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Evaluation of Cell Velocity Regulation in a Microfabricated Adhesion-Based Cell Separation Device

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Abstract

This paper reports the characterization of adhesion-based cell velocity regulation in a prototype microfabricated cell separation device for regenerative medicine. The principle of cell sorting is based on immunoreaction for accurate recognition of target stem cells. Target-cell specific antibody is immobilized on the micro channel wall to form a selectively adhesive surface, where a new class of functionalized parylene is used as the surface material for antibody immobilization. The flowing velocity of sample cells in a prototype microfabricated cell separation column is examined under the microscope. The measurement results show that the cell velocity is reduced by 40 % due to the effect of antigen/antibody interaction.

Keywords: Stem cells, Cell sorting, Functionalized parylene, Antigen/antibody interaction

1 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. The major candidate cell to be used for the regeneration of tissue is embryonic stem cell [1], although its use often encounters ethical concerns. Among adult stem cells, mesenchymal stem cells (MSCs), derived from bone marrow or peripheral blood, show the ability to differentiate into various tissue cells [2]. The number density of MSC in all cells in peripheral blood is around 10^{-8}, so that an efficient and accurate cell separation method is needed for the extraction of these cells.

Previously, we have proposed an adhesion-based cell separation principle (Fig. 1) [3]. Antibodies immobilized on the channel wall specifically bind to their counterpart antigens on the target-cell membrane, so that the target cells rolling along the wall are decelerated. The deceleration of the target cells results in streamwise separation of a cell mixture plug into plugs containing target and non-target cells. The present approach requires no target-cell marking process prior to separation, necessary in conventional cell sorting techniques such as fluorescence activated cell sorting [4] or magnetic cell sorting [5].

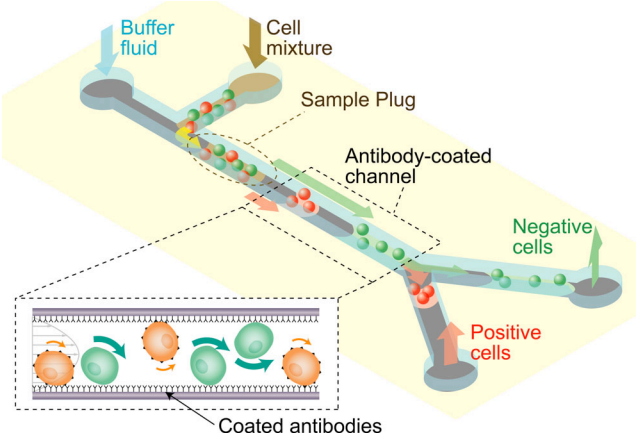

Figure 1. Schematic of the adhesion-based cell separation principle.

In the present study, we evaluate the cell-velocity regulation performance in our prototype antibody-coated cell separation device through cell velocity measurement under the microscope.

2 CELL DECELERATION MECHANISM

In the present cell separation principle, the target cell is decelerated by transient attachment and detachment of bonds between antigens on the cell membrane and antibodies immobilized on the channel wall. The velocity of the decelerated cells depends on the balance between fluid shear and antigen/antibody binding force. The magnitude and distribution of the binding force also depends on the shape of cell membrane in the vicinity of the antibody-coated surface. The mechanism of cell rolling is rather complicated, and requires a simple model of the surrounding flow, cell membrane deformation, and chemical reaction.

Dembo *et al.* [6] has proposed a two-dimensional membrane peeling model (Fig. 2) to derive a mathematical description of the cell rolling phenomena. The streamwise and wall-normal directions are taken as the x- and y-directions, respectively. The position along the contour of the membrane is tracked by the arc-length coordinate s, and the origin of the whole coordinate system is fixed on the point of contact, which is the upstream-most point of the attached region. At

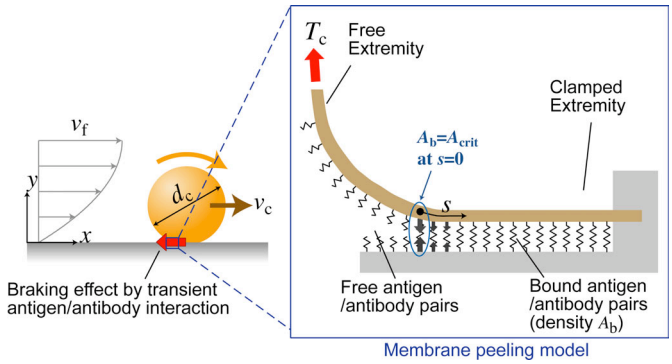

Figure 2. Schematic of the membrane peeling model [6].

one extremity ($s \to +\infty$), it is presumed that the membrane is firmly clamped to the surface. Tension T_c is applied at the other extremity ($s \to -\infty$) at specified orientation with respect to the surface.

Association and dissociation of antigen/antibody pairs are expressed by a simple kinetic relationship [7], assuming that the cell rolling phenomena is reaction-limited (i.e., diffusion time scale of antigens on the cell membrane are negligibly small compared to that of antigen/antibody binding). The local area density of bound molecule pairs A_b is dependent on the distance between the antibody-coated surface and the cell membrane. The bonds are modeled as a Hookean spring with spring constant κ and undisturbed length λ, and binding force is assumed to act only in the wall-normal direction.

The kinetic model treats the antigen/antibody binding as a reversible reaction with forward and reverse reaction rates $K_f(Y)$, $K_r(Y)$, expressed by the following form [7];

$$\mathrm{f} \underset{K_r(Y)}{\overset{K_f(Y)}{\rightleftharpoons}} \mathrm{b}, \tag{1}$$

where f denotes the free state and b the bound state. The continuity equation for the area density of antigen/antibody bonds $A_b(s, t)$ includes the membrane peeling velocity v_c explicitly,

$$\frac{\partial A_b}{\partial t} = v_c \frac{\partial A_b}{\partial s} + K_f\left(A_{tot} - A_b\right) - K_r A_b, \tag{2}$$

where A_{tot} is the total bond density of free and bound antigen/antibody pairs.

The cell membrane shape ($X(s)$, $Y(s)$) can be described by the force balance in tangential and normal directions,

$$\frac{\partial}{\partial s}\left(T + \frac{1}{2} M_b C^2\right) = A_b \kappa \left(Y - \lambda\right) \frac{\partial X}{\partial s}, \tag{3}$$

$$M_b \frac{\partial^2 C}{\partial s} - CT = -A_b \kappa \left(Y - \lambda\right) \frac{\partial Y}{\partial s}, \tag{4}$$

where M_b is the bending modulus of the cell membrane, and T, C the membrane tension and curvature, respectively.

Through numerical simulations with equations (2) ~ (4), Dembo *et al.* [6] discovered that the membrane peeling velocity v_c at a steady state could be described as a function of T_c. With an order estimation that T_c is proportional to the flow velocity v_f, the model equation is formulated as a function of v_f [6];

$$v_c \approx p_1 \left(1 + \frac{p_0}{v_f}\right)\left(1 + p_2 \frac{v_f}{p_0}\right) \ln\left(\frac{v_f}{p_0}\right). \tag{5}$$

Here, p_0 is a parameter introduced to normalize v_f and includes the ratio between the membrane tension T_c and its critical value, where the cell is trapped at the antibody-coated wall. The ratio between membrane stiffness and the spring constant of antigen/antibody bonds is represented by p_1, and p_2 is a nondimensional parameter representing the effect of non-specific repulsion between adhesion molecules.

The model equation shows the presence of a critical flow velocity at which the cell is captured on the antibody-coated surface. For higher velocities, cells start to roll and move downstream due to larger shear stress on the cell surface.

3 MATERIAL SELECTION

In order to immobilize antibodies on the micro channel walls, we select aminomethyl-functionalized parylene (diX AM, KISCO) [3]. Figure 3 shows the molecular structure of the diX AM dimer, where only one of the monomers has the aminomethyl group. Coating is made through a simple vapor deposition process (vaporization at 120 ~ 180 ºC, pyrolysis at 700 ºC, chamber pressure 80 mTorr).

The advantage of the use of functionalized parylene for biomolecule immobilization is three-folds. Firstly, parylene is biocompatible in nature, secondly, the entire surface of three-dimensional structures are conformally coated, and thirdly, amino group is available on the coated surface straight after vapor deposition.

We investigate the presence of amino group on the surface of the diX AM by conjugating fluorescent molecules on the surface of parylene-coated glass substrates. NHS-rhodamine, dissolved into pH 8 bicine-buffered saline, is incubated on the parylene-coated substrate for an hour at 30 ºC. After washing the surface with distilled water, fluorescence intensity is evaluated by analyzing filtered microscopic images. Fluorescence intensity of diX AM surface is order of a magnitude larger than that of a glass substrate, on which

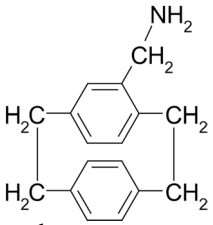

Figure 3. Molecular structure of diX AM (poly(aminomethyl-[2,2]paracyclophane)) dimer.

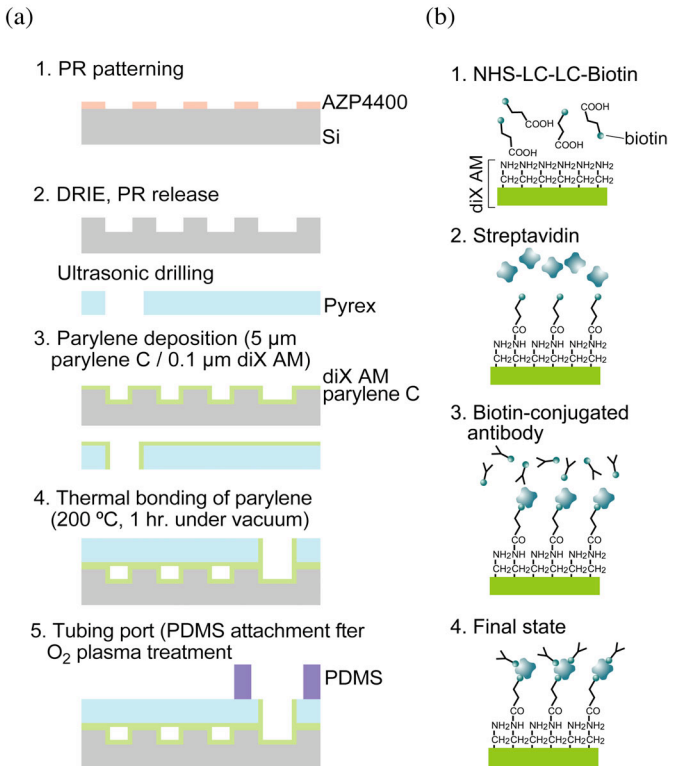

Figure 4. Fabrication process of the present cell separation device. (a) Microfabrication process of the micro channel structure, (b) antibody-immobilization on diX AM surface.

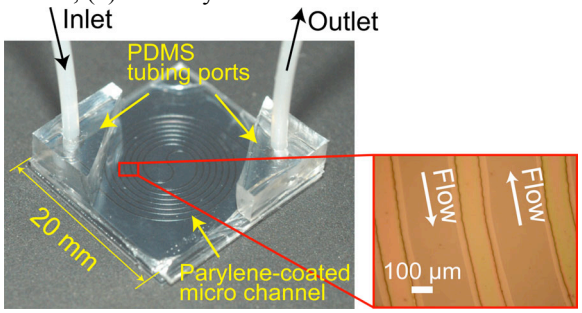

Figure 5. Microfabricated prototype cell separation column.

NHS-rhodamine is non-specifically adsorbed. It is confirmed that diX AM provides amino-rich surface, and is suitable for use in the cell separation column.

4 FABRICATION OF THE ANTIBODY-COATED MICRO COLUMN

For efficient separation, the cells in the sample suspension need to roll along the antibody-coated walls. In the present study, the micro channel depth is designed as 40 µm, which is approximately twice the diameter of a typical cell (e.g., 20 µm for monocytes). The micro channel is spiral-shaped in the streamwise direction in order to obtain long separation length with minimal surface area. 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. Here, the channel length is made sufficiently long, i.e., 400 mm. The channel width is chosen as 300 µm to achieve µL-order channel volume in the present study, although much larger volume can be achieved by simply increasing the gap between neighboring channels.

Figure 4a shows the microfabrication process of the current antibody-coated micro column. Firstly, 50 µm-deep micro channel structure is etched into silicon by deep RIE. The whole surface of the micro channel structure is then conformally coated with a 5 µm-thick cushioning layer of parylene C, followed by deposition of a 0.1 µm-thick diX AM layer to provide amino-functionalized surface. A pyrex glass lid with inlet and outlet fluidic ports is coated with the same parylene C/diX AM layer, and bonded to the channel structure with the thermal bonding technique [8]; the substrates are clamped and treated at 200 ºC in a vacuum oven for 1 hour to have sufficient bonding strength. Finally, PDMS tubing ports are bonded to the pyrex glass lid after surface treatment with low-power oxygen plasma.

Figure 4b shows the schematic of the antibody immobilization procedure. The micro channel walls are first biotinylated by conjugating NHS-LC-LC-biotin to the surface amines. NHS-LC-LC-biotin is dissolved into DMSO, and next into bicine buffer in the same manner as the surface visualization experiment described above. The biotin solution is introduced into the micro column with a syringe pump, and incubated for one hour at 30 ºC. Streptavidin and biotin-conjugated antibody solutions, each of them dissolved into PBS-buffered saline (pH 7.4), are successively incubated in the same manner to form the antibody-coated surface.

5 EXPERIMENTAL

In order to demonstrate the effectiveness of the present cell separation method, the cell velocity under the effect of antigen/antibody interactions is measured. Human umbilical vein endothelial cell (HUVEC) is used as the model target cell, and mouse anti-human CD31 (PECAM1) antibody is selected as the counterpart antibody. Figure 6 shows the surface CD31 expression of HUVEC evaluated with autoMACS (Miltenyi Biotec GmbH) [5]. HUVEC shows almost 100 % positive CD31 expression, thus we can assume that the motion of all cells in the sample will be affected by antigen/antibody interaction.

HUVEC is suspended in PBS-buffered saline (pH 7.4) with a number density of 10^5 cells/mL, which corresponds to the volume concentration of approximately 5 x 10^{-2} %. The sample cell suspension is introduced into the prototype cell separation column (Fig. 5) using a syringe pump, and microscopic examination is conducted at the position 10 mm downstream of the inlet. The focal plane of the microscope is set at the bottom wall of the micro channel. Since the depth

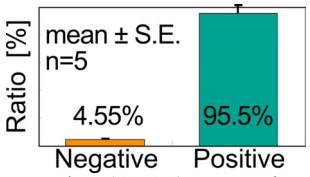

Figure 6. Surface marker (CD31) expression of HUVEC.

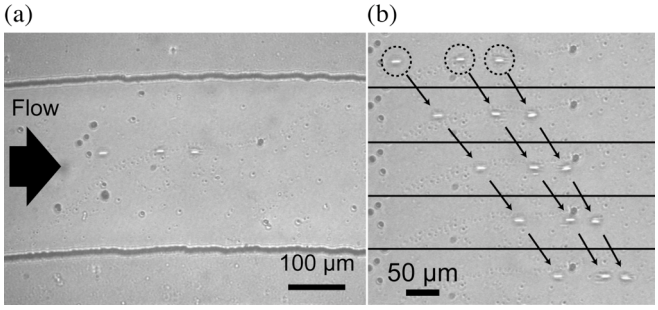

Figure 7. Snapshots of cell motion in the adhesion-based cell separation column.

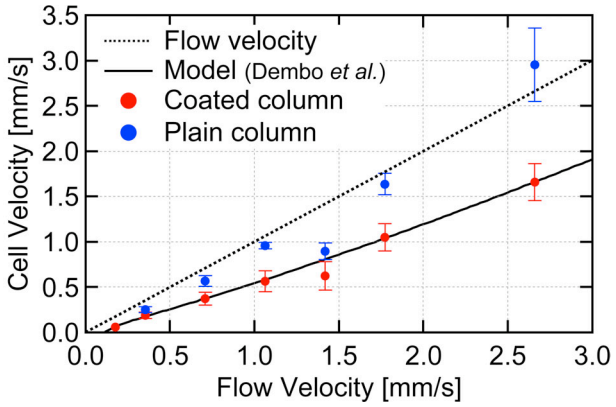

Figure 8. Cell velocities in coated and plain cell separation columns. The dotted line shows the bulk mean velocity of the fluid.

of focus is 8 μm, only cells rolling on the bottom wall are observed. Microscopic images are taken at the frame rate of 33 fps with a CCD camera, 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 measured cell velocities in the antibody-coated and plain cell separation columns under various flow velocities. Cell velocities observed in the plain column are in good agreement with the fluid velocity, showing that the effect of nonspecific binding is negligibly small. On the other hand, HUVEC velocity in the coated column is 60 to 70 % of that in a plain column. Cell velocity decrease in the coated micro column was observed throughout the flow rate range investigated in the present study, and this fact demonstrates the effectiveness of the present cell separation method.

Parameters p_0, p_1 and p_2 in the model equation (5) were determined with the experimental data using a least-square method, and is shown by a solid line on Fig. 8. From the fit curve, we expect that the cells under influence of antigen/antibody interaction keep flowing at flow velocities higher than 0.1 mm/s, while cells are likely to be trapped at lower flow velocities.

6 CONCLUDING REMARKS

A prototype adhesion-based cell separation column by transient antigen/antibody interaction is developed. A new class of aminomethyl-functionalized parylene is selected as the surface material to form selectively adhesive micro channel walls by immobilizing CD31 antibodies on the surface. It is found in flow-through experiments using HUVEC that the cells are decelerated by 40 % due to the antigen/antibody interaction. This shows the effectiveness of our prototype micro column for use in adhesion-based cell separation.

Acknowledgements

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