Cell separation by an aqueous two-phase system in a microfluidic device

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We generated an aqueous two-phase laminar flow in a microfluidic chip and used the system to isolate leukocyte and erythrocyte cells from whole blood cells. The microfluidic system reduced the effect of gravity in the aqueous two-phase system (ATPS). Poly(ethylene glycol) (PEG) and dextran (Dex) solutions were used as the two phases, and the independent flow rates of the solutions were both 2 μL/min. When hydrophobic and hydrophilic polystyrene beads were introduced into the microfluidic device, the hydrophilic beads moved to the Dex layer and the hydrophobic beads to the interface between the two phases. In the case of living cells, Jurkat cells and erythrocytes moved more efficiently to the PEG and Dex layers, respectively, than they move in a conventional ATPS. When whole blood cells were inserted into the microfluidic chip, leukocytes could be separated from erythrocytes because erythrocytes moved to the Dex layer while leukocytes remained outside of this layer in the microfluidic system. The reported microfluidic chip for the whole blood cell separation can effectively be integrated into a Micro Total Analysis System designed for cell-based clinical, forensic, and environmental analyses.

Introduction

Cellular-level analysis helps researchers understand the mechanisms of disease in tissues, organs, and other parts of the body.[1–4] Cell separation technology is very important in biochemical and medical fields for use in gene analysis, screening for cell surface molecules, sorting stem cells, and similar analyses.[1,5] Completely separating specific cells has always been difficult because cells are living organisms with different sizes and shapes.[6] Fluorescence-activated cell sorting[7] (FACS) and magnetic-activated cell sorting[8] (MACS) have been intensely investigated as cell separation technologies. They show high selectivity for the target cells, but they use antibodies that can change the surface characteristic of the cells due to biding of cell surface epitopes on the cells. In addition, only limited kinds of cells can successfully be separated by using antibodies, because antibodies have many types of signals, such as apoptosis.[5]

Cell separation techniques and devices have been reported that use conventional techniques and microfabrication technology for the development of highly capable separation systems.[9–15] An electrophoresis device[16,17] and FACS-[18] and MACS-based devices[19,20] have also been developed. These devices are miniaturized versions of conventional technologies, which employ antibodies, electrical separation, and a microscope system.

Density-gradient centrifugation and an aqueous two-phase system (ATPS)[21,22] for cell separation can be applied for the analysis of intact cells because only aqueous solution is used, and there is no need for conjugate (*e.g.* fluorescent) antibodies to isolate target cells. By using only aqueous solutions, a liquid–liquid system enables the enrichment of a product with high yields in a single extraction step. ATPS was developed primarily for the purification of chemicals, but the system has also been employed for cell separation. The system uses an aqueous, liquid–liquid biphasic system consisting of two polymers, or a polymer and a salt. In previously used ATPS methods,[21,22] cells move into one of the layers because of affinity between the cell surface and solutions owing to differences in size, charge, and hydrophobicity. However, this system shows lower selectivity as a result of gravitational force and needs a large volume of ATPS solution. Therefore, it is desirable to have a cell separation technique that has a high selectivity and high yield, is easy to use, and does not damage cells.

We selected the microfabrication technology for use in this study for several reasons. Because a microfluidic reaction channel is so small, a low volume of reactants is required for analysis, and the channel is not affected by gravitational force. Moreover, a two-phase interface can easily be made in a microfluidic channel with laminar flow. It is well known that a stable laminar flow can be obtained in a microchannel because of its low Reynolds number.[23–25] Our goal is to develop a new system of cell separation using a microfluidic device and ATPS to realize good selectivity and isolation efficiency without the necessity for conjugation of fluorescent reagents or antibodies. A two-phase interface in a microfluidic channel using laminar flow of two different solutions, poly(ethylene glycol) (PEG) and dextran (Dex),[26] which possess low biological activity, was prepared (Fig. 1A). We then determined whether the microfluidic device could separate beads with different surface modifications or different types of cells into different phases. We also tested whether the device could separate erythrocytes and leukocytes from whole blood into two different phases.

Experimental

Preparation of the microfluidic device

The microfluidic device was made of poly(dimethylsiloxane) (PDMS) (Dow Corning Toray Silicone, Tokyo, Japan), which is

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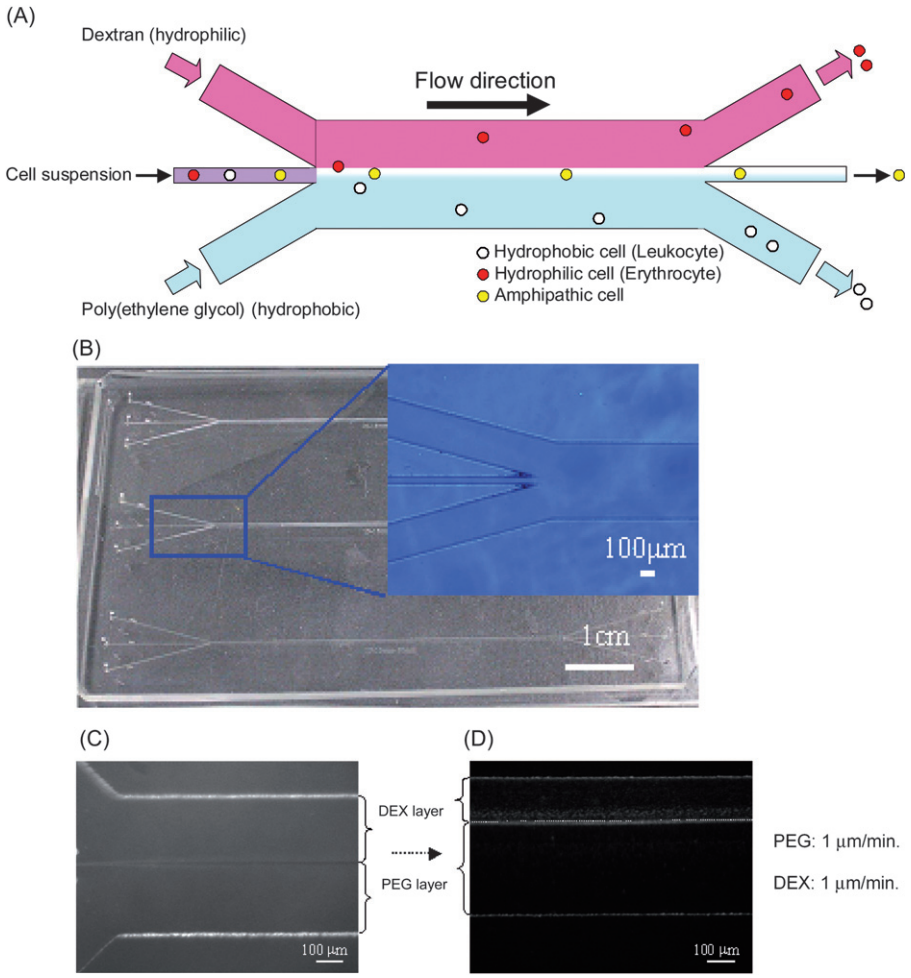

Fig. 1 Schematic illustration of microfluidic device coupled with an aqueous two-phase system for cell separation (A). Photograph of a microfluidic device for cell separation (B). The aspect of interface at the introductory part (C) and the terminal part (D) of the microfluidic device.

easy to handle; in addition, its transparency permits microscopic observation of cells.[27] The basal plate, which was made of a silicon wafer, was washed by ultrasonication, first in acetone and then in hydrofluoric acid. A thick film of SU-8 (MicroChem, USA) was then formed over the basal plate using a spin-coater. The basal plate was pre-baked at 65 °C for 15 min and at 95 °C for 20 min; the plate was then masked and exposed to ultraviolet irradiation for 10 s. After a post-bake at 65 °C for 5 min and at 95 °C for 2 min, the basal plate was submerged in SU-8 developer (MicroChem, USA) to create the final template. Next, microfluidic channels were transcribed from the template into PDMS, which was the thermosetting resin. Finally, the PDMS with the microfluidic channels was irradiated with oxygen plasma, enabling it to stick to the PDMS basal plate. The microfluidic device had a reaction channel with a depth of 100 μm, a width of 500 μm, and a length of 5 cm, with three inlets and three outlets. Two inlet channels had a depth of 100 μm and a width of 235 μm, and the third inlet channel had a depth of 100 μm, and a width of 30 μm (Fig. 1B).

Conditions were monitored at PEG and Dex solution channel tilt angles of 10°, 15°, 30°, and 45° to determine the angle that would produce the optimum laminar flow.

Isolation of beads using the microfluidic device

A two-phase interface of PEG and Dex solutions was prepared using the laminar flow in the reaction channel on the chip.[16] The concentration of the PEG solutions, which had average molecular weights of 3350 (Sigma-Aldrich, Missouri, USA), 6000 (Nacalai Tesque, Kyoto, Japan), and 8000 (Valeant Pharmaceuticals International, California, USA), were varied in steps from 1 to 10% (w/v), respectively. The concentration of the Dex solutions, which had average molecular weights of 40 000 (Wako Pure Chemical Industries, Osaka, Japan) and 500 000 (Amersham Bioscience Corp, New Jersey, USA), were also varied in steps from 1 to 10% (w/v), respectively. Polystyrene beads (6 μm i.d.; Polyscience, USA), which were either carboxylated or unmodified, were suspended in PEG and Dex solutions with the same concentrations described above, that is, 1–10%. The selected polystyrene beads were employed as a model for human cells, because their specific gravity is similar to that of cells. The unmodified polystyrene beads were hydrophobic, but the carboxylated beads were hydrophilic and were thus used as models for hydrophilic cells. The PEG solution was put into the chip through its inlet, and the Dex solution was introduced from the other inlet. In the reaction channel, the two-phase interface was created using the laminar flow of PEG

and Dex solutions. Next, the polystyrene bead suspension was introduced through the sample inlet. A glass syringe (Hamilton, USA) and a syringe pump (Eicom, Japan) were used for all sample introductions. The observation was performed using a fluorescence microscope (Olympus, Tokyo, Japan) and a CCD camera (Carl Zeiss, Germany). Optimum conditions were monitored by alternating the flow rate from 0.1 to 3 mL/min.

Sample cells

We used human blood cells, which are not adhesive and can be manipulated to determine separation conditions. Normal erythrocyte and leukocyte cells and acute lymphocytic T cell leukemia cells (Jurkat, Clone E6-1; American Type Culture Collection, Virginia, USA) were also used. Jurkat cells were used as a model of leukocytes because normal human leukocytes occur in very low numbers in human blood. We prepared 1×10^5 cells/mL of erythrocyte and Jurkat, respectively. In experiments with whole blood cells, we used pure human blood cells. Ethical approval and informed consent were obtained before blood donation. Normal erythrocyte and leukocyte cells were also isolated using a conventional density-gradient centrifugation method as a control experiment.

Isolation of cells using microfluidic device

To isolate cells, a two-phase interface was generated using the laminar flow of PEG and Dex solutions. All cell samples were suspended in a phosphate buffered saline solution (PBS) (0.1 M pH 7.4), respectively. To confirm partitioning of cell, the erythrocyte and Jurkat cell suspension were then introduced through the sample inlet, independently. A fluorescence microscope and a CCD camera were used to observe the cells that were recovered from the outlet channels. Erythrocytes were observed in light microscopy. Leukocytes and Jurkat cells were stained by YOYO-1 (Molecular Probes, Oregon, USA), which intercalates into double-strand DNA, and were thus distinguishable from erythrocytes. The recovered cells were counted by hemocytometry, and the results were compared with those obtained by existing conventional methods.

Results and discussion

We investigated the optimized concentration of two-solutions in ATPS to separate sample. In particular, polystyrene beads with different surface characteristics could be successfully separated in PEG (MW 6000, 6%)–Dex (MW 40 000, 10%) with conventional ATPS.

We tested several different tilt angles for optimum formation of laminar flow. At angles of 30° and 45°, no two-phase interface formation was observed; the PEG and Dex solutions formed an emulsion that could be observed as the flowing solution became turbid. This result suggested that the force exerted by the solution at the inlet channel was stronger than the force exerted at the outlet channel. At a 10° angle, a space formed where there was no liquid solution. This phenomenon was attributed to limitations of the photolithographic method at 20 μm, which may have caused the microchannel shape to be transcribed incorrectly. At a 15° tilt angle, a stable two-phase interface in connection with laminar flow was observed from the introductory part of microfluidic device to terminal of it (Fig. 1C and 1D).

The flow rate of the solution also profoundly affected the formation of the ATPS. When the flow rate was 0.1–1 μL/min, two-phase interface formation was not observed and emulsions formed in the microchannel. This result indicated that the diffusion coefficient of the solution was larger than the flow rate. An emulsion was formed where the PEG solution interacted with the Dex layer, which raised the possibility of a decline in laminar flow separation. When the flow rate was more than 1 μL/min, a stable two-phase interface of PEG and Dex solutions in a laminar flow was achieved.

The flow rate of the sample suspension applied to the chip was determined by the PEG and Dex solution flow rates and concentrations. The flow rate of three-phase interface varied depending on the flow rate and concentrations of the PEG and Dex solutions. When the sample suspension was applied, a three-phase interface, formed by the PEG and Dex solutions and the sample suspension with a laminar flow, was observed in the reaction channel at a each flow rate of more than 2 μL/min and concentrations of 6% PEG and 10% Dex. This result indicated that the flow rate was higher than the diffusion rate. We speculated that the PEG–Dex solution was diluted by the sample solution, and thus we increased the concentration of PEG to avoid the dilution effect. When the each flow rate ranged from 1 to 2 μL/min and concentrations were 7% PEG and 10% Dex, a stable two-phase interface was observed in the microfluidic chip. The optimum flow rate and solution concentrations including the sample suspension were then determined to

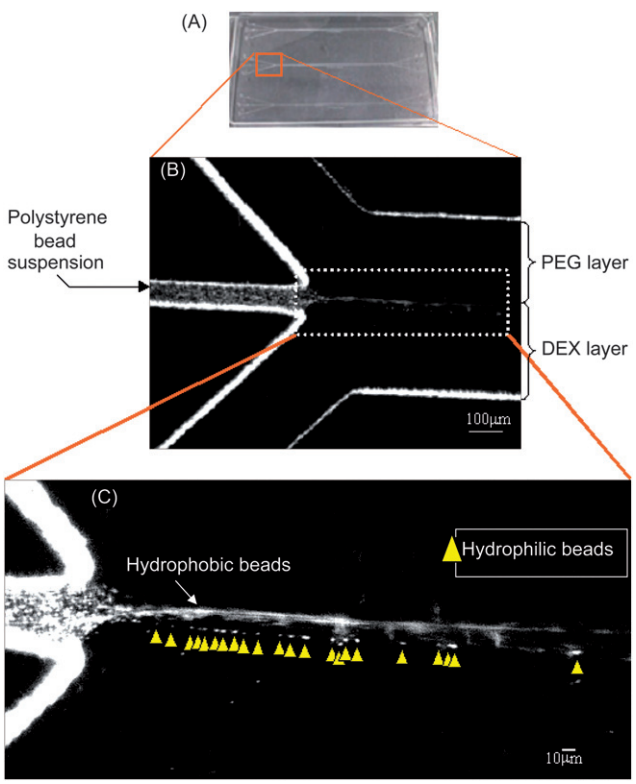

Fig. 2 Isolation of hydrophilic and hydrophobic beads by the microfluidic device-based ATPS (A). Superimposed photograph of introductory part of channel; the introduction of the sample *via* the center inlet was confirmed (B). The partitioning of two types of particle was confirmed from the superimposed white-dashed line region (C). The yellow triangles showed hydrophilic beads.

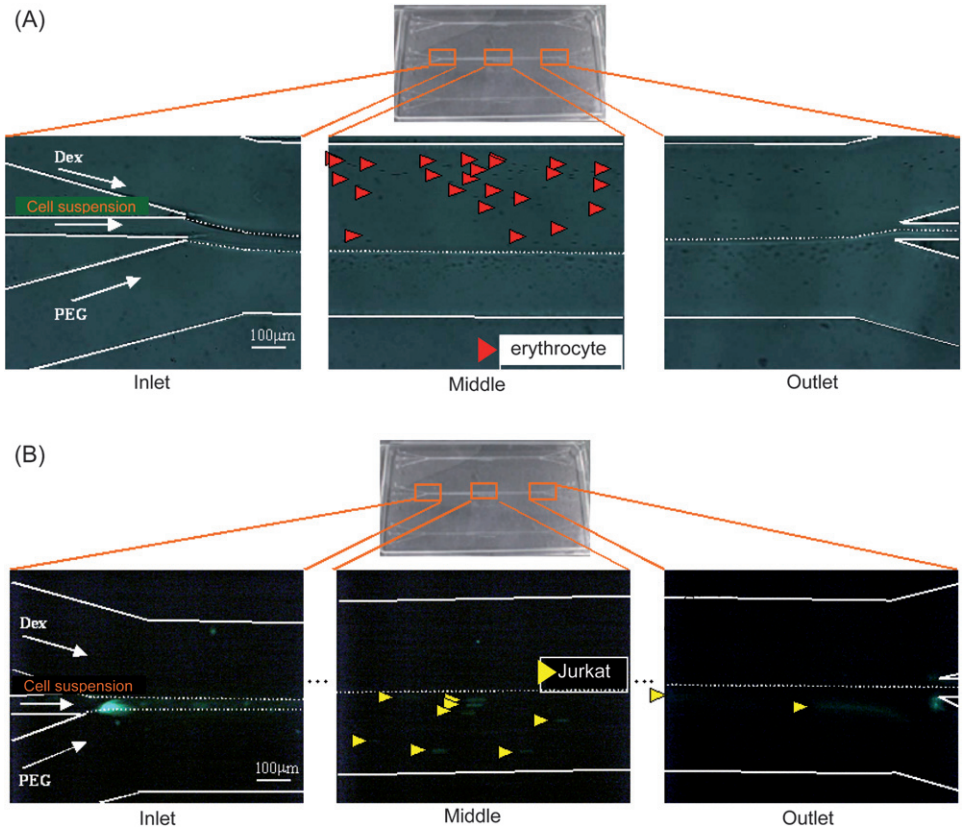

Fig. 3 Separation of (A) erythrocytes and (B) Jurkat cells using the microfluidic device-based ATPS. Superimposed photograph showed the introductory part, middle part and terminal part of the microfluidic device. The dashed line showed the three-phase interface (PEG, sample and Dex layer). The red and yellow triangles showed erythrocyte and Jurkat cells, respectively.

be 2 μL/min and 7% PEG and 10% Dex even though this was a slightly faster flow rate and a richer concentration than those used without the sample.

Fig. 2 shows the partitioning of artificial particles using the ATPS flow. Experiments were carried out under the above optimized conditions. The Reynolds number for this condition is 1.0×10^{-1}, which indicated that a stable laminar flow was obtained. When the two types of polystyrene beads were introduced to the chip (Fig. 2B), only the carboxyl-modified hydrophilic beads moved into the Dex layer. Hydrophobic beads did not move from the two-phase interface (Fig. 2C). This movement suggested that the carboxylic groups on the bead surface interacted with Dex and that this interaction pulled these beads into that layer of the flow. Therefore, using a microfluidic device coupled with the ATPS, we were able to isolate a sample depending on the character of the sample surface and eliminate the effect of gravity.

When a sample of normal human erythrocytes was tested, they gradually moved into the Dex layer (Fig. 3A, red triangles). The interaction of the sugar chain on the erythrocytes with Dex might have caused this phenomenon. In contrast, the Jurkat cells moved into the PEG layer when they were introduced into the chip (Fig. 3B, yellow triangles). We could clearly confirm fluorescent Jurkat cells at the middle part of the channel. The diffusion of cells may occur at the terminal part of it. The negative charge on the membrane of the Jurkat cells might have repulsed the Dex layer, promoting their incorporation into the PEG layer. About 99% of the sample erythrocytes and 96% of the sample Jurkat cells could be recovered from the Dex and PEG layers, respectively. For comparison, about 97% of erythrocytes and 15% of Jurkat cells could be recovered from the Dex and PEG layers, respectively, with conventional ATPS. An additional 17% and 68% of the Jurkat cells could be recovered in the Dex

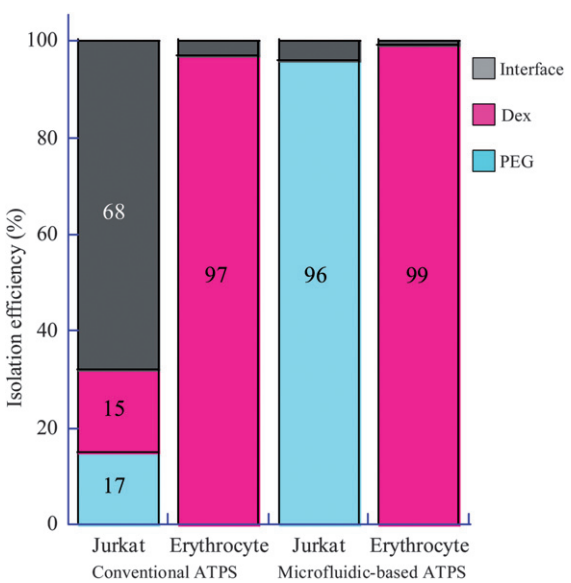

Fig. 4 Comparison of isolation efficiency between conventional and microfluidic device-based ATPS.

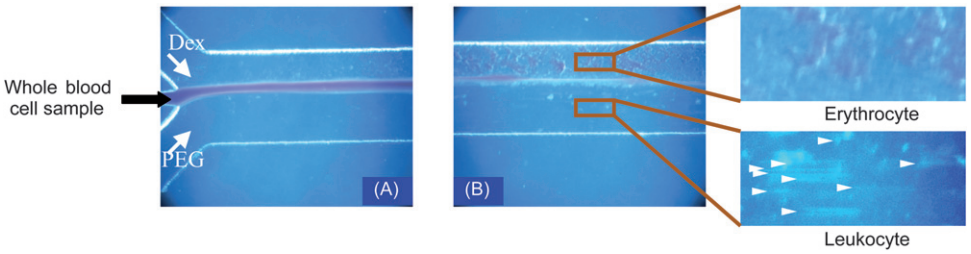

Fig. 5 Separation of whole blood cells using the microfluidic device-based ATPS. Superimposed photograph at introductory part (A) and middle part (B) of the microfluidic device.

and interface layers, respectively. The Jurkat cells were recovered from Dex with conventional ATPS as a result of the effect of gravity. Thus, our microfluidic device-based ATPS was superior to conventional ATPS (Fig. 4), especially in terms of the isolation efficiency of Jurkat cells.

Finally, we carried out isolation of whole blood cells to confirm whether our system can work using a raw sample. Fig. 5 shows the separation of the cells when whole blood was introduced into the chip. The erythrocytes moved into the Dex layer while the leukocytes moved into the PEG layer. The erythrocytes aggregated at the terminal position of the microfluidic device. Therefore, we were not able to count cells as numerical data. We speculate that erythrocytes aggregate each other because the inhibition of cell aggregation by Dex[28] was weak in a huge number of erythrocytes. The different cells in the whole blood sample could be separated using the microfluidic device-based ATPS.

Conclusion

We developed a new and highly effective cell separation system, which we call a microfluidic device-based ATPS. When whole blood cells were introduced into the microfluidic channel, leukocytes could be separated from erythrocytes because erythrocytes moved into the Dex layer while the leukocytes remained outside this layer in the microfluidic system. This rapid and effective cell separation system with parallel detectors is a promising candidate for point-of-care tests, which require rapid, miniaturized devices that are easy to handle. In addition, the system can be effectively integrated into a Micro Total Analysis System designed for cell-based clinical, forensic, and environmental analysis.

Further investigations are necessary for a recovery condition of whole blood cells and the use of diseased cells, for example, leukemic cells.

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