supplying the building blocks alternately onto the solid surface capturing the analyte. A certain number of reaction cycles build a large dendritic structure, which can easily be detected, on the analyte molecule. However, the need for iterative incubation steps is a major drawback of the conventional DA methods.

To minimize the number of incubation steps, we recently invented laminar flow-assisted dendritic amplification (LFDA) for microfluidic solid-phase immunoassays.⁷ We employed FITC-labeled streptavidin (F-SA) and biotinylated anti-streptavidin (B-anti-SA) as the DA building blocks. These building blocks were simultaneously supplied onto surface-bound, biotinylated secondary antibody from laminar streams generated by a Y-shaped microchannel. The dendritic structures were continuously built along the 3-phase (the two laminar streams and the solid surface) contact line. In that work, only one analyte was detected using a single Y-shaped microchannel. However, LFDA is potentially applicable to simultaneous amplification of multiple binding spots aligned along the microchannel. Toward that extension, knowledge of spatial distribution of LFDA product, especially that along the microchannel, is indispensable.

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In this paper, we report spatial distribution of LFDA product for the first time. We fabricated Y-shaped microchannels using poly(dimethylsiloxane) (PDMS). After imperfect blocking of the microchannel walls, F-SA and B-anti-SA solutions were pumped into the microchannel by an externally applied pressure difference. The LFDA was started by nonspecific adsorption. We investigated spatial distribution of the fluorescence intensity with the course of time. Along the microchannel, the peak intensity decreased almost linearly with increasing logarithm of the distance, and the peak width was broadened in accordance with the 1/3 power law, which has been described in relation to lateral diffusion near the top and the bottom walls of microchannels.31-33

**Experimental section**

**Microchip design and fabrication**

Fig. 1a shows the layout of the microchannel. The microchip consisted of a 30 × 20 × 2 mm PDMS part, containing three identical microchannel patterns, and a 30 × 30 × 2 mm PDMS flat slab. A cross-sectional view around the inlets is illustrated in Fig. 1b. Fabrication of the microchip was similar to that described previously.6 Briefly, a negative master for molding of PDMS was fabricated on a silicon wafer with ultrathick photoresist (SU-8 25, MicroChem, Newton, MA, USA). The surface of the master was passivated by exposure to CHF$_3$ plasma in a plasma etcher (RIE-10NR, Samco International, Kyoto, Japan). Prepolymer of PDMS (Sylgard 184, Dow Corning, Midland, MI, USA; base:curing agent = 10:1) was cast onto the master with a frame for holding the prepolymer. The cured PDMS was peeled off from the master, and outlet access holes with 1 mm diameter were punched using a metal pipe. Silicone tubes with 1 mm outer diameter were inserted into the access holes, and glued with PDMS. To make the inlet openings, the PDMS part was cut with a knife. The PDMS part was reversibly bonded to a PDMS flat slab, which had been molded from a flat silicon master. The assembled PDMS microchip was placed onto a glass slide (76 × 52 × 1 mm) to keep mechanical stiffness during experiments.

**LFDA experiment**

The microchip was degassed in a vacuum desiccator (model VS, As One, Osaka, Japan) at 10 kPa for 0.5–1 h. Immediately after removal of the microchip from the desiccator, the outlet silicone tube was pinched with a paper clip. The microchip was placed on the stage of an inverted fluorescence microscope (TE2000-U, Nikon, Tokyo, Japan). Two aliquots of 1% (w/v) bovine serum albumin (BSA, Wako Pure Chemical Industries, Osaka, Japan) in phosphate-buffered saline (PBS, pH 7.5, Cambrex Bioscience, Walkersville, MD, USA), 3.0 µL for each, were dispensed at both the inlet openings using a micropipette. In 2–3 min, the microchannel was filled with the BSA solution by the power-free pumping mechanism.34 At this moment, most of the BSA solution remained at the inlet openings, because the calculated volume of the microchannel is only 0.20 µL. Then we connected the outlet silicone tube to a vacuum pump (DAL-5D, Ulvac, Chigasaki, Japan) via a vacuum regulator (VR200-G, Koganei, Tokyo, Japan), and removed the paper clip from the tube. By applying a vacuum pressure (10 kPa below the atmospheric pressure) to the outlet, the remaining BSA solution was pumped into the microchannel. After the pressure at the outlet was brought back to the atmospheric pressure, 8.0 µL of 100 µg mL$^{-1}$ FITC-labeled streptavidin (F-SA, Sigma, St. Louis, MO, USA) in PBS containing 1% BSA was dispersed at the left inlet, and 8.0 µL of 100 µg mL$^{-1}$ biotinylated rabbit anti-streptavidin (B-anti-SA, Rockland Immunochemicals, Gilbertsville, PA, USA) in PBS containing 1% BSA was dispersed at the right inlet. LFDA was started by applying the same vacuum pressure as described above. This moment is defined as time $t = 0$. The experiments were carried out at room temperature (~25 °C).

During the flow, fluorescence images of the microchannel were taken every 1 min using the inverted fluorescence microscope equipped with a 100-W mercury lamp, a dichroic mirror block (excitation 465–495 nm and emission 515–555 nm), a 10× objective lens, and a cooled CCD camera (CoolSNAP HQ2, Photometrics, Tucson, AZ, USA). The exposure time of the CCD camera was varied between 50 and 1000 ms for the optimal imaging conditions, depending on the fluorescence intensity. An intensity profile plot across the microchannel was obtained using image analysis software (Image J 1.38x, National Institutes of Health, USA). The data were normalized by subtracting background intensity at the outside of the channel and by dividing the remainder by the exposure time of the CCD camera.
Results and discussion

To study the spatial distribution of LFDA, we designed and fabricated the Y-shaped microchannels shown in Fig. 1a. The microchannel had a uniform rectangular cross section with a width of $w = 110 \, \mu m$ and a height of $h = 22 \, \mu m$. The channel after the confluent point had a serpentine shape with a length of $L = 75 \, mm$, but only the first straight portion ($y < 10.1 \, mm$) was investigated in this work. As shown in Fig. 1b, geometry of the inlets is the same as that in our previous report.\(^7\) However, the material has been changed from the PDMS-glass hybrid microchip to the all-PDMS microchip, to avoid the possible complication caused by the different adsorption properties of glass and PDMS. The solutions dispensed at the two inlets were pumped into the microchannel by applying a vacuum pressure (10 kPa below the atmospheric pressure) to the outlet.

Figs. 1c and 1d depict the LFDA experiment. The initial seeding of the LFDA was accomplished by nonspecific adsorption of F-SA or B-anti-SA onto the PDMS surfaces, which had been blocked with 1\% BSA in advance. We previously reported that 1\% BSA plus 0.1\% casein-based commercial reagent yielded better blocking performance to PDMS surfaces than 1\% BSA alone.\(^7\) In the current study, we intentionally used the unoptimized blocking solution (1\% BSA alone) to obtain a moderate degree of nonspecific adsorption. Preliminary experiments comparing these two blocking solutions are presented in the ESI (Fig. S1).\(^+\) On the nonspecifically adsorbed seed molecule (F-SA or B-anti-SA), a dendritic structure was built by continued supply of F-SA and B-anti-SA from the laminar streams (Fig. 1d). We took fluorescence images showing three kinds of F-SA superimposed: adsorbed onto the top wall, adsorbed onto the bottom wall, and flowing in the bulk solution. Because the entire microchannel could not be imaged at the same time with satisfactory quality, independent experiments were run for different positions of interest ($y$).

The pressure difference $\Delta P = 10 \, kPa$ between the inlets and the outlet pumped the 16.0 \, \mu L volume (8.0 \, \mu L of the F-SA solution plus 8.0 \, \mu L of the B-anti-SA solution) in 17.9 $\pm$ 0.8 min (mean $\pm$ SD of 10 experiments). From these values, the flow rate after the confluent point was calculated as $Q = 14.9 \pm 0.7 \, \mu L \, s^{-1}$, which corresponds to the mean flow velocity $U = Q/\left(wh\right) = 6.2 \pm 0.3 \, mm \, s^{-1}$. The measured $Q$ roughly agreed with the estimation from the following equation:\(^{35}\)

$$Q_{calc} = \frac{\Delta P}{\pi \eta L} \left(1 - \frac{2h}{\pi w} \tanh \frac{\pi y}{2h} \right),$$

where $\eta$ is the viscosity of the fluid. Assuming $\eta = 0.89 \times 10^{-3} \, Pa \, s$ (water at 25°C), we obtain $Q_{calc} = 12.6 \, \mu L \, s^{-1}$.

Fig. 2 shows representative fluorescence intensity profile plots across the microchannel. The peaks at the center of the channel were caused by LFDA. At $y = 10.1 \, mm$ (Fig. 2b), the peaks are lower and broader than those at corresponding times at $y = 0.17 \, mm$ (Fig. 2a). The profiles at the early stages of LFDA are magnified in the insets, where the fluorescence from the F-SA bulk solutions can be seen as the raised regions in the left side. When LFDA is monitored in real time (as in this study), the bulk fluorescence level limits the detection capability of LFDA; we cannot detect the amplified signal until the signal exceeds the bulk fluorescence level. This event is referred to as the initiation of LFDA hereafter. In addition, the bulk fluorescence level gives the calibration factor $k$ of our setup to convert the measured fluorescence intensity $I$ to the amount of F-SA per unit area $\sigma$: $\sigma = kI$. We calculated as $k = 4.2 \, ng \, mm^{-2} (a.u.)^{-1}$. The unit of $I$ is arbitrary, but consistent throughout this paper. The $k$ value will be used for estimation of the amount of adsorbed F-SA later.

Fig. 3a shows the time course of the peak heights at various distances. After the initiation of LFDA at $t = 4–5 \, min$, the peaks grow exponentially except for some saturation effect. This saturation might be caused by molecular crowding effect between and within the dendritic structures. Fig. 3a clarifies that the peak lowering with increasing distance $y$ was caused by the different growth rates, not by the difference in initiation timing of LFDA. We suppose that the lower growth rate at the larger $y$ was caused mainly by progress of reaction between F-SA and B-anti-SA in the bulk solution. As reported previously,\(^7\) when F-SA and B-anti-SA aggregate together, they basically lose their binding capability onto the surface. In the current study, the aggregation must have progressed as they were mixed by diffusion, and this lowered the concentrations of free (LFDA-effective) F-SA and B-anti-SA. (Note that the diffusion itself does not lower the concentrations at the center position. Theoretically, without chemical reaction, the concentrations at the center position are kept at 1/2 of the inlet concentrations regardless of the progress of diffusion.) We suppose that the adsorption in the upstream region caused only little depletion effect in the downstream region. As we will discuss later, we can estimate the amount of F-SA adsorbed onto the channel walls ranging from $y = 0$ to 10.1 mm (see Fig. 5b). The F-SA adsorption during the last 1 min ($t = 14–15 \, min$) was 2.8 ng, which is ten times as much as the adsorption during $t = 5–6 \, min$ (0.26 ng). Despite the 10-fold increase in the adsorption, the peak growth rate at $y = 10.1 \, mm$
(Fig. 3a, slope of the plot) did not change very much during the same period (t = 5–15 min). This means that the growth of the peak was not primarily affected by the adsorption in the upstream region. This aspect is significant when LFDA is applied to multiple binding spots aligned along a microchannel; if amplification at a downstream spot were much affected by the amplification at the upstream spots, this crosstalk would make the data analysis complicated. Fig. 3b summarizes the spatial distribution of the peak heights. We have found that the peak height decreased almost linearly with increasing logarithm of y, with a small anomaly around y = 1 mm. Currently, we do not have simple explanation for the physical basis behind this dependency, which might reflect kinetics of aggregation between F-SA and B-anti-SA in the bulk solution.

We also evaluated the peak widths by measuring the full width at half maximum. At y = 10.1 mm, reliable measurements were possible only for the peaks at t = 11 min or later because of the significant interference by the bulk fluorescence (see Fig. 2b, curves (A) to (C)). Throughout the late period (t = 11–15 min), the peak width did not much vary at each y. Fig. 4 shows them at t = 11 and 15 min in a log–log plot. The data were well fitted with a straight line with a slope of 0.335. This means that the peak was broadened in accordance with the 1/3 power law, which has been described in line with the “butterfly” effect of transverse diffusion between laminar streams in microchannels.\[31-33\] This effect, caused by the parabolic velocity profile over the z direction, was first studied by Ismagilov et al.\[31\] They proved that the diffusive broadening near the top and the bottom walls of the microchannel obeys the 1/3 power law, instead of the classical 1/2 power law, when the following conditions are met: 1 \ll y/h \ll Pe; where Pe is the Péclet number, Pe = U h/D; and D is the diffusion coefficient. Recently, Salmon and Ajdari\[34\] have carried out a numerical study and have refined the above conditions as 10^{-5} < y/(Pe h) < 10^{-1} at the limit of high Pe. Our experimental conditions roughly fall within this regime. (Pe = 2200 and y/(Pe h) = 0.0035–0.21, where we assume that the diffusion coefficients of F-SA and B-anti-SA are equal to the value reported for streptavidin: 10^{-6} D = 6.2 \times 10^{-7} \text{cm}^2 \text{s}^{-1}.) Overall, our results imply that the peak broadening of LFDA is dominated by diffusion of F-SA and B-anti-SA near the surface. In their pioneering work,\[35\] Ismagilov et al. mentioned that the 1/3 power law should be useful to predict the resolution of laminar flow-assisted microfabrication.\[25\] To our knowledge, Fig. 4 is the first experimental evidence of their prospect.
Next, we evaluated the peak areas by numerically integrating the intensity profile plots over \( x \). Contribution from the bulk fluorescence level has been eliminated by subtracting the area value at \( t = 1 \) min, which amounted to 33 ± 4 a.u. \( \mu \)m, from each area value. The results are shown in Fig. 5a. The variation of the peak area over \( y \) is much smaller than that of the peak height (Fig. 3b). The effects of peak lowering and peak broadening cancelled out to some extent. Because of the smaller spatial variation, the peak area may provide a better measure than the peak height, for practical applications in which multiple spots are amplified using a single microchannel. However, the anomaly around \( y = 1 \) mm, which was also seen in Fig. 3b, became more obvious. We have not evaluated the statistical significance of the anomaly in Fig. 3b. However, even without the anomaly in Fig. 3b, in other words, if the peak height had been expressed as \( a - b \log y \), the peak area would have had a maximum value somewhere, because the peak area would have been proportional to \( (a - b \log y)^{b/3} \), which has a maximum value at \( y = \exp(ab - 3) \).

Finally, we estimated the amount of F-SA adsorbed onto the surfaces in the whole range by integrating the area–\( y \) curves (interpolated with straight lines) over \( y \). The unit was converted by the calibration factor \( k \). By taking differences per 1 min, we plotted F-SA adsorption rate in Fig. 5b, along with the fraction in the total F-SA flowed, 44.7 ng min\(^{-1} \), in the right axis. As discussed earlier, the adsorption rate grew 10-fold during the last 10 min, and reached 6% of the total F-SA flowed. Although we have not established a clear criterion, this depletion effect may pose a limitation on the channel length and the amplification time of LFDA for practical applications.

Conclusions

We have described the spatial distribution of LFDA product. We found an apparent linear-log dependency of the peak height on the channel distance. The physical basis behind this dependency remains an open question. Further study with numerical simulation may help better understanding of this phenomenon. On the other hand, the peak width clearly exhibited the 1/3 power dependency on the channel distance. Our results have provided the first experimental evidence indicating that the 1/3 power law also holds even when a 3-phase reaction is involved. For the peak area, the lowering effect and the broadening effect canceled out to some extent, and relatively small variation along the channel was observed. The knowledge obtained here will be useful for designing an LFDA device with multiple binding spots along a single microchannel.

References