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An Easy-to-Use Polystyrene Microchip-based Cell Culture System

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In this study, we developed an integrated, low-cost microfluidic cell culture system that is easy to use. This system consists of a disposable polystyrene microchip, a polytetrafluoroethylene valve, an air bubble trap, and an indium tin oxide temperature controller. Valve pressure resistance was validated with a manometer to be 3 MPa. The trap protected against bubble contamination. The temperature controller enabled the culture of *Macaca mulatta* RF/6A 135 vascular endothelial cells, which are difficult to culture in glass microchips, without a CO_2 incubator. We determined the optimal coating conditions for these cells and were able to achieve stable, confluent culture within 1 week. This practical system is suitable for low-cost screening and has potential applications as circulatory cell culture systems and research platforms in cell biology.

Keywords Microchip, microfluidics, cell assay, bubble trap, endothelial cell, cell-response monitoring

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Introduction

Integrated chemical systems known as micro total analysis systems (μ-TAS)[1,2] or lab-on-a-chip[3] have become popular because of their low reagent and sample consumption, low space requirements, and short analysis time. μ-TAS has many on-chip applications such as in flow injection analysis, solvent extraction,[4] microreactors,[5] immunoassays,[6] and microfluidic cell culture systems. The increasing costs of drug development have made inexpensive screening systems highly desirable. Microfluidic cell culture devices are a promising technology for low-cost bioassay systems. They offer high reaction efficiency in a low volume because the liquid microspace inside a microchip is suitably scaled[7] and enables kinetic measurement under continuous flow.[8] The microchip cell culture system enables continuous and rigorous flow control, which is advantageous in vascular biology studies.[9-11] Vascular dysfunction[12] is associated with major diseases such as diabetes and cancer, and microfluidic cell culture systems are excellent tools for elucidating the cellular mechanisms underlying these diseases. A microfluidic cell culture system has been developed as a perfusion system that mimics the *in-vivo* environment[13,14] and facilitates evaluation of shear stress.[15-17]

As Meer *et al.*[18] noted, almost all studies on microfluidic cell culture systems thus far have been proof-of-principle experiments that require substantial personal effort and intervention by the researcher. For instance, air bubbles in the tubing cause serious problems such as wash-out and poor reproducibility. Therefore, standardization and simplification are required to optimize the microfluidic cell culture technology.

In the current study, we designed and evaluated a new easy-to-use microchip-based cell culture system for the culture of the Rhesus retinal vascular endothelial cell line RF/6A19 (cell size: 10 – 20 μm without protrusions, 50 – 100 μm with protrusions). First, we built a closed fluidic system with an indium tin oxide temperature controller, enabling culture without a CO_2 incubator, as well as real-time microscopic observation. The pressure capacity of the valve was validated. An air bubble trap was designed to remove air bubbles that emerge from the medium. We then measured the cell adhesion rates on polystyrene (PS) microchips, which are inexpensive, biocompatible, and transparent. We also tested various coating reagents to improve cell adhesion.

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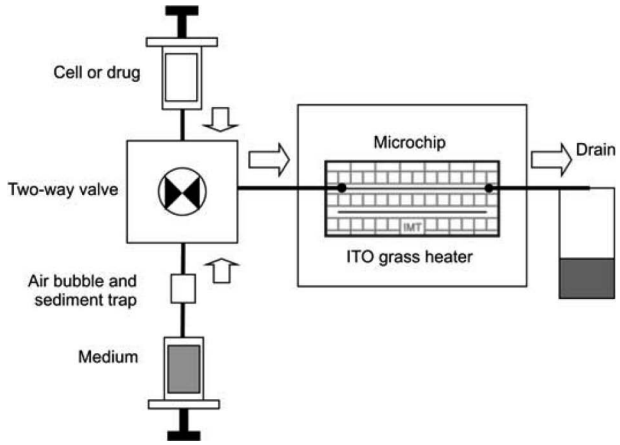

Fig. 1 Scheme for the microchip-based cell assay.

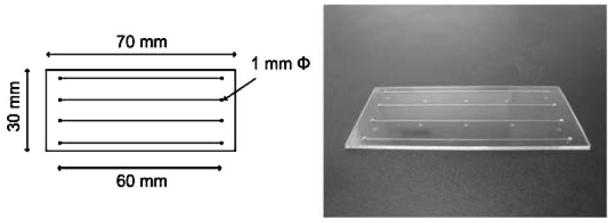

Fig. 2 Cell culture using the microchip system.

Experimental

System concept

We aimed to perform stable cell culture on a microscope, without CO_2 incubation and with minimal tubing. Our system included a PS microchip for cell culture, a valve for medium exchange, and a syringe pump. Capillary tubes were used to connect the syringe pump to the drain bottle. An indium tin oxide (ITO)-coated temperature controller IOK-41 (Institute of Microchemical Technology Co., Ltd., Kawasaki, Japan) was placed under the microchip for incubation on the microscope stage (Fig. 1).

Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from MP Biomedicals (Santa Ana, CA); streptomycin and penicillin from Meiji Seika Pharma (Tokyo, Japan); papain from Worthington Biochemical (Lakewood Township, NJ); fetal bovine serum (FBS) from Life Technologies (Carlsbad, CA); Cellmatrix type IV from Nitta Gelatin (Osaka, Japan); poly(ethylenimine) solution from Sigma-Aldrich (St. Louis, MO); poly-L-lysine and poly-D-lysine from Wako Pure Chemical Industries (Osaka, Japan); and NUCLEAR-ID(R) Blue/Red cell viability reagent from Enzo Life Sciences (Farmingdale, NY).

Fabrication of the microchip

PS microchips were manufactured by injection molding at Sumitomo Bakelite Co., Ltd. (Tokyo, Japan) (Fig. 2). Each chip comprised 2 PS plates: the cover and base plates (each 1-mm thick). The culture channel was 6-cm long, 300-µm wide, and 100-µm deep.

Glass microchips for validation controls were fabricated by photolithography and wet-etching techniques, as reported previously.[20] In brief, the Pyrex substrates Au and Cr were sputter-coated, and a positive photoresist was spin-coated over the substrates; the channel was exposed to ultra-violet (UV) light through a custom metal mask. After photolithography, the substrates were etched with hydrofluoric acid, and the substrate and cover plate with inlet port were then bonded at 670°C.

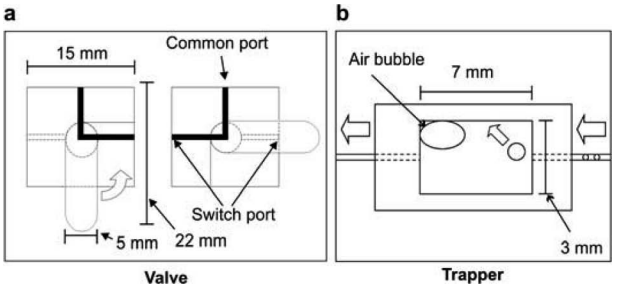

Fig. 3 (a) Valve for medium change (top view). (b) Trap to remove air bubbles (side view).

Fabrication of the valve

A valve for medium exchange was fabricated from a polytetrafluoroethylene (PTFE) base and an L-shaped switching shaft (Fig. 3a). The switching shaft was fabricated from cross-linked PTFE (diameter, 5 mm) and was inserted through a hole in the base (diameter 5 mm). The medium/reagent flow direction was determined by using a two-way stopcock, with a switching volume of 0.6 µL. Polyetheretherketone (PEEK) tubes (internal diameter, 0.3 mm; external diameter, 0.5 mm) were used to connect the elements of the chip holder.

Fabrication of the air bubble trap

An air bubble trap was fabricated from a glass tube and silicone tube. The details of the design are described in "*Optimization of the bubble trap*" in the Results and Discussion section. The internal diameter and length were 3 and 6 mm, respectively, and the volume was 42.41 µL (Fig. 3b). Trapped air bubbles rose to the top of the trap.

Cell culture in dishes

M. mulatta RF/6A 135 vascular endothelial cells were obtained from Cell Bank, RIKEN BioResource Center (Tsukuba, Japan), and were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 IU/mL penicillin, and 25 µg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Cell culture in the microchip

RF/6A 135 cells were introduced into the microchip after channel pretreatment as follows. First, the channel was washed with 0.1 M NaOH, followed by 70% EtOH, for the dissolution of proteins and for sterilization, respectively; the channel was then washed with sterilized distilled water. Next, the channel was filled with coating reagents for 2 h to coat the inner surface. The channel was then washed with sterilized distilled water. After pretreatment, cells were introduced as follows. First, 20 µL of a 1.0×10^6 – 10^7/mL cell suspension was prepared from dish cultures. Next, the cell suspension was introduced *via* the cell port of the valve. After introduction of the cells, the valve was turned to the stop port. After 2 h of incubation to allow cell adhesion to the channel walls, the valve was turned to the medium port, and medium perfusion was started at 0.1 – 1 µL/min and 37°C using the ITO temperature controller.

Cells were counted in all visible areas of the microchannel by using a tally counter. Cell attachment ratios were evaluated by using the *t*-test.

Coating reagents

After 6 h of culture, cell attachment ratios were evaluated on coated and non-coated PS chips and non-coated glass. The coating time was 2 h. Optimum conditions were determined by measuring the number of adherent cells. Cells were introduced at a density of 10^6/mL.

Measurement of pressure capacity

The valve pressure capacity was validated by using a PT-20 manometer (Nagano Keiki Co., Ltd., Tokyo, Japan). The PT-20 manometer was attached to the system, and the flow was increased until leaking was observed; the PT-20-recorded pressure at the leaking point was defined as the pressure resistance of the system.

Results and Discussion

Valve pressure capacity

The combination of a PTFE body and shaft yielded a valve pressure resistance of 0.7 MPa. To increase the pressure resistance, we exchanged the PTFE shaft for a cross-linked PTFE shaft, which yielded a resistance of 3 MPa. This improvement was attributed to the greater elasticity and anti-deformation and anti-abrasive properties of cross-linked PTFE, which provided better seal and contact performance.

Conventional methods require exchange canalization for assay completion, but this is not necessary in our system. Our use of a valve plus an air bubble trap was sufficient for microchip cell culture.

Optimization of the bubble trap

In this system, medium flowed into the microchip through a microsyringe pump at 0.2 µL/min in the injection mode, in order to reproduce *in-vivo* conditions, as described by Jang *et al.*[21] Air bubbles are generated from the medium and cause serious damage to cells; therefore, we added an air bubble trap to this system. The size and volume of air bubbles in the medium were measured with a microsyringe after 7 days. The diameter of the air bubbles was less than 1 mm, and their volume was 1% the syringe volume. The trap size was estimated from the rising rate (calculated by Stokes' law), and was expressed as *V*:

$$V = \frac{1}{18} \times \frac{gd^2}{v} \quad (1)$$

where *g* is the gravitational acceleration, *d* is the diameter of the air bubbles, and *v* is the dynamic coefficient of the viscosity of medium.[22] In this experiment, *V* was estimated to be 7.8 mm/s. The total volume of naturally occurring air bubbles during 1 week of culture was estimated to be 20.16 mm^3. Thus, a trap with a diameter of over 3 mm and a length of over 7 mm was needed for the estimated volume of air bubbles to be half the internal volume of the trap. The trap removed air bubbles from the medium effectively. Without the trap, cell viability was <20% after >6 h of culture; with the trap, cell viability was 83% after >6 h of culture ($n = 3$), and confluence was achieved after 1 week.

Fig. 4 Image of cells cultured in the microchip. (a) Polystyrene (PS) microchip, 1.3×10^6 cells/mL; (b) PS microchip, 2.6×10^6 cells/mL; (c) PS microchip, 7.0×10^7 cells/mL.

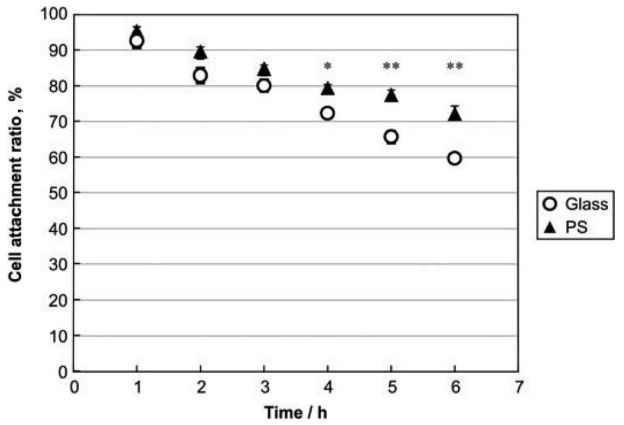

Fig. 5 Comparison of the performances of glass and PS. $*p < 0.05$, $**p < 0.01$.

Microscope culture

The other advantages of this system include the ability to culture cells directly on a microscope stage with an ITO glass temperature controller (the measurement precision was 0.3°C), thereby eliminating the need to change the medium and move the system for microscopic observation. Conventional microchannel systems experience washout during transport. Another advantage of our closed fluidic system is that CO_2 gas is not required during incubation. During cell culture, the pH of the medium in the microsyringe remained unchanged, as confirmed by the color of the medium.

RF/6A 135 vascular endothelial cell culture in the microchip

In a preliminary experiment, a glass channel that was 6-cm long, 100-µm wide, and 40-µm deep was used; however, this system clogged, and cells could not be injected into the system (data not shown). In the subsequent experiment, we achieved smooth injection by using a channel that was 300-µm wide and 100-µm deep.

Microchannel injection was performed with culture densities of 1.3×10^6/mL, 2.6×10^6/mL, and 7.0×10^7/mL [Figs. 4(a) – 4(c)]. Only a few cells were successfully injected at densities of 1.3×10^6/mL and 2.6×10^6/mL; immediate adhesion and confluence was achieved at 7.0×10^7/mL.

A comparative analysis of the performance of PS microchips and glass chips during validation is shown in Figs. 5 and 6.

Cell adhesion was evaluated by injecting a few cells (10^6/mL) into the glass and PS chips and counting the adherent cells in all visible parts of the microchannel. The cell adhesion rate before medium perfusion was defined as 100%. The cell adhesion rate was 70% in bare PS microchips and 57% in bare glass chips (Fig. 5; $p < 0.05$, $p < 0.01$, respectively). Therefore, we concluded that the PS microchips are better than glass microchips.

Figure 6 shows a comparison of the coating reagents tested in the study. Of the four coating reagents tested, Cellmatrix was the best (cell attachment ratio is 83%), but no significant differences were found between non-coated and coated PS chips by variance analysis ($p > 0.05$). In addition, glass cannot be coated with Cellmatrix (data not shown).

The viability of the cells after 1 week of culture is shown in Fig. 7. In flask culture, RF/6A 135 cells multiplied approximately two times per day and became confluent within a few days. In microchip culture, the cells became confluent after 2 days, as measured in the same number per unit area as the flask culture. The cells remained alive after 1 week of culture in the microchip, similar to the flask culture. If the cells had died during culture, they would have peeled off and flowed out of the microchannel in the medium. In a preliminary experiment, we assayed live cells using the NUCLEAR-ID(R) Blue/Red cell viability reagent. All adhesive cells were colored blue (live).

In the UV region, the transmittance of the PS substrate decreases abruptly below approximately 300 nm. Therefore, this system cannot be used in experiments involving UV light. PS has good transmittance in the visible-light region and has been widely used in the manufacture of flasks and microplates. However, proteins can adsorb on PS. Therefore, for experiments involving proteins, flasks and microplates made with PS need to be blocked for nonspecific protein adsorption before use.

Our study demonstrated that PS chips may be used for the adhesion culture of vascular endothelial cells, regardless of the coating reagent. This system was optimized for real-time monitoring, kinetic assay, and screening. This system may be used for kinetic measurements to monitor protein expression; moreover, the system enables recovery[11] in small volumes. This system may also be used for primary cell culture.[11] Primary cells require delicate culture conditions, and the PS substrate can be used with several coating reagents. Moreover, the temperature controller limits temperature fluctuations. On the other hand, single cells and non-adherent cells are difficult to culture using this system because they tend to be eliminated in the flow-through.

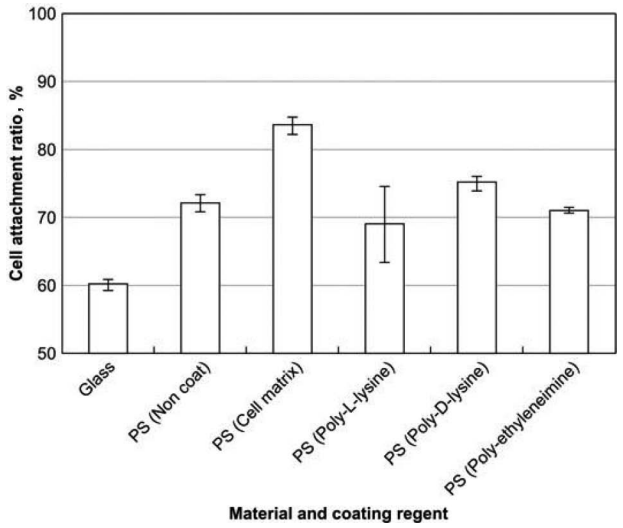

Fig. 6 Comparison of materials and coating reagents. Data represent the mean ± S.E. ($n = 3$).

Conclusions

We constructed a microfluidic system comprising a temperature controller, culture chip, valve, and air bubble trap. We achieved successful microchip culture for 1 week without contamination by air bubbles and cell debris. Our system reduces cell and reagent use; all components, except the disposable microchip, are manufactured from heat-resistant material and can be autoclaved (121°C). The system enables low-cost, easy-to-use microchip-based cell culture. In the future, systematization and automation will further add to the practicality of the system.

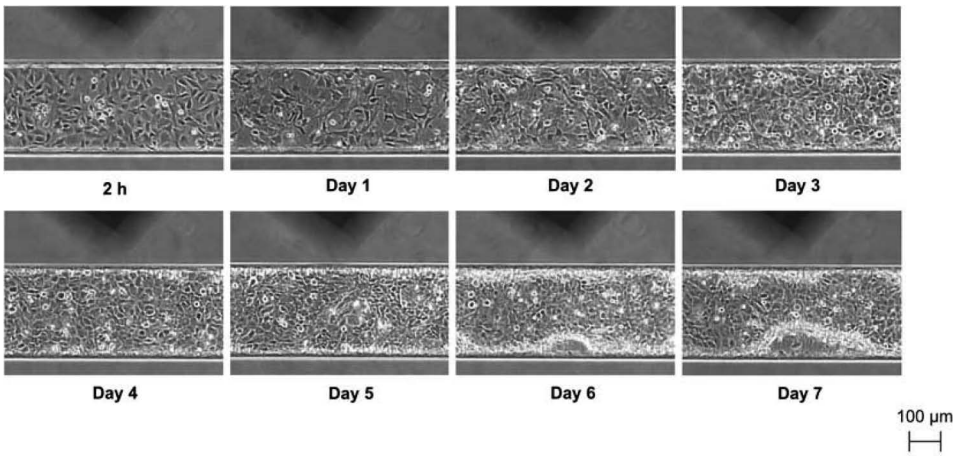

Fig. 7 Image of cells cultured in the microchip for over 1 week. Inverted triangle at the upper position indicates that these images were captured at the same point.

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