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ADHESION-BASED CELL SORTER WITH ANTIBODY-IMMOBILIZED FUNCTIONALIZED-PARYLENE SURFACE

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ABSTRACT

We report the development of a micro cell sorter for extraction of rare cells from a limited amount of sample. The separation principle is based on specific adhesion with the aid of surface-immobilized antibodies. Antibody-immobilized channel walls are fabricated with a versatile technique using functionalized parylene. Performance of the cell sorter is characterized through cell velocity measurements, in which deceleration of positive cells up to 60 % is observed. Cell separation with a mixture containing positive and negative cells is also demonstrated.

INTRODUCTION

Stem cell therapy is a rapidly evolving biomedical technology, in which multipotent stem cells are cultured *in vitro* and transplanted to regenerate damaged or deficit tissue. Ethical issues with the use of ES cells are increasing the importance of efficient extraction of adult stem cells from our bodies. However, these undifferentiated cells are very rare and difficult to identify. Therefore, the development of an efficient and accurate cell separation method of rare cells from cell mixture, such as monocyte fraction centrifuged from blood, is necessary.

Conventional cell sorting principles often employ surface marker (antigen) recognition by binding their counterpart antibody. Well-established techniques such as fluorescence activated cell sorting (FACS) [1] or magnetic cell sorting (MCS) [2] utilize antigen/antibody binding for labeling target cells. However, the applicability of these methods to regenerative medicine is in doubt considering the unwanted effect of fluorescent molecules or magnetic beads on cell cultivation and transplantation. In the present work, we develop a novel label-free adhesion-based micro cell sorting device with surface antibody immobilized on functionalized parylene.

Figure 1 shows the schematic of the present cell sorting principle. The cell sorter consists of a micro column with antibodies immobilized on the entire inner surface. Antibodies specifically bind to their counterpart antigens on the target-cell membrane, so that the target cells rolling along the walls are decelerated. Sample cell suspension is introduced as a plug, with its width focused at the inlet of the spiral-shaped cell sorting column to avoid unwanted effects of flow velocity distribution.

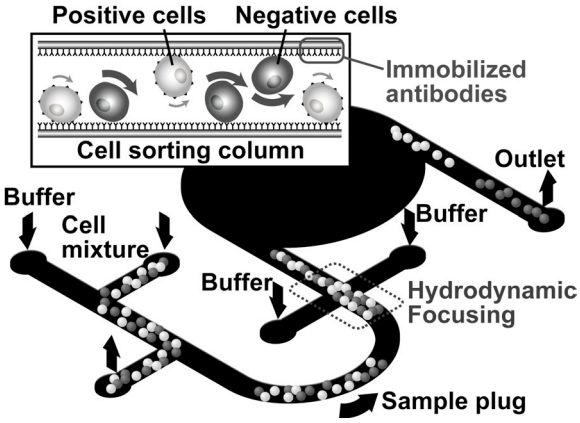

Fig. 1 Schematic of the adhesion-based cell sorter.

SURFACE FUNCTIONALIZATION

We have previously proposed a biomolecule immobilization method using diX AM (poly(aminomethyl-[2,2]-paracyclophane), Kisco), a class of amino-functionalized parylene [3]. The advantage of using diX AM for immunoreactive surfaces is two-folds. Firstly, amino-rich surface is provided as-deposited on conformally-coated three dimensional structures. Secondly, diX AM is highly biocompatible in nature, which is also the case for other classes of parylene, showing almost no non-specific adsorption of cells (Fig. 2ab).

The surface amino group on diX AM surface is visualized with NHS-rhodamine as shown in Fig. 2c. The fluorescence observed on diX AM surface is an order of magnitude larger than that on glass, also with a uniform distribution throughout the surface.

DESIGN AND MICROFABRICATION

In the present study, it is required that the sample cells roll along the antibody-coated micro channel walls. Therefore, the micro channel depth is fixed as 40 μm, which is approximately twice the diameter of a typical monocyte. The channel width is chosen as

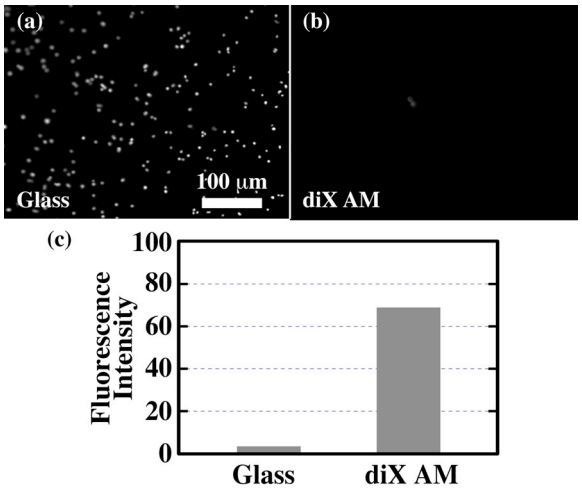

Fig. 2 Characterization of diX AM surface. (a) and (b) show non-specifically adsorbed human umbilical cord vein endothelial cells (HUVEC) after 1 hour incubation at room temperature. (c) Fluorescence intensity of NHS-rhodamine immobilized on glass and diX AM.

500 μm, to treat a sufficient volume of sample cells. The length of the plug formation channel and cell sorting column is chosen to be 100 mm and 600 mm, respectively. With this configuration, a 2 μL plug can be completely separated into positive and negative cell types in less than an hour at the flow rate of 1 μL/min.

Figure 3 shows the fabrication process for the present micro cell sorter. Firstly, micro channel structures are etched into the silicon substrate using deep reactive ion etching. A pyrex substrate with ultrasonically drilled fluid inlets/outlets is used as the lid. Amino-functionalized surface is formed by successively coating the entire surface of the two substrates with 5 μm-thick parylene-C and 0.1 μm-thick diX AM. Thermal bonding of the amino-parylene surfaces [4] is accomplished by clamping the substrates for an hour at 200 ^{o}C in a vacuum oven. Inlet and outlet fluid ports are made of PDMS blocks, firmly bound to the pyrex glass lid after oxygen plasma treatment. Figure 4 shows the micro cell sorter prototype developed in the present study.

Antibody immobilization on the channel walls is performed by successive introduction and incubation of biomolecule solutions with a syringe pump (Fig. 5). The micro channel walls are first biotinylated by conjugating NHS-LC-LC-biotin to the surface amines. NHS-LC-LC-biotin is dissolved into dimethylsulfoxide, and next into bicine buffered saline (pH 8). The biotin solution is introduced into the micro column and incubated for one hour at 30 ºC. Streptavidin and biotin-conjugated CD31 antibody solutions, each of them dissolved into PBS

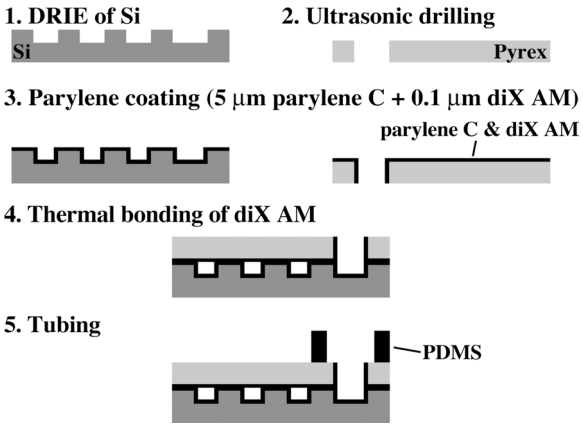

Fig. 3 Microfabrication process of diX AM channel structure.

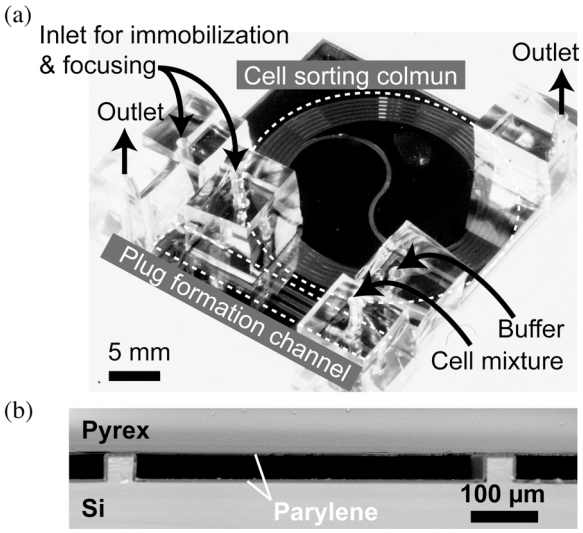

Fig. 4 (a) Microfabricated adhesion-based cell sorting device, (b) SEM image of the parylene micro channel cross section.

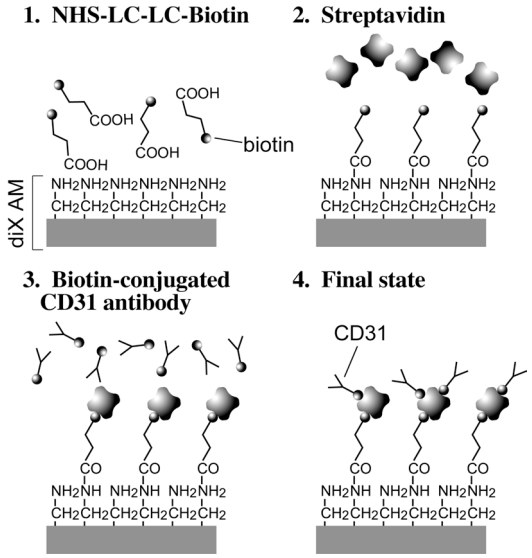

Fig. 5 Schematic of the CD31 antibody immobilization procedure on diX AM surface.

(pH 7.4), are successively incubated. The procedure described above is performed by introducing solutions from the buffer inlets for hydrodynamic focusing, leaving the plug formation micro channel plain. Although CD31 is used in the present study, various kinds of proteins and other biomolecules can be immobilized using the present material.

DECELERATION BY SPECIFIC ADHESION

We characterize the cell deceleration effect in the adhesion-based cell sorter through microscopic velocity measurements of human umbilical vein endothelial cells (HUVEC) and human leukocytes (HL60) rolling along the bottom wall.

Mouse anti-human CD31 (PECAM1) antibody is selected as the counterpart antibody. Figure 6 shows the CD31 expression of HUVEC and HL60 evaluated with autoMACS (Miltenyi Biotec) [2]. HUVEC is almost 100 % positive against CD31 antibody, whereas HL60 shows only 1.7 % positive expression. Therefore, we can expect that the motion of all HUVEC in the sample will be affected by antigen/antibody interaction.

Cells are stained with SYTO24 and suspended in PBS (pH 7.4) at the number density of 10^5 cells/mL,

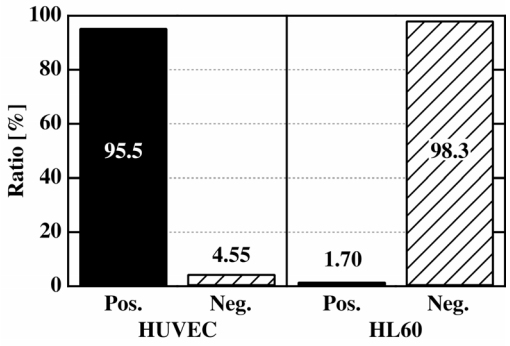

Fig. 6 CD31 expression of HUVEC and HL60.

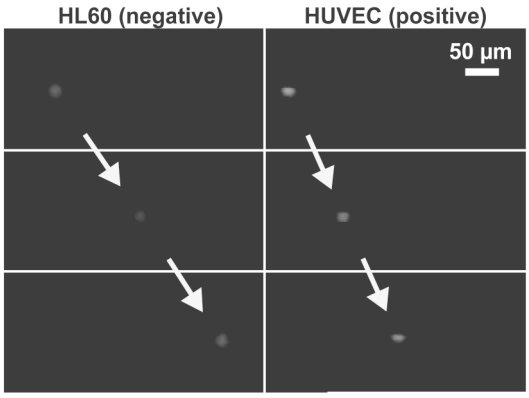

Fig. 7 Fluorescence microscopic images of cells flowing in the CD31-immobilized micro column. The bulk mean flow velocity is 0.2 mm/s.

which corresponds to the volume concentration of approximately 0.05 %. The sample cell suspension is continuously introduced with a syringe pump, and microscopic examination is conducted at the position 10 mm downstream of the column inlet. The focal plane of the microscope is set at the bottom wall of the micro channel. Microscopic images are taken at the frame rate of 6 fps, and the cell velocity is calculated from the displacement of the cell center between successive images (Fig. 7). The number of cells analyzed is 50 at each flow condition.

Figure 8 shows the time trace of instantaneous HUVEC velocity measured in the antibody-coated and plain micro columns. HUVEC is markedly decelerated with the effect of antigen/antibody binding compared to those flowing in a plain micro channel, showing the effectiveness of the present cell sorting principle. The velocity of HUVEC in both micro channels are almost constant, indicating that the cell deceleration phenomena reaches steady state within the 10 mm distance from the inlet to the test section.

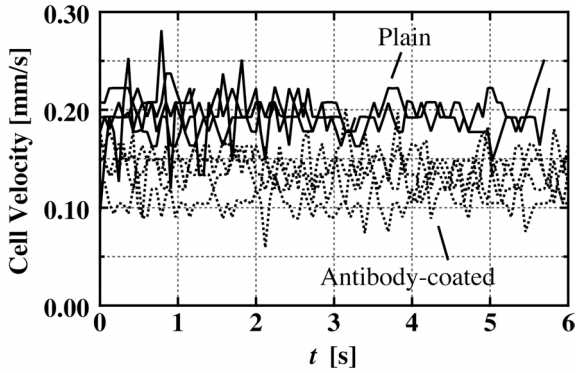

Fig. 8 Time trace of HUVEC velocity in antibody-coated and plain cell sorting columns. The bulk mean flow velocity of 0.2 mm/s.

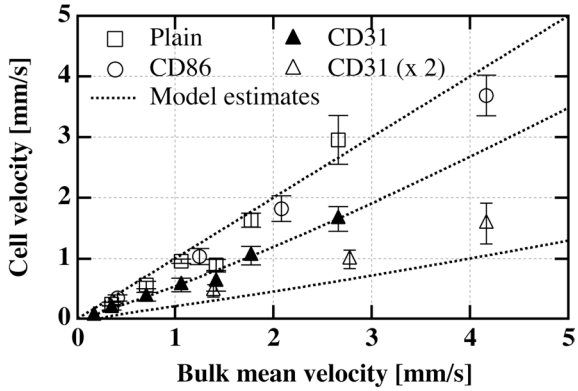

Fig. 9 Time-averaged HUVEC velocity in micro columns with various surface antibody immobilizations. The dotted lines show model estimates using a semi-analytical membrane-peeling model [5].

Figure 9 shows the average cell velocities obtained in devices with different surface treatment. HUVEC velocity in a positive antibody-coated micro channel is as small as 30 % of the flow velocity, in good agreement with the semi-analytical membrane-peeling model [5]. On the other hand, the velocity observed in a negative antibody-coated micro channel is almost the same as that in a plain channel, thus the effect of non-specific adhesion can be neglected.

CELL SEPARATION

Finally, the cell sorting performance is examined by sample plug separation experiments. HUVEC and HL60 are mixed at 5 x 10^5 cells/mL total concentration, at the ratio of 1:1. The mixture is then introduced into the plug formation channel from a syringe. The cells sink due to gravitational force, so that all cells flowing in the cell sorter is expected to roll along the antibody-immobilized surface. The flow is driven by a syringe pump at the total flow rate of 1.2 μL/min., with the volume ratio between the main inlet and the two hydrodynamic focusing buffer inlets being 2:1:1. The corresponding bulk mean flow velocity in the cell sorting column is 1 mm/s.

The separation performance is evaluated by counting the number of cells passing the test section, located downstream of the spiral cell separation column. Two separate experiments are made, and nuclei of either HUVEC or HL60 are labeled with a fluorescent dye (SYTO 24).

Figure 10 shows the time trace of the number of cells. The sample cell plug is successfully separated into plugs of each cell type, with the residence time of HUVEC being 1.5 times longer than that of HL60, which is in good agreement with the cell velocity measurement result (Fig. 9, solid symbols).

In the present study, the throughput of the device is approximately 10^3 cells per hour due to the low cell concentration and the low flow velocity presently employed. Note that the throughput of the present cell sorter is proportional to the sample concentration and flow rate, each of which can each be increased by at least an order of magnitude. Therefore, we expect the throughput to become 10^5 cells/hr. or higher at more practical experimental conditions.

CONCLUSIONS

A novel specific-adhesion-based cell sorter has been developed with a versatile antibody immobilization technique using amino-functionalized parylene. It is demonstrated that the antibody-positive cells could be significantly decelerated by specific adhesion in the present device. It is shown in a cell separation experiment that a 2 μL plug of HUVEC and HL60 cell mixture is separated into plugs of each cell type in an hour. Considering the merit of label-free sorting and the potential for higher throughput, the present cell sorter could become a powerful alternative to FACS or MCS.

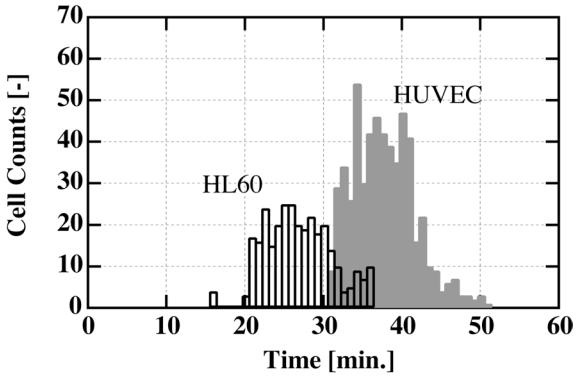

Fig. 10 Time trace of the number of cells counted at the test section of the cell sorting device.

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