Development of micro immunoreaction-based cell sorter for regenerative medicine

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Abstract

Development of micro cell sorters for stem cell extraction is reported. The principle of cell sorting is based on immunoreaction for accurate recognition of target stem cells. A micro immunomagnetic cell sorter (μ-IMCS) consisting of a lamination micro mixer and a separation reservoir with external permanent magnet was fabricated with soft lithography. Experiments with human umbilical cord vein endothelial cells (HUVEC) and anti-human CD31 antibody show that μ-IMCS is capable of trapping target cells within much shorter processing time compared to conventional IMCS. From cell separation experiments with HUVEC and human mesenchymal stem cells (hMSC), the accuracy of cell separation was comparable to conventional IMCS, and the potential use μ-IMCS in real applications is certified. Following the performance examination of μ-IMCS, a concept of single-step cell separation method utilizing the affinity of cell-surface antigen and its counterpart antibody is introduced. For this, microfabrication of a cell separation channel and antibody coating of the micro channel walls are made successfully.

1. Introduction

Regenerative medicine is a promising biomedical technology in the near future, in which multipotent stem cells are cultured to proliferate indefinitely or differentiate into various tissues and transplanted to damaged or deficit tissues and/or organs. Embryonic stem cell is currently a major candidate for regeneration of tissues, but its use often encounters ethical concerns. On the other hand, mesenchymal stem cell derived from adult bone marrow or blood can also differentiate into various kinds of cells [1], but is a very rare cell. Therefore, a safe, inexpensive and highly efficient method for separating stem cells from whole blood or bone marrow is necessary.

The recent progress in research of micro total analysis systems (μ-TAS) has revealed the possibility of acceleration, miniaturization, and cost reduction in biochemical processes. The most preferable way to distinguish stem cells from other kinds of cells is by their specific set of surface markers (antigens), which play an important role in immunological reactions in our body. Microfluidic cell sorting devices capable of utilizing immunoreaction for target cell recognition such as fluorescence activated cell sorter (FACS) [2] have already been developed, but most of them require bulky external optical systems, or electric fields which may affect the multipotency or plasticity of stem cells.

In the present study, we introduce two kinds of micro cell sorters with immunoreaction-based target cell recognition. Prototype cell sorters are microfabricated and their performance is examined in detail.

2. Micro immunomagnetic cell sorter

2.1 Concept

Immunomagnetic cell sorting (IMCS) is known as an alternative method to FACS with the same order of accuracy, but higher throughput and lower cost. IMCS consists of two steps: (1) marking of target cells with magnetic beads with antigen/antibody reaction, and (2) separation of marked target cells with magnetic force. In conventional IMCS, the first step is the bottleneck because the mixing of cells and magnetic beads rely on diffusion in a bulky container.

Figure 1 shows the concept of μ-IMCS. Cell sample

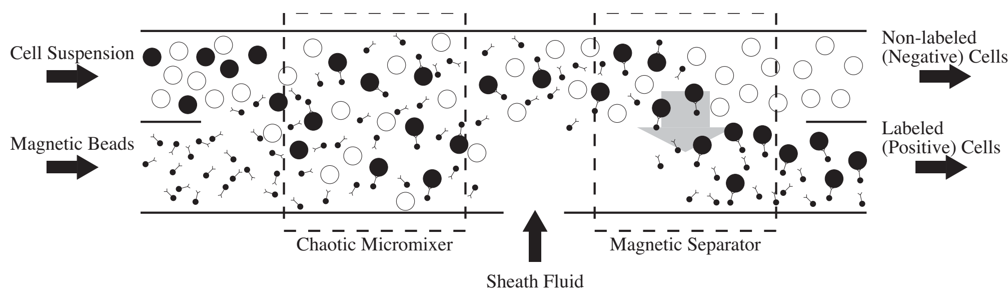

Figure 1. Schematic of μ-IMCS.

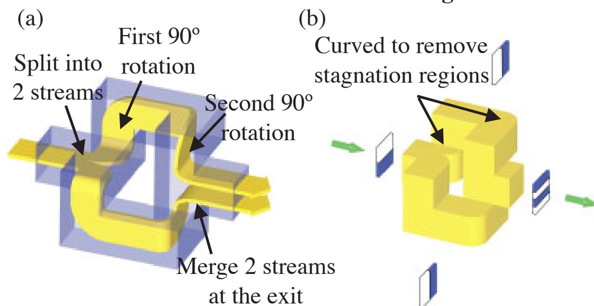

Figure 2. (a) Lamination micro mixer with 180° rotation. (b) Geometry of a single mixer unit.

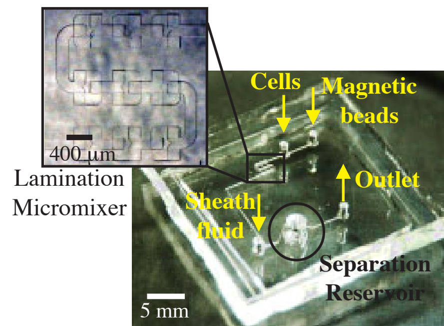

Figure 3. Prototype μ-IMCS.

and magnetic bead solution are separately introduced into a micro channel. Magnetic beads are coated with antibody, which attaches to specific surface antigens on the target cells. The two streams are completely mixed in the micro mixer in order to attach the magnetic beads to the target cells. Subsequently, the cell-beads complexes are separated into the buffer fluid by an external magnetic field.

Since the mixing of cells and magnetic beads is the crucial factor in the performance of μ-IMCS, we develop a passive micro mixer [3], where mixing enhancement in a micro channel is expected.

2.2 Design and fabrication

The principle of mixing in the passive micro mixer is based on lamination of layers in the fluid stream. Figure 2 shows the geometry of a single mixer unit. The flow is split into two streams at the inlet, and merged at the outlet to double the number of layers in the flow. For n mixer units, the number of layers at the outlet is 2^n, which means the thickness of a single layer becomes $1/2^n$ for a channel with constant cross sectional area. Since cells and magnetic beads have larger specific density, the geometry of the mixer is designed in such a way that the flow is inverted 180° at each unit to reduce sedimentation loss of cells and magnetic beads. Corners and stagnation regions of the mixer are rounded so that stagnant flow regions are minimized. In order to make the thickness of one layer smaller than the diameter of magnetic beads, the channel cross section is designed as 200 × 200 μm^2, while the number of mixer units is chosen to be 9.

The separation section consists of a 5 × 5 mm^2 reservoir and an external permanent magnet. Target cells that are labeled with magnetic beads are pulled up toward the top wall and trapped, while the non-target cells flow out of the reservoir with the rest of the flow. After all the non-target cells are collected at the outlet, the permanent magnet is removed manually to let the target cells flow out.

Figure 3 shows the prototype μ-IMCS. The mixer with the configuration shown in Fig. 2 was made through soft lithography [4]. By combining three layers of polydimethylsiloxane (PDMS), the mixer and separator sections are fabricated on a single device. SU-8 molds are made on a silicon wafer by using standard lithography techniques, and each PDMS layer is cast from the master molds. The surfaces of each layer are treated with oxygen plasma, and permanent bonding is made after alignment of the layers under the microscope. The separation reservoir is fabricated on the bottom layer, and a hole for insertion of the permanent magnet is opened manually in the top layer.

2.3 Cell trapping experiment

A combination of human umbilical cord vein endothelial cells (HUVEC) and mouse anti-human biotin-conjugated CD31 (PECAM1) monoclonal antibody, which attaches specifically to HUVEC [3], is used to evaluate the performance of μ-IMCS. CD31 antibody is bound to the surface of HUVEC with conventional incubation method before infusion into the μ-IMCS. Streptavidin-conjugated magnetic beads of 1 μm (Dynal Biotech Inc.) are used as markers that specifically attach to the CD31 antibodies on HUVEC via streptavidin/biotin binding in the micro mixer.

The number density of HUVEC in the cell suspension is chosen as 1.09 x 10^6 cells/ml, and the volume concentration of the magnetic beads are chosen as 0.15 %. The flow is driven by a syringe pump, and the total flow rate of cell suspension and magnetic bead suspension is kept as 5.9 μl/min, 11.8 μl/min and 23.6 μl/min, which correspond to the Reynolds number of 0.5, 1 and 2 in the

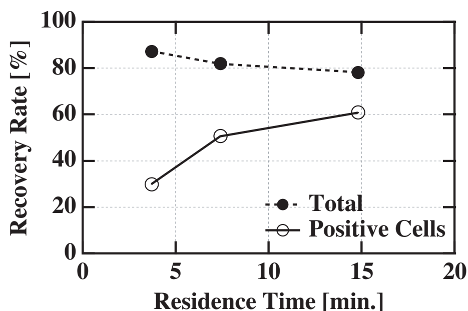

Figure 4. Effect of residence time of the sample solution on cell trapping performance.

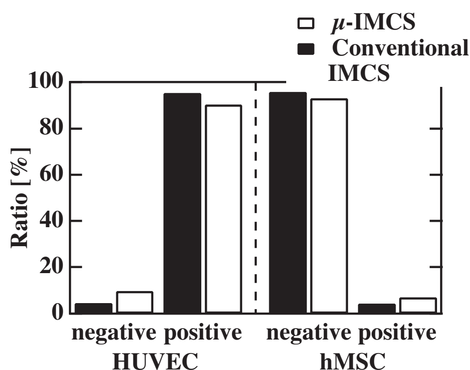

Figure 5. Cell separation performance of μ-IMCS.

mixer section, respectively. The trapped (positive) and non-trapped (negative) portions of the cells are collected separately, and counted with a hemacytometer.

Figure 4 shows the effect of residence time of sample fluid on the recovery rate, which is defined as the ratio of the number of cells collected at the outlet and the number of cells infused in the inlet. The recovery rate of positive cells increases with increasing the residence time, in other words, with decreasing the flow rate. From our CFD analysis [3], it is shown that the flow rate does not affect the mixing performance in this flow rate range. It is concluded that a long enough time is needed to get sufficiently many magnetic beads attached to a cell, so that the magnetic force acting on the target cells should be strong enough to trap them. The residence time at the highest recovery rate achieved is 15 minutes, and this is still shorter than the incubation time required in conventional IMCS (around 1 hour). On the other hand, the recovery rate of total cells shows a slight decrease with increasing the residence time. The cause is most likely to be the increase of sedimentation loss in the channel between the mixer section and the separation reservoir. Higher total recovery yield may possibly be achieved through improvement in the design of the whole system.

2.4 Cell separation

In order to compare the performance of μ-IMCS with conventional IMCS, a mixture of HUVEC and human mesenchymal stem cells (hMSC, CD31 negative) were infused into the μ-IMCS. The experimental conditions are similar to those described in the previous section. The volume concentration of cells is 0.04% with the ratio of the number of HUVEC:hMSC being 1:1.24. The flow rate is 11.8 μl/min, corresponding to the Reynolds number of unity in the mixer section. The inner part of hMSC is dyed with green fluorescent protein (GFP) in order to distinguish the two kinds of cells when counting.

Figure 5 shows the ratio of the number of positive and negative cells counted at the outlet, for both HUVEC and hMSC. For comparison, a cell separation experiment with conventional IMCS (autoMACS, Miltenyi Biotec GmbH) was made. It is shown that the cell separation performance of μ-IMCS is comparable to that of the conventional IMCS. With the superiority in processing time above conventional IMCS, μ-IMCS should have a potential to be used in real applications with less cost.

3. Micro affinity chromatography column for cell sorting

3.1 Concept

In the μ-IMCS described above, multiple steps are required for cell sorting, and a micro mixer and a separator should be integrated on a single device. In this section, a new single-step cell sorting method is introduced. It imitates the mechanism of the behavior of leukocytes in our body [5]. Leukocyte-specific antibody is expressed at the endothelium close to sites of inflammation or injury, and leukocytes slow down with transient attachment and detachment between the antibodies and the surface markers of the leukocytes. The velocity of cells rolling over the antibodies depends on the balance between shear rate of surrounding flow and antigen/antibody binding force, mediated by cell membrane deformation [5].

Figure 6 shows a schematic of the present cell separation method. Antibodies that specifically attach to target cells are immobilized on micro channel walls, and the velocity of the target cells are affected by antigen/antibody interaction, resulting in the separation of target and non-target cells in the streamwise direction of the flow. By coating the antibody on the channel walls with a sufficient surface density, it is expected that the target cells slow down to a terminal velocity, at which they could be completely separated from the non-target cells. Also, it is possible to sort multiple cell species according to the affinity of each cell species to the antibody on the wall, or more specifically,

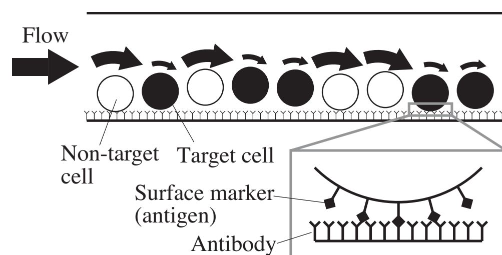

Figure 6. Schematic of cell separation with surface-coated antibody.

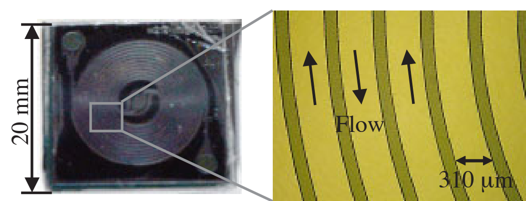

Figure 7. Microfabricated cell separation channel.

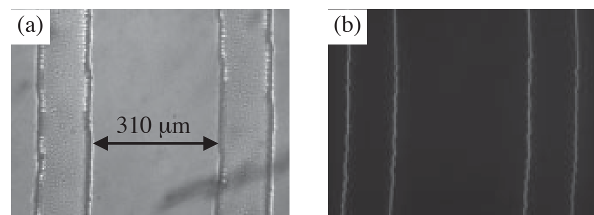

Figure 8. Channel with quantam-dot coated walls. (a) Magnified view, (b) epifluorescent image.

the number of the specific antigen on the cell membrane of each kind of cell.

3.2 Design and fabrication

Figure 7 shows a prototype cell separation micro channel. For all the target cells to be affected by antibodies on the wall, the channel should be designed so that all cells flow in the vicinity of the channel wall. Thus, the depth of the cell separation channel was made 50 μm, which is approximately twice the diameter of typical rare cells found in blood, such as hMSC. Because of possible clogging of cells in the channel, its width was made sufficiently large, i.e., 310 μm in this study. The desired channel length would vary depending on the surface density of antibodies on the wall, and also that of antigens on the target cell membrane, which depends on the kind of target cell. Here, the channel length is varied as 40 ~ 200 mm. The channel is spiral-shaped in order to obtain long length with minimal surface area.

As the material of channel walls, functionalized parylene [6] is selected, for its biocompatibility, ease of MEMS fabrication, and possible antibody conjugation. First, channel structure is made with standard lithography processes of SU-8 on a silicon wafer, and parylene is coated on SU-8 to form the channel wall. Pyrex glass, also coated with parylene, is used to cover the channel by thermal bonding of parylene [7]. Fluid inlet and outlet ports are opened on the pyrex glass wafer with ultrasonic tool, and inlet/outlet tubes are attached to the fluid ports by bonding PDMS to the pyrex glass after oxygen plasma treatment.

After fabrication of the whole channel, anti-human CD31 antibody is immobilized on the channel walls by successive incubation of reagent solutions in the channel as follows. Biotin is immobilized onto the functionalized parylene walls, followed by streptavidin conjugation, and finally biotin-conjugated CD31 antibody. The attachment of biotin on the channel walls is confirmed by visualization of the immobilized biotin with Qdot 655 streptavidin conjugate (Quantam Dot Corp.), as shown in Fig. 8. Strong fluorescence can be seen on the channel walls. Although it is not directly confirmed, considering the strong affinity between biotin and streptavidin, immobilizing the antibody on the wall is confirmed to be accomplished. Also, from the fact that the channel walls are entirely coated with biotin, the surface density of antibody is expected to be easily controlled by altering the concentration of the antibody solution, so that cell separation is possible regardless of cell species, so far as the specific antigen is known.

4. Conclusions

Two kinds of immunoreaction-based microfluidic cell sorters are designed, fabricated and tested. The μ-IMCS consisting of a microfabricated lamination mixer and a reservoir with external permanent magnet is successfully fabricated. The results from experiments with human cells show that the cell separation performance of μ-IMCS is comparable to conventional IMCS, and its processing time is much shorter. With improvement in total recovery yield, μ-IMCS is expected to become a promising device for stem cell extraction from a patient's body.

The concept and design of an affinity chromatography column for cell separation utilizing antigen/antibody interaction, which enables single step separation of target cells, is also introduced. The fabrication of the spiral micro channel is described, and successful immobilization of biotin on the channel walls is confirmed.

Acknowledgements

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