A Multi-level Microchannel Integrated Microfluidic Device for Extraction and Separation of Microparticles

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A Multi-level Microchannel Integrated Microfluidic Device for Extraction and Separation of Microparticles

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Abstract

Microfluidic devices facilitate the separation of microparticles, blood samples, and even microorganisms by miniaturizing the centrifugal, magnetic, hydrodynamic, electrokinetic, or dielectrophoretic mechanisms on a chip. Most microfluidic devices are fabricated using standard photolithography technology so that they are limited to a single, uniform microchannel depth. However, multi-level microchannels (MLMs) can significantly enhance the separation performance and efficiency. In this work, a simple method is described for fabricating MLMs by combining a polydimethylsiloxane (PDMS) grey-scale photomask (PGSP) and standard photolithography technology. The PGSP adjusts the total amount of UV absorption in photoresist via a wide range of dye concentrations, which in turn adjusts the degree of cross-linking of the photoresist. This enables the fabrication of a multi-depth photoresist master for microfluidic PDMS replica devices. Using the device, it was demonstrated that an MLM-integrated microfluidic device can filter and accumulate both polystyrene microparticles (5.4 μm, 9.2 μm, and 12.0 μm in diameter) and yeast cells in a Korea traditional rice wine (Makgeolli) by size. It was also demonstrated that a pneumatic pressure controller makes it possible to sequentially extract the separated microparticles and yeast cells from the device. In addition, another microfluidic separation device utilizing chemotaxis of microorganisms was developed to extract motile microorganisms (e.g. *Escherichia coli*) from immotile ones (e.g. yeast). For this, the concentration gradient across the microchannel was well produced along the microchannel and motile *E. coli* cells were successfully separated from immotile yeast cells. Because the PGSP-based soft-lithography technology provides a simple but powerful fabrication method for MLMs, it is believed that the fabrication method can be widely used for micro total analysis systems that benefit from MLMs. Furthermore, both the microfluidic separation devices that can actively modulate filter gaps and extract motile from immotile microorganisms using chemotaxis could be utilized for biotechnological applications such as the filtration, concentration, and extraction of mammalian cells and microorganisms.
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Chapter I

Introduction

1.1 Microparticle separation

The separation of microparticles is defined as the physical fractionalization and/or isolation of homogeneous microparticles from a heterogeneous mixture by size, shape, deformability, various intrinsic properties, and so on. Microparticle separation is essential for applications in industry, medicine and biochemistry [1-3] particularly, it has high potential for mammalian and bacterial cell separation for developments in biology [4] and microbiology [5]. While conventional particle separation methods mainly depend on mechanical membrane filtration, microfabrication technologies are demonstrating unprecedented capabilities in the separation of microparticles and cells [6, 7]. In particular, microfluidic devices provide various methods for separation and filtration over wide ranges of microparticle sizes [8-12], shapes, and deformabilities [4, 13].

1.2 Separation technologies

Most commonly, microfluidic separation devices can be categorized into passive and active devices from the viewpoint of separation mechanisms, as shown in Table 1. For passive methods, spiral channel networks [9], pillar structures [14, 15], slanted microfluidic obstacles in a microchannel [11], non-Newtonian fluids [12], and inertial focusing [8, 10, 16] are used for microparticle separation and/or filtration. These methods determine the range of microparticle sizes to separate or filter at the design and fabrication step. Because of this, the dynamic range of separation of microparticles is relatively narrow and fixed, although passive separation is a continuous, high-throughput technique. For active methods of separation, various separation mechanisms such as dielectrophoresis [17, 18], acoustic waves [19, 20], centrifugation [21, 22], and magnetic forces [23, 24] are used for the separation, sorting, and capture of microparticles. Although these methods allow more controllable separation and can function with a wider dynamic range of microparticle sizes, they require an additional external experimental setup with unwanted effects in terms of the portability and the miniaturization of the microfluidic separation devices.

Most passive devices for microparticle separation use physical trapping and penetration mechanisms of microparticles by and through filter gaps, respectively. However, more controllable techniques that actively adjust the filter gaps in microfluidic devices are required. As mentioned earlier, the filter gaps in microfluidic devices are typically established at the design and fabrication
step and are not easily controlled. However, a device was demonstrated that employed a pressure controller to adjust the filter gap[25]. To date, many passive devices have been designed and fabricated to separate microparticles on a chip according to their size. Most of these separation devices were fabricated using standard soft-lithography technology, which limits the device to a single-level, uniform microchannel network. Although, there is a high potential for multi-level microchannels (MLMs) to significantly enhance the separation performance and efficiency.

In general, MLMs are fabricated with multiple photomasks and repeating photolithography processes such that the number of photomasks required is equivalent to the number of levels of microchannels because a multi-level master is necessary to create polydimethylsiloxane (PDMS) replica devices[5]. Also, a two-step photolithography process was reported, but with restrictions in manipulating the channel width[26]. To resolve the problems with repeated lithography steps, direct laser writing was utilised not only on a metallic nano-film[27] but also on a silicon wafer[28]. A 9-level microchannel network was fabricated by replicating a silicon master for an artificial lung. In addition, a maskless exposure system was employed to produce 3D multi-level patterns. Although the laser writing and the maskless exposure system create multi-level masters, they are expensive and incompatible. In other words, these alternatives require expensive equipment as well as skilful staff, rendering these processes unsuitable for combination with standard soft-lithography technology. For this reason, the development of a simple soft-lithography technology that facilitates the fabrication of MLMs is essential.

1.3 Chemotaxis for cell separation in mixtures

The importance for efficient cell separation has recently led to the development of various separation technologies in microfluidics. Aside from an abovementioned microparticle separation method, there is a state-of-the art in microfluidics-based cell method depending on the chemical, chemotaxis. Chemotaxis is a well-organized event in which cells move along a chemical gradient[29]. This mechanism plays a key role in investigating and separating the several movable bacterial cells by using the chemotactic response[30, 31]. Based on the these research, most of microfluidic devices have been studied to the specific components such as, concentrator, separator by applying the chemotactic response of bacterial cells, because they can generate various concentration gradients by injecting fluid flows that contain diffusible molecules. Especially, a flow-based Y-shaped microfluidic device is widely used format in this field because it is easy to make the two faced laminar flows but not directly mixed[32, 33], allowing the migration behavior of soluble molecules from one flow stream to diffuse into the other to generate concentration gradients along the microchannel[34].
Table 1 Microparticle separation technologies.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mechanism</th>
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<tbody>
<tr>
<td><strong>Active</strong></td>
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<tr>
<td>Flow cytometry (FACS)[35]</td>
<td>Fluorescence</td>
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<tr>
<td>Magnetic force</td>
<td>In/homogeneous magnetic field</td>
</tr>
<tr>
<td>Mechanical filters[36]</td>
<td>Physical size</td>
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<tr>
<td>Dielectrophoresis</td>
<td>Inhomogeneous electric field</td>
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<tr>
<td>Electrophoresis</td>
<td>Homogeneous electric field</td>
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<tr>
<td>Centrifugation</td>
<td>Centrifugal force</td>
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<tr>
<td>Acoustic force</td>
<td>Ultrasonic standing waves</td>
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<tr>
<td><strong>Passive</strong></td>
<td></td>
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<tr>
<td>Pillar, weir[37, 38] and slanted structures</td>
<td>Laminar flow</td>
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<tr>
<td>Pinched flow fractionation</td>
<td>Hydrodynamic force</td>
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<td>Hydrodynamic filtration</td>
<td>Hydrodynamic force</td>
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<tr>
<td>Inertial force</td>
<td>Shear-induced and wall-induced lift</td>
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<tr>
<td>Non-Newtonian fluids</td>
<td>Fluids properties</td>
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<tr>
<td>Hydrophoretic filtration[39]</td>
<td>Pressure field gradient</td>
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</table>
Fig. 1.1 Microorganism sorting mechanism by using chemotaxis. Chemotaxis is the phenomenon of somatic cells, bacteria, and other single-cell direct their movements according to certain chemicals in their environment. This is important for bacteria to find food (for example, glucose) by swimming towards the highest concentration of food molecules[40].
2.1 Separation device using PDMS based grey-scale photomask

In this work, we report a simple but powerful microfabrication method to form MLMs by combining a polydimethylsiloxane grey-scale photomask (PGSP) and standard soft-lithography technology[41]. Generally, a grey-scale is an image, which the value of each pixel is carried only intensity of information. This technique commonly used black-and-white are composed exclusively of shades of grey, varying from black at the weakest intensity to white at the strongest[42]. In microfluidics, a grey-scale photolithography technique has been suggested by using a microfluidic photomask with positive photoresist on a silicon wafer[41]. However, to provide a more convenient and compatible fabrication method with soft-lithography technology for MLM-integrated PDMS replica devices, we modify the technique to use a negative photoresist on a glass substrate, as shown in Fig. 2.1. We also employ a manual pneumatic pressure controller that independently raises and lowers the filter barrier by attaching an individual PDMS chamber on top of each filter barrier. We tested the MLM-integrated microfluidic device for its functionality in filtering and concentrating microparticles by the filter gaps based on their size. We also checked that it sequentially sorted the microparticles along the microchannel by raising the filter barrier, which results in the sequential extraction of them from the MLM-integrated microfluidic device. Lastly, we demonstrate that the same method can be used for the separation by size and extraction of yeast cells in grain-fermented alcohol for additional biochemical and microbiological assays.

Fig. 2.1 Concept of the grey-scale photolithography
2.2 Experimental setup and methods

2.2.1 Reagents and microparticle and yeast

A dye (Allura red AC, Sigma-Aldrich, Korea) was diluted with distilled water to bestow proper concentrations. A phosphate buffered saline (PBS, Sigma-Aldrich, pH = 7.4) solution and a 1% Pluronic surfactant (F-127, Sigma-Aldrich) were used to the every experiments. The purchased microparticles (Spherotech, Korea) were 5.4 µm, 9.2 µm, and 12.0 µm in diameter. Yeast cells were obtained from a Korea traditional rice wine called Makgeolli (pH = 4.5–5.0). The solution accumulated specific size microparticles was fabricated by centrifuging 1 mL of Makgeolli at 1000 rpm for 30 s and then adding 0.5 mL of the supernatant solution to 0.5 mL of the PBS solution (refer to Fig. 2.11)[43].

2.2.2 Fabrication procedures

2.2.2.1 Fabrication of the microfluidic photomasks

The device fabrication consisted of three steps: photomask fabrication, MLM fabrication, and pneumatic chamber fabrication (see Fig. 2.2). First, a microfluidic photomask was devised by using normal soft-lithography technology as used in our previous work[43]. As illustrated in Fig. 2.2 (A)–Step 1, a master was patterned on a silicon wafer using a negative photoresist (SU-8, 2050, MicroChem, Newton, MA, USA) and then the microfluidic channels were prepared by replicating the master pattern with PDMS. The PDMS mould was bonded using an oxygen plasma treatment with a cover glass under 50 sccm of O_2 at 50 W for 5–10 s (Cute-MP, Femto Science, Korea). The PDMS microchannels, at 75 µm deep and 100 µm wide, were used as a photomask these are used the PGSPs, which minimise the use of multiple metal- or film-based photomasks that is commonly adjured to produce MLMs in other study[5, 26]. The effect of the microchannel width in a PGSP on the filter barrier was investigated in Fig. 2.6. Our microfabrication process requires only one film-based photomask for MLMs, while, in the aforementioned previous work, MLMs fabrication involved one photomask for each level. This respect is additionally dealing with in below paragraph.

2.2.2.2 Fabrication of the multi-level height devices

To fabricate the MLM-integrated microfluidic device, the SU-8 photoresist was spin-coated on a glass wafer at 500 rpm for 5 s and 3500 rpm for a continuously 30 s to obtain an approximately 20 µm thickness layer. It was pre-baked for 5 min on a hot plate and the PGSP prepared in the mentioned earlier first step was attached to the backside of the glass wafer. All of the microchannels of the PGSP
were washed of impurities with a PBS solution and then filled with dye solutions of different concentrations. Next, the glass wafer was exposed to a collimated UV light using a mask aligner (MA6, SUSS MicroTec, Germany) through the PDMS photomask, as shown in Fig. 2(A)–Step 2. Because the photoresist was negative, it was cross-linked from the bottom side of the glass substrate to the top of the glass. After the UV exposure (90–100 mJ/cm²), the glass substrate was heated on a hot plate at 95°C for the post-exposure bake for 4 min and then developed with an SU-8 developer (MicroChem, Newton, MA, USA). The depolymerised photoresist was removed, resulting in a patterned, multi-depth SU-8 master. After being rinsed and dried, the master on the glass substrate was annealed again on a hot plate at 150°C for approximately 2 min to reinforce the surface uniformity of the SU-8 master, as shown in Fig. 2.3. The PGSP adjusted the total amount of UV absorption of the photoresist by using a wide range of dye concentrations. Because the dye adjusts the degree of cross-linking of the photoresist, the fabrication of multi-depth photoresist masters for a microfluidic PDMS replica device is possible. Subsequently, the SU-8 master was silanised with trichloro(3,3,3-trifluoropropyl)silane (Sigma-Aldrich, Korea) in a vacuum jar for 1 h to minimise the adhesion between the substrate and the PDMS pre-polymer.

Lastly, the microfluidic chambers were fabricated in PDMS in the same manner as the first soft-lithography process and then attached to the top of the MLM-integrated device by using the same oxygen plasma treatment. The chambers were separately connected to a 3 mL syringe via an air-tight tube so that the pressure in the chamber was individually manipulated manually. Because the top chamber layer (5 mm) is much thicker than the MLM layer (20 µm), a negative pressure easily raised the filter barrier. In this manner, each filter barrier was raised and lowered by manually controlling the pneumatic pressure in the chambers.

2.2.2.3 Conventional fabrication method of the multi-level height microfluidic devices

Typically, photolithography technology is offer to advantage in 2D micro patterns. However, when we apply to these protocols at desired MLMs device, it was consumed multiple photomasks and repeating photolithography processes such that the number of photomasks required is equivalent to the number of levels of microchannels. First, the positive photoresist was spin-coated on a silicon wafer to obtain a thin-layer. After the collimated UV exposure on the silicon wafer using a mask aligner, the positive photoresist was spin-coated on a wafer to obtain the second layer. In particular, the photomasks was blocked the UV onto the first layer. Repeatedly, the microstructures were fabricated in the same manner as the first and second photolithography by using a mask aligner. As shown in Fig. 2.2(B), these are containing the whole processes to fabricate the several height microstructures. Because it has the high-resolution of precise microstructure, this method can be
possible to apply the specific field. Therefore, in order to fabricate the multi-level microfluidic separator, a novel fabrication technology for linked-microchannel is essential.
Fig. 2.2 Microfabrication processes: microfluidic photomask (Step 1) and multi-level microchannel fabrication (Step 2). (A) A microfluidic channel was fabricated by using a standard soft-lithography technology and used as a PGSP. The photoresist coated on the frontside surface of the glass substrate was UV-exposed but the UV intensities were adjusted by the PGSP, which was closely bonded on the backside of a glass substrate and filled with a dye solution with different concentrations, resulted in a multi-level photoresist master. (B) The fabrication method for multi-height microchannel by using the conventional photolithography technology.
Fig. 2.3 The microscopic images of re-flow of the SU-8. (A) Before and (B) after the hard bake of the re-flow by heating on a hot plate at 150°C for fabricate to uniform surfaces.
2.2.3 Experimental procedure and data analysis

The separation channels were rinsed with a PBS buffer solution and then coated with Pluronic surfactant (F-127, 1% in PBS) to minimize non-specific binding between the polystyrene microparticles and glass/PDMS surface. The residue of the surfactant in microchannels was gently rinsed with the PBS (about 200 µl) for approximately an hour. Polystyrene microparticles were agitated in a sonicator (5510E-DTH, Bransonic, USA) and then injected into the separation channel via a Tygon tube. A microscope (IX71, Olympus, Japan) equipped with a CCD camera (Clara, Andor Tech, CA, USA) and Metamorph 7.7 (MDS Analytical Technologies, Sunnyvale, CA) was used to take the images of microparticles and yeast. All images processing and quantification of the polystyrene microparticles and yeast cells were performed using Metamorph and Image J (NIH, USA) and the results were plotted using Origin 8.0 (OriginLab, Northampton, MA, USA).

2.3 Results and discuss

2.3.1 Calibration of penetrated UV amount after a PGSP

We characterised the master thicknesses produced by a PGSP in order to fabricate an MLM-integrated separation device. Firstly, we measured coherent UV intensities directly after an I-line filter (365 nm) using an intensity meter (SUSS MicroTec, Germany), as shown in Fig. 2.4(A). We tested a wide range of dye concentrations and then measured the corresponding UV intensities to determine the final concentrations, which ranged from 12% to 30%. As expected, the UV intensities decreased almost linearly as the concentration of the dye solution increased. Fig. 2.4(B) shows the blockage of UV light by the PGSP shown in the inset in percentages. In the same manner, a higher dye concentration corresponded with a higher the blockage. For example, for a 30% dye concentration, the blockage was measured to be approximately 68%; for a 12% dye concentration, the blockage was approximately 10%. To confirm that the dye in the solution does not significantly bleach under the UV light, Fig. 2.4(C) demonstrates the variation of the UV intensities through the PGSP over 180 s, but no variations were observed.
Fig. 2.4 Characterization of master thicknesses fabricated by using a PGSP. (A) UV intensities were measured and decreased as the concentration of dye solutions increased. (B) A high concentration of dye solutions blocks more light than a low concentration so that the blockage is almost linearly proportional to the dye concentration. The inset shows different concentrations of dye solutions in the PGSP. (C) UV intensities through the PGSP do not change over time up to 180 sec within which exposure process can be easily finished. (D) Calibration data between the dye concentrations and the photoresist thicknesses under different UV intensities as described in the graph.
Fig. 2.5 The microscope images of a multi-level PDMS obstacles created by using the grey-scale photolithography processes. The scale bar is 50 μm.
Fig. 2.6 The relationship between the width of a PGSP (width of a PDMS microchannel used as photomask) and the flatness/shape of microfabricated filter barriers when three widths of a PGSP were tested. (A) 100 μm, (B) 50 μm, and (C) 25 μm in width under three different dye concentrations (left, middle and right).
2.3.2 Calibration of master thickness using a PGSP

To optimise the fabrication of a multi-level master, we quantified the height of the master, which is denoted as the filter gap, with respect to the dye concentration, as shown in Fig. 2.5. This relationship can be described by Beer’s law; the cross-linked photoresist thickness can be estimated from the percentage of UV transmittance (T). Because both the PDMS and the water are approximated to be transparent (\( T_{PDMS} = 92.0\text{–}93.3\% \) and \( T_{water} > 99.97\% \)) the transmittance can be estimated to be \( \log T = -\varepsilon C_d H_L \), where \( \varepsilon \) and \( C_d \) are the absorptivity and the concentration of the dye, respectively, and \( H_L \) is the height of the microchannel (75 µm) filled with a dye solution[41]. The UV intensities through the PGSP gradually decrease as the dye concentration increases, so the thickness is an exponential function of \( C_d \), as shown in Fig. 2.4(D). This is also why the filter gap decreases exponentially with respect to the dye concentration. In fact, we used a short range of dye concentrations (10% to 18%) to account for the exponentially decaying filter gap. Using the calibration data, we selected a proper range of dye concentrations to fabricate MLMs to filter and concentrate microparticles and yeast cells. On the basis of the Fig. 2.4(D) and 2.5, we demonstrated the device composed multi-height microchannel by using different dye concentration. Moreover, the effect of the microchannel width in a PGSP on the filter barrier was investigated in Fig. 2.6, the PGSP width are 100 µm, 50 µm, and 25 µm, respectively, and the sharpness of the filter barrier gradually increase as the width of PGSP decrease, so we choose the PGSP width over the 50 µm for flat filter barriers. Meanwhile, Fig. 2.7 shows that PDMS mould fabricated by using PGSP can sequentially separate the microparticle due to the filter barriers up to 5 µm.

2.3.3 Filtration and accumulation of microparticles with a MLM

The SU-8 master was then replicated with PDMS in the same manner as abovementioned processes. In Fig. 2.7, the SEM images, depicts a series of filter barriers ranging from 5 µm to 16 µm that were fabricated in a PDMS channel. The PDMS replica and a glass slide were treated with oxygen plasma under the same conditions as before for permanent bonding. As shown in Fig. 2.8, to separate and extract the microparticles in mixture we design and install the separator and external equipment, respectively. Then, we tested the filtration of three microparticles (5.4 µm, 9.2 µm, and 12.0 µm) using an MLM-integrated device. As shown in Fig. 2.9(A), the microparticles that crossed the line a–a’ were continuously filtered at each V-shaped barrier and accumulated over time. Because the first filter gap (\( V_1 \)) was approximately 11 µm, the 12.0-µm microparticles were filtered while the smaller microparticles penetrated the first filter gap and crossed the line b–b’.
Fig. 2.7 (A) A scanning electron microscope (SEM) image of a multi-level PDMS microchannel for separator created by using the grey-scale photolithography processes. (B) Close-up SEM images of each filter barrier ranging from 5 µm to 16 µm in height, adjusting the microparticles size.
Fig. 2.8 Fabrication of a microfluidic device with a multi-level microchannel for microparticle separation. (A) A schematic illustration shows the principal fabrication process for a multi-level microchannel using a PGSP. (B) A real image of the MLM-integrated microfluidic device shows an inlet and an outlet for loading a microparticle suspension and three pump connections for controlling the filter gaps.
When the smaller microparticles confronted the second filter gap ($V_2$) that was approximately 8 µm, the 9.2-µm microparticles were filtered in the same manner as the first filter gap. Only the 5.4-µm microparticles still flowed along the microchannel and crossed the line $c'c$, until they met the third filter gap. Because the third filter gap ($V_3$) was approximately 4 µm, the 5.4-µm microparticles were filtered and continuously accumulated. The filtration efficiency ($\eta$) is defined as follows:

$$\eta = \frac{N_{\text{before}} - N_{\text{after}}}{N_{\text{before}}} \times 100 \% \quad (1)$$

where $N_{\text{before}}$ is the total number of microparticles flowing along a microchannel before a filter gap and $N_{\text{after}}$ is the number of microparticles flowing after the filter gap. For example, the efficiency of the first gap was calculated by counting the number of microparticles crossing the line $a'a$ before the filter gap and that crossing the line $b'b$ after the filter gap when a homogenous mixture of 12.0-µm microparticles were loaded and observed for 20 min; the efficiency of the first filter was 89%. In the same manner, the efficiencies of the second and third filter gaps were calculated by loading 9.2-µm and 5.4-µm microparticles, resulting in efficiencies of 85% and 94.5%, respectively. Theoretically, the efficiency is 100%, but the errors are mainly caused by the non-uniformity of the filter barrier across the microchannel and partially caused by the non-uniformity of the microparticle size.

In addition to individual microparticle filtering and accumulation experiments, we performed an experiment with a mixture of three microparticles used above to analyze the efficiency of the filter gaps. Before the first gap, three microparticles that crossed the line $a'a$ along the microchannel were observed and counted, as shown in Fig. 2.9(B). The 12.0-µm microparticles were unable to penetrate through the first filter gap, so only the 9.2-µm and 5.4-µm microparticles were observed at the line $b'b$ before second filter gap. The 12.0-µm microparticles were rarely observed. We repeated the same analysis before and after the lines of the second and third filter gap and found out that the MLMs successfully filtered and caused the non-uniformity of the filter barrier across the microchannel and partially caused by the non-uniformity of the microparticle size.

In addition to individual filtering experiments, we performed another experiment with a mixture of three microparticles of different densities (5.4 µm, 16 %w/v; 9.2 µm, 15 %w/v; 12.0 µm, 14 %w/v) to analyse the efficiencies of the filter gaps. Before the first gap, the three types of microparticles that crossed the line $a'a$ were observed and counted, as shown in Fig. 2.9(B). The 12.0-µm microparticles were unable to penetrate through the first filter gap, so only the 9.2-µm and 5.4-µm microparticles were observed at the line $b'b$ before second filter gap. The 12.0-µm microparticles were rarely observed. We repeated the same analysis before and after the lines of the second and third filter gaps and determined that the MLMs successfully filtered and concentrated microparticles continuously.
Fig. 2.9 The results of microparticles concentration using the multi-level filter gap fabricated by grey-scale technology. (A) We demonstrated that the microparticles were filtered by the filter gap over time while other smaller microparticles penetrated through the filter gap. (B) The number of microparticles that is flowing along the microchannel and crossing the line a–a’, b–b’ and c–c’, respectively. (C) The composition of each microparticle at the filter gaps.
Fig. 2.10 Fabrication of a microfluidic device with a multi-level microchannel for microparticle separation. (Extraction 1) The structure of a multi-level microchannel facilitates separation of microparticles by physically filtering them by size. (ii–iv) The filtered and concentrated microparticles were sequentially extracted from the device along the microchannel by controlling the pneumatic pressure in the chamber on top of each filter gap to lift up the filter barriers at the same time. (Extraction 2) The concentrated microparticles were extracted from the device by controlling the pressure, individually.
After these measurements, we emptied the inlet reservoir, which was filled with a mixture of microparticles and then loaded a buffer solution that contained no microparticles. This step removed most of the microparticles flowing along the microchannel and enabled us to count the number of microparticles accumulated by the filter gaps. The resulting particle composition of the mixture accrued at each filter gap, denoted as (i), (ii), and (iii), is shown in Fig. 2.9(C). The composition ratios of the microparticles at the filter gaps were determined by counting the number of the microparticles of each size and then dividing these numbers by the total number of the microparticles of the given size. The composition shows a relative percentage of the microparticles at each filter gap for 20 min so that it also implies the separation efficiency. In other words, a heterogeneous suspension of the microparticles was separated by the filter gaps according to particle size and the final compositions was 92.5 %, 88.0 %, and 95.0 % for the first, second, and third filter gaps, respectively. Errors between the theoretical and experimental composition were caused by the non-uniformities of both the filter barrier and the as-purchased microparticles.

2.3.4 Sequential extraction of the separated microparticles

To extract the separated microparticles at the filter gaps from the device, we need to check that it sequentially sorted the microparticles along the microchannel by raising the filter barrier. And then we demonstrated that the proposed MLM can be used for the filtration and accumulation of microparticles. An inlet reservoir was evacuated and refilled with a buffer solution to remove all the microparticles in the microchannel. Next, the filter gap was sequentially controlled by utilizing a negative pneumatic pressure. In Fig. 2.11(A) describes the process of releasing the concentrated microparticles by raising the filter barrier. For example, as soon as the third filter barrier was raised by the negative pressure in the top chamber, the microparticles were driven by the flow for several seconds while the other filter barrier remained unaltered. Because the top chamber was attached to the top of each filter gap, a negative pneumatic pressure decreased the chamber volume, which enlarges the filter gap. This enables the concentrated microparticles to penetrate the filter gap. Next, both the third and second filter barriers were raised simultaneously to allow the 9.2-µm microparticles to pass along the microchannel for 8 s. Lastly, the 12.0-µm microparticles were extracted along the microchannel in the same manner by raising all the filter barriers higher than 12.0 µm. It was possible to individually control the pneumatic pressure in the chambers by manually pulling the piston of a 3-mL syringe. As a result, the trapped microparticles were sequentially extracted along the microchannel through the outlet.
Fig. 2.11 Sequential extractions of separated microparticles along the microchannel from the device. (A) Microparticles of 5.4 μm are filtered and concentrated by the third filter gap but they are driven to flow downstream as soon as the third filter gap was lifted up. In the same manner, microparticles of 9.2 μm are extracted downstream along the microchannel by lifting the second and third filter barrier up. Microparticles of 12.0 μm are lastly extracted by lifting all the filter barriers simultaneously. (B)–(D) The number of the microparticles at the first (V₁), second (V₂) and third filter gap (V₃) before and after extraction.
Fig. 2.12 (A) The preparation of a yeast cell suspension. (i) The yeast cells in the Makgeolli were observed to be dense and aggregated. Moreover, the suspension was mixed with grain-particles that were various in sizes. Grain-particles were removed by using a centrifuge. That is, supernatant solutions are prepared by using a different rpm of the centrifuge such as (ii) 500 rpm, (iii) 1000 rpm, (iv) 1500 rpm, (v) 2000 rpm, and (vi) 3000 rpm, respectively, for 30 sec. (B) Shows serial-diluted cell suspensions. (i) The same solution of the supernatant obtained at 1000 rpm for 30 sec. (ii)–(ix) Various cell suspensions were prepared with different mixing ratios of the cell suspension to a PBS buffer from (ii) 0.1 mL to 0.9 mL to (ix) 0.8 mL to 0.2 mL.
The numbers of microparticles were counted and compared with each other at the first, second, and third filter gaps before and after the extraction process. We used equation (1) again to quantify the extraction efficiency. For example, Fig. 2.11(D) presents the number of microparticles trapped by the third filter gap, denoted as ‘Before’ on the left side of the graph, and the number of microparticles remaining after the extraction, denoted as ‘After’ on the right side of the graph. The majority of the microparticles were clearly extracted downstream, implying that the filter gap was raised higher than the largest microparticle (>12.0 µm). That is, 98% of the 5.4-µm microparticles were released by the third filter gap and extracted downstream. Although the numbers of 12.0-µm and 9.2-µm microparticles are negligible at the third filter gap, the extraction efficiency was 83% and 99%, respectively. In addition, when the third and second filter barriers were raised simultaneously, 94% of the 12.0-µm, 95% of the 9.2-µm, and 72.5% of the 5.4-µm microparticles were extracted (Fig. 2.11(C)). Lastly, when all three of the filter barriers were raised simultaneously, 97% of the 12.0-µm, 93% of the 9.2-µm, and 93% of the 5.4-µm microparticles were successively extracted (Fig. 2.11(B)).

2.3.5 Application to separation of yeast cells by size

We demonstrated that the MLM-integrated microfluidic device provides a simple but powerful method for separating and extracting a heterogeneous population of microparticles by size. We further applied the device to the separation and the extraction of yeast cells, which are found in a Korea traditional rice wine called Makgeolli, as shown in Fig. 2.13(A)-(i). We purified the yeast cells by using a centrifuge to eliminate the powdered grains and the aggregated cells, as described in the Materials and methods section (refer to Fig. 2.12). Next, we loaded the pre-treated yeast cell suspension in the device (Fig. 2.13(A)-(ii)). As shown in Fig. 2.13(B), the yeast cells were filtered and separated by the filter gaps in a similar manner as the microparticles in Fig. 2.9. Because three filter gaps were used in the yeast separation experiment, the yeast cells were divided into three groups. Their histograms were obtained and shown in Fig. 2.13(D)-(F). In terms of size ranges, the largest group ranged from 9.5 µm to 14.5 µm, the intermediate group ranged from 4.8 µm to 11.2 µm, and the smallest group ranged from 2.9 µm to 7.1 µm. In addition, the filtration efficiency was calculated using equation (1); it was 72% for the first, 56% for the second, and 52% for the third filter gap. The efficiency values for yeast were much lower than those of the microparticles. This difference in efficiency can be attributed to the stiffness difference between polystyrene (E = 3.0–3.6 GPa) and yeast (ca. E = 107 MPa)[44]. Stiff microparticles are better filtered by the PDMS filter barrier than the more compliant yeast cells.
Fig. 2.13 Separation of yeast cells in a Korea traditional rice wine (Makgeolli). (A) A suspension of yeast cells that were extracted from Makgeolli and ranged from about 14.5 µm to 2.9 µm in size. (B) (i–iii) Microimages showing filtered and concentrated yeast cells in the device, resulting in separation by size under flow rate of reference, under 2 times flow rate (iv–vi) and 4 times flow rate (vii–ix), respectively. (C) After sequential extractions of the separated yeast cells. Histograms of yeast cells filtered at (A) the first, (B) the second and (C) the third gap. (N=50)
We hypothesised that the efficiency of yeast cells is highly affected by the flow rate because they are much more compliant than polystyrene. To verify the hypothesis, we studied the flow efficiency for three flow rates. First, the rate of 0.45 μL/min used in the microparticle separation experiments above was employed, which corresponds to an average flow velocity of 50 μm/s in the microchannel (Fig. 2.13(B) (i)-(iii)). Next, the flow rate was doubled (0.9 μL/min, Fig. 2.13(B) (iv)-(v)) and quadrupled (1.8 μL/min, Fig. 2.13(B) (vi)-(ix)) to investigate the effect of fluid shear stresses on the filtration and the penetration of the yeast cells. An analysis of the qualitative results indicates that more yeast cells accumulate by the filter gap when the flow rate was doubled, but they can deform to penetrate the filter gaps under the quadrupled flow rate. In addition, because a flow rate is linearly dependent on the fluid pressure in the microchannel, the elastomeric PDMS microchannel can deform. This will in turn enlarge the filter gaps. The combination of the flow rate and the enlargement of the filter gap affect the net filtration efficiency and the squeezing phenomena. Further study is beyond the scope of this work. In the same manner, the separated yeast cells under the reference flow rate were sequentially extracted from the device, as shown in Fig. 2.13(C).

2.4 Discussion

Typically, the filter gaps were microfabricated as a rectangular shape. However, a PGSP enables the production of parabolic filter gaps by using concentration gradients of a dye solution along the microchannels of the PGSP; the method for generating concentration gradients of a dye solution in a microfluidic channel can be easily found in literature. In addition, as briefly discussed in the previous section, PDMS microchannels can easily deform in the presence of high pressure (i.e. high flow rate), which is directly related to the throughput of the separation device. However, we propose several methods to prevent the deformation of the MLM-integrated separation device developed in this work as follows. First, we believe that the pneumatic pressure control in the top chamber can actively and precisely adjust the filter gap under high flow rates, as similarly demonstrated by Chang et al.[25]. Second, a PDMS replica can be replaced with another hard polymer material, as well-reviewed and characterised by Sollier et al.[45_ENREF_45]. One unique feature of the MLM-integrated device as compared to other filtration and/or separation microfluidic devices is that the proposed device can separate microparticles of as many sizes as the filter gaps in the device. We again note that the filter gaps can be easily fabricated with a PGSP and the soft-lithography delineated in this work. Another novel feature of the MLM device is that it can also accumulate as many microparticles as required and sequentially release them by manipulating the filter gaps.

Meanwhile, we fabricated the microstructure that autonomously accumulate to the microparticles, and gradually extract to by lifting-up at each barriers. Moreover, it can be adjust the filter gap specifically by pushing the upper PDMS layer. However, most of the devices consisted in barrier and
based on push-type method have the slit gap on the edge between PDMS and surface due to the boundary constraint. Although various attempts were trying again to solve these leakage problems, it is difficult to fill the minute slit. Because our device is also not uniform at the edge of structure owing to the boundary constraint, in the event of push the upper PDMS using positive pressure for close the microchannel, it can be generate the tiny silt. But, by means of technology based on the dye concentration, it can be build the parabolic-shape filter barrier caused by fine gradient from inlet to center of microchannel. Therefore, we can fabricate the microstructure by using the various dye concentration less tiny silt than conventional push-type microfluidic devices. It was possible to sufficiently make the long-parabolic microchannel in single device by using the fully-long microchannel.
Chapter Ⅲ

3.1 Chemotactic microfluidic device for cell separation in mixtures

In this study, it was demonstrated that the microfluidic device developed can sequentially extract the microorganisms along the microchannel by drifting the main stream, which results in the chemotactic extraction from a mixture. Moreover, it could be extracted in a high efficient manner and offer the advantages as follows:

1) A series of continuous processes to extract microorganisms without the exclusive equipment
2) A novel method for sorting specific microorganism groups by using their intrinsic chemotactic characteristics and motility toward the chemoattractant molecules
3) Extraction processes can be repeated until functional microorganisms are obtained

The ultimate objective of this experiment is to eliminate the unnecessary fungi and particles from the sample containing the several microparticles by using the motility of the bacterial cells. In addition, this method can effectively take the attached bacteria out from the soil particles using the chemotaxis.

As shown in Fig. 3.1, the device consists of two regions for different functional sorting processes. The first region for sorting generates chemical concentration gradients which attract cells in the extraction region. The microorganisms collected from the first region can be further separated based on their size and/or other intrinsic characteristics in the second region. In other words, the most important advantage of this device is to both separate motile cells from immotile cells and to separate the microorganisms by using the chemotaxis property. Therefore, it is believed that the device can be widely used in microfluidic microorganism and/or microparticle separator.

The design of the bottom-layer of the microfluidic device in Fig. 3.1 has three input microchannels. The solution containing microorganisms was injected into the center channel and chemoattractants and a buffer solution were infused into the side channels, respectively. The hydrostatic pressure was utilized to control the relative height of the inlet solutions to acquire the consequent velocity. Meanwhile, to additionally separate the lured motile cells, the top-layer was fabricated to have micro-pillar structures. In this thesis, it is focused to develop the bottom-layer for isolation of motile cells by using chemoattractants and the additional separation would be future work.
Fig. 3.1 Schematic illustration of a microfluidic device for extraction of motile bacteria (blue) from immotile fungi (green) in a microorganism mixture obtained from a micro-ecosystem.
3.2 Experimental setup and methods

3.2.1 Materials and preparation of cells

In this experiment, *E. coli* strain MG1655 was used, which is derived from K12 (a wild type strain). A small colony of *E. coli* grown on a Luria broth (LB) solide medium plate was inoculated into 5 mL of tryptone broth (TB, 1 % Bacto Tryptone and 0.5 % NaCl) media and luria broth (LB, 1.5 % LB and 1 % NaCl). The *E. coli* cells were then grown in a rotary shaking incubator (32°C and 150 rpm) to mid-log phase and required about 8 hours for the OD<sub>600</sub> (optical density at 600 nm) reading to be about 0.4. Before the cell motility was observed, cells were centrifuged at 5000 rpm for 2 min, the supernatant liquid was removed and the pelleted cells were suspended again in fresh (TB) media and (LB) media (30min) and final OD<sub>600</sub> adjusted to a value of 0.5. Yeast cells were obtained from a Korea traditional rice wine called Makgeolli (pH=4.5-5.0). 1 mL of Makgeolli was centrifuged at 1000 rpm for 30 sec and then the supernatant solution of 0.5 mL was added in PBS solution, as shown in Fig. 2.12, previous chapter.

3.2.2 Fabrication of the microfluidic device

Microfluidic devices were fabricated by means of standard soft-lithography technique. Briefly, a SU-8 photore sist (Microchem 2025, Newton, MA, USA) master approximately 20 μm thick was fabricated using standard photolithographic procedures. The surface was silanized using trichloro (3, 3, 3-trifluoropropyl) silane (Sigma Aldrich, Korea) in a vacuum jar for an hour. Subsequently, Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) polymer mixed in the ratio of 10:1 with the curing agent was cast onto the fabricated silicon master to form a replica of the device. The PDMS devices were treated with oxygen plasma under 30 sccm of O<sub>2</sub> and 50W for 30s and bonded with a glass substrate prior to the experiments (see Fig. 3.1).

3.2.3 Experimental procedure and data analysis

All microchannels were washed with a PBS buffer solution to remove impurities and then coated with Pluronic surfactant (F-127, 0.01 % and 1 %) to minimize non-specific binding between the cells and glass surfaces. The residue of the surfactant was subsequently rinsed with the TB buffer solution (about 200 µl) for about 1 hour. A syringe pump (#703007, Harvard Inc.) was used to drive syringes consistently filled with the nutrient and cell. A fluorescence microscope (IX71, Olympus, Japan) equipped with a CCD camera (Clara, Andor Tech, CA, USA) and Metamorph 7.7 (MDS Analytical Technologies, Sunnyvale, CA) was used to take the fluorescence and bright field images of microparticles and yeast. All image processing and quantification of the *E. coli* and yeast cells were
performed using Metamorph and Image J (NIH, USA) and the results were plotted using Origin 8.0 (OriginLab, Northampton, MA, USA).
Fig. 3.2 Characterization of the concentration gradients along the microchannel by using the fluorescein isothiocyanate (FITC). (A) A range of velocity of the buffer and FITC as chemoattractants was tested to measure the generated concentration gradient across the microchannel. (B) The fluorescent intensities verse channel positions at different flow velocity. (C) The result of the diffusion at each channel position from the channel junction.
3.3 Results and discussion

3.3.1 Characterization of the device using FITC

The designed microfluidic device was characterized prior to conducting the experiments on cell separation. In particular, molecular diffusion along the microchannels was visualized and the fluorescence intensities were measured using FITC in PBS buffer solutions. It was found that diffusion was greatly affected by the flow rate and the length of the microchannels. As shown in Fig. 3.2(A), a range of velocities of the buffer and FITC solutions was tested and the generated concentration gradients quantified to determine an optimal velocity for chemotactic separation. Graphs in Fig. 3.2 (B) and (C) show fluorescence intensity vs. channel position at different flow velocities, and the results of diffusion at each channel position from the junction of the channel. A suitable velocity (100 μm/s) was chosen for every experiment involving a chemical diffusion. On the basis of the selected velocity, it was found that before 100 μm along the ordinate, the fluorescence signals corresponded to higher concentrations near the reservoir. It was confirmed that high fluorescence intensities affected the FTIC source. After 100 μm, the lower region of the main channel was shown to have a higher concentration than the upstream region.

3.3.2 Separation of bacterial cells

This experiment was conducted under the same conditions used for measuring the properties of the device, such as flow velocity and diffusion rate, using the FITC and buffer solution. The chemoattractant, cells, and buffer solution were injected in sequence into each inlet reservoir. To control the flow velocity, the reservoir height was set to different values ranging from 0 to 30 mm. After a transient-state of chemical diffusion, E. coli cells cultured in TB medium could be seen actively swimming toward the attractant side (Fig. 3.3(A)-left channel), exhibiting a chemotactic response and migration. Meanwhile, a nonmotile E. coli cell tinged with red, cultured in LB medium, was swept in the direction of wastes. E. coli cells cultured in both TB and LB media in the microchannel filled with M9 solution (Fig. 3.3(A)-right channel) did not exhibit any migration excepting for random motions. Therefore, as shown in Fig. 3.3(B), the ratio of these two groups, motile and nonmotile cells, showed a relative response between the chemoattractant and buffer solution. If a relative point was over 1.0, it indicated that bacterial cells were swept toward the extraction channel, while a relative point under 1.0 signified that the cells were washed away in the direction of wastes.

On average, most of the E. coli cells cultured in TB medium were over the reference point 1.0, corresponding to microchannels 3.4, 2.6, 2.9, 3.5, and 4.3. On the other hand, all of the RFP E. coli...
cells cultivated in LB medium were observed in the microchannel around point 1.0. These results showed that the GFP and RFP cell groups had a relatively different chemotactic response-generated chemical gradient in the microchannel. In addition, the number of cells attracted to the extraction channel was quite constant for 30 minutes, as shown in Fig. 3.3(C), indicating that they were not attached to the surface of the substrate. In other words, although this device posed problems with respect to counting the entire extracted number of cells because of exclusion of the concentrator, it could continuously extract and send cells in a specific direction for connecting the other structures and/or layers.

3.3.3 Yeast and bacterium mixture separation

The conditions for this experiment were the same as those for the earlier one, except for the composition of the mixture of cells used for separation. The separation of a mixture of RFP and GFP E. coli cells by chemotaxis, demonstrated earlier, involved only a single (bacterial) species. Hence, separation of cells of a single bacterial species (E. coli) on the basis of motility was achieved in that experiment. This study was conducted to investigate the separation of cells from a mixture of two different cell types, bacterial (E. coli) and yeast. As seen in Fig. 3.4, bacterial cells, by virtue of their being actively motile in solution, migrated toward the chemoattractant channel (green arrow), while the nonmotile yeast cells remained in the fluid stream at the center regardless of the concentration gradient (black arrow). Thus, it was possible to separate specific cell types on the basis of a single property: motility. Moreover, the device developed has been shown to separate 3 different cell types: nonmotile/motile E. coli and yeast cells.
Fig. 3.3 Bacterial cell separation by using the chemotactic response (motility). (A) GFP-expressing *E. coli* were cultured in TB to confer high motility while RFP-expressing ones were cultured in LB media to confer low motility. The former migrate toward the high concentration region (left fluid stream) while the latter remain in the middle fluid stream, indicating that the device can be used to separate microorganisms according to their motility (chemotactic response). (B) The ratio of the GFP- and RFP-expressing cells shows different responses to the chemoattactant and buffer stream. (C) The number of GFP- and RFP-expressing cells attracted and repelled to the extraction channel (left fluid stream).
Fig. 3.4 The result of yeast and bacterial cell sorting in a microfluidic device. Motile bacterial cells (*E. coli*) migrate toward the high concentration region (left fluid stream) while immotile yeast cells remain on the fluid stream at center regardless of the concentration gradient.
3.4 *Future work*

In this study, it was demonstrated that the separation of *E. coli* and yeast cell mixture can be achieved by using a microfluidic device. Since the device consisted in double-layer, the separated results also divided by two groups. First, the microbiota group, which was movable in the microchannel included the random migration, were possible to separate by using the chemotaxis. Second, the group of the immotile cell, they could be secondary extraction by using specific properties such as size, shape, deformability, various intrinsic properties, and so on. Besides, it was possible to extract the particular cells from the mixture. In this chapter, we mainly described in terms of methodology for divided by the former and the latter. And we were dealing with the physical separation technique by using the multi-level height or the micro-separators applied effective technologies to extract other size particles in chapter II. Therefore, the technology, which extracts to target cell in motile cell group will be developed for materialization of a multifunctional device.
Chapter IV

Conclusion

An MLM-integrated microfluidic separation device has been developed to separate microparticles and microorganisms on a chip by modifying a grey-scale photo-mask technique. Compared with earlier MLM fabrication methods, the proposed technique seems to facilitate better separation as it can be easily combined with the standard soft-lithography technology, widely used for PDMS-based microfluidic devices, and metal- or film-based photo-mask processes enable the easy production of MLMs. With the help of the MLM-integrated device, microparticles of diameters 5.4 µm, 9.2 µm, and 12.0 µm could be physically filtered and concentrated by filter gaps that were easily controlled by the dye concentrations. The separated microparticles were sequentially extracted from the device by actively raising or lowering the filter barrier, which was achieved by attaching an individual air chamber on top of each filter barrier to control the pneumatic pressure. The device was tested for separating and extracting yeast cells found in grain-fermented alcohol, and it was confirmed that the cells were separated and extracted according to their size in the same manner as microparticles, but with a lower efficiency.

In addition, another microfluidic separation device utilizing the chemotactic response of microorganisms was developed to separate motile microorganisms (e.g. E. coli) from nonmotile ones (e.g. yeasts). For this, the concentration gradient across the microchannel was well characterized along their length. The device was then tested for separation of actively motile E. coli cells, cultured in TB medium, from nonmotile cells of E. coli cultured in LB medium. The results indicated that motile and nonmotile cells of E. coli could be easily separated using the device. Besides, under identical conditions, motile cells of E. coli were successfully separated from nonmotile yeast cells using chemotaxis.

It is believed that this MLM fabrication method could be broadly applied to other microfluidic devices that benefit from multi-level features. Furthermore, both the microfluidic separation devices—the ones that can actively modulate filter gaps and those that can separate motile and nonmotile microorganisms by chemotaxis—may be utilized for biotechnological applications such as filtration, concentration, and extraction of mammalian cells and microorganisms.
V. REFERENCES


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