Microfluidic PDMS (Polydimethylsiloxane) Bioreactor for Large-Scale Culture of Hepatocytes

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Microfluidics could provide suitable environments for cell culture because of the larger surface-to-volume ratio and fluidic behavior similar to the environments in vivo. Such microfluidic environments are now used to investigate cell-to-cell interactions and behaviors in vitro, emulating situations observed in vivo, for example, microscale blood vessels modeled by microfluidic channels. These emulated situations cannot be realized by conventional technologies. In our previous works, microfluidic channels composed of two PDMS (poly(dimethylsiloxane)) layers were successfully used for Hep G2 cell culture. To achieve physiologically meaningful functions in vitro, a culture with a larger number of cells and higher density must be performed. This will require bioreactors with larger surface areas for cell attachment and sufficient amounts of oxygen and nutrition supply. For those purposes, we fabricated a bioreactor by stacking 10 PDMS layers together, i.e., four cell culture chambers, and a chamber dedicated to the oxygen supply inserted in the middle of the 10-stacked layers. The oxygen supply chamber is separated from the microfluidic channels for the culture medium perfusion by thin 300-μm PDMS walls. The high gas permeability of PDMS allows oxygen supply to the microfluidic channels through the thin walls. On the basis of the measurement of glucose consumption and albumin production, it is shown that cellular activity exhibits a gradual increase and saturation throughout the culture. We clearly observed that in the case of the microfluidic bioreactor for large-scale cultures, the oxygen chamber is indispensable to achieve longer and healthy cultures. In the present bioreactor, the cell density was found to be about $3-4 \times 10^7$ cells/cm^3, which is in the same order of magnitude as the conventional macroscale bioreactors. Consequently, by stacking single culture chambers and oxygen chambers in between, we could have a scalable method to realize the microfluidic bioreactor for large-scale cultures.

Introduction

Culturing cells up to higher density and larger numbers is one of the most important tasks to achieve physiologically meaningful functions, which are required for tissue engineering (*1*, *2*). To reach this goal, continuous nutrition and oxygen supply and waste removal through the culture medium have to be ensured (*3*). However, in conventional cell culture formats such as dishes and macroscale bioreactors, it is quite difficult to realize the delivery of a sufficient amount of those substances throughout the cultured tissue. This is due to the difficulty in designing and fabricating large complex bioreactors in which the cells are fed by a spatially homogeneous distribution of the fluid flow (*4*). Microsystems technology enables us to realize microfluidic channels suitable for such oxygen and nutrition supply (*5*, *6*). In this context, new approaches to microfabricated bioreactors are more and more investigated (*7*).

The advances in microsystems technology have been used to fabricate two- or three-dimensional structures to cultivate various types of mammalian cells with various materials, such as silicon, silicone elastomer, and biocompatible (*8*, *9*) and biodegradable polymers (*10*−*12*). In particular, silicon- and silicone elastomer-based bioreactors have also been developed for liver cell cultures in perfusion circuits (*13*, *14*). In silicon bioreactors, typical tissue structures reorganized from injected spheroids were observed during perfusion cultures (*15*). A silicone elastomer, i.e., PDMS (poly(dimethylsiloxane)) (*16*), has also been used to build such bioreactors. PDMS is a favorable material because of its biocompatibility and high gas permeability (*17*−*19*). Endothelial cells were successfully cultured as an attempt of vascular system reconstruction (*13*, *20*). In our previous works, we demonstrated that PDMS could also be used to culture liver cells (*21*). We observed that Hep G2 cells clustered and self-organized in the microfabricated PDMS bioreactors. The cells were stably kept alive as a result of the oxygen that can diffuse through the material itself.

In this paper, we introduce a microfabricated PDMS bioreactor for large-scale cultures. The bioreactor is composed of multiple layers of PDMS with dedicated

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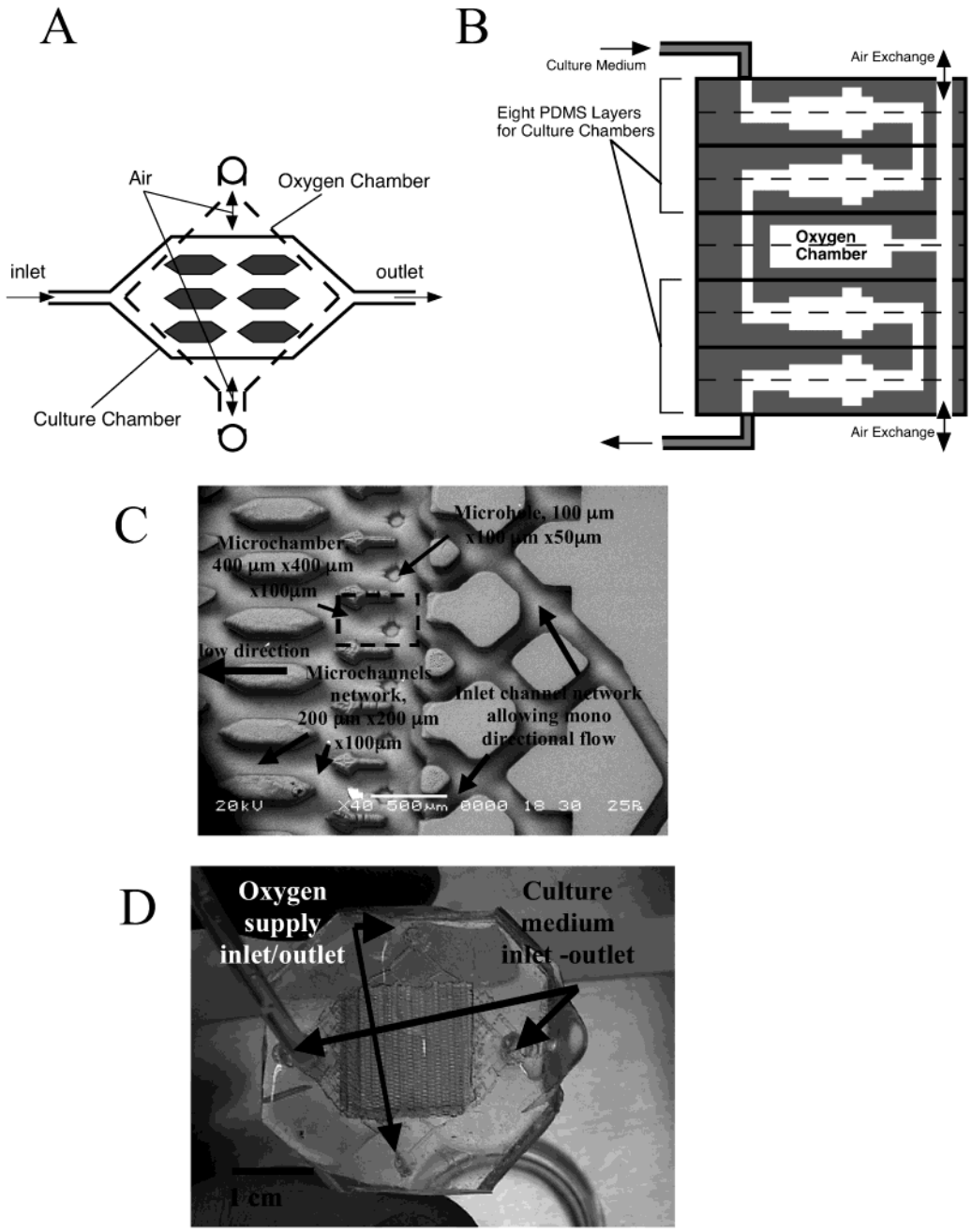

Figure 1. Structure of the multilayer bioreactor with an oxygen chamber (MLO): (A) schematic drawing of the oxygen chamber (dotted line) superimposed over the culture chamber, viewed from the top of the layers; (B) cross-sectional view of the bioreactor. The oxygen chamber is located in the middle of the layers of the culture chambers and is directly connected to the outside; (C) SEM view of the microstructure in the PDMS layer for the culture chamber, composed of microchambers, microholes, and microchannels; (D) photo of the bioreactor with connecting tubes for culture medium perfusion and holes for the oxygen chamber.

structures for oxygen supply and cell culture and is applied to continuous perfusion culture of Hep G2 cells.

Materials and Methods

Description of Bioreactors. The bioreactor was fabricated by combining a microfluidic network and an oxygen chamber as shown in Figure 1A. The overall structure of the bioreactor is shown in Figure 1B, which shows a cross-sectional view of the bioreactor. The bioreactor is composed of 10 stacked PDMS layers. Eight layers, with microfluidic networks, constitute cell culture chambers, whereas two layers form the oxygen chamber in the middle of the bioreactor. The microfluidic network in each PDMS layer contains two kinds of particular structures, an inlet channel network to distribute the culture medium homogeneously and microstructures for the cell attachment. The latter includes microholes (100 μm × 100 μm × 50 μm) and microchambers (400 μm × 400 μm × 100 μm) as shown in Figure 1C. The total height of the structure is about 300 μm. Two PDMS layers are bonded together to form a cell culture chamber of 600 μm height. The outlet of each microfluidic network is connected to the inlet of the lower ones to realize a uniform flow through the whole bioreactor. An oxygen chamber is inserted in the middle of the eight PDMS layers and is connected to the outside air. The oxygen chamber and the microfluidic network are separated by thin 300-μm walls, which could allow oxygen diffusion from the oxygen chamber into the cell culture chambers.

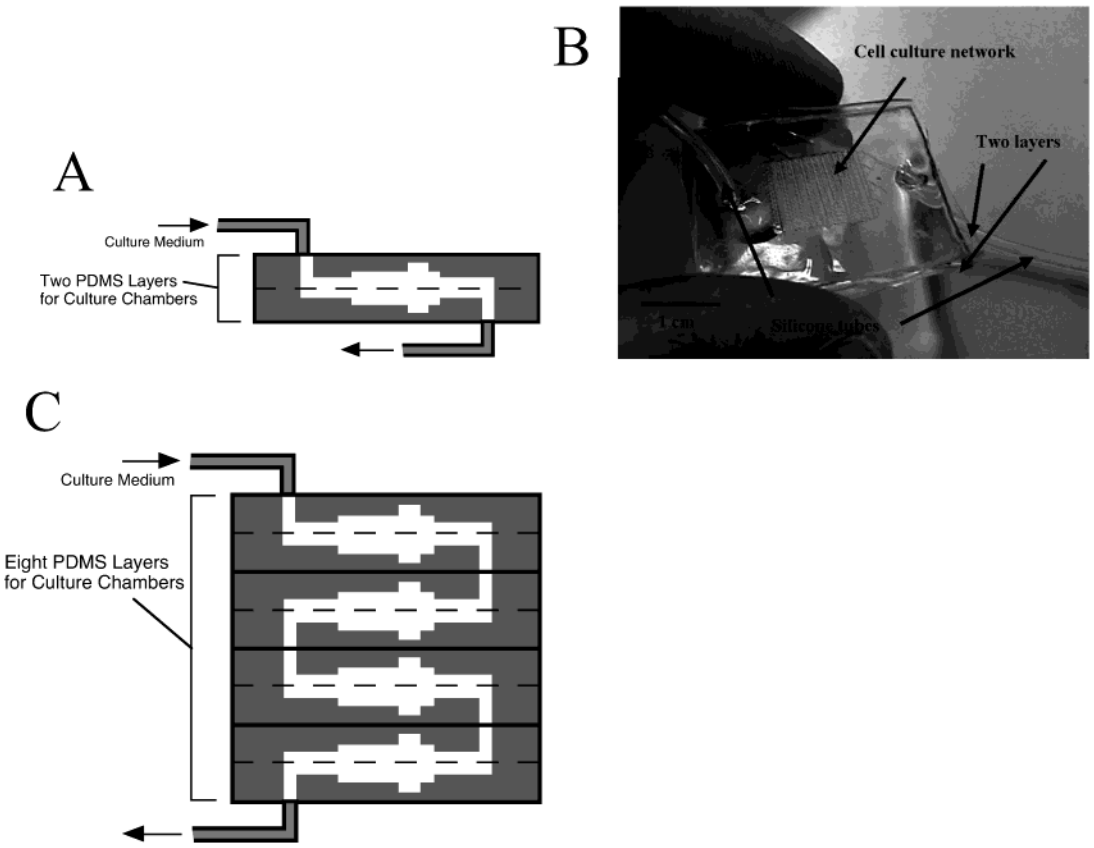

Figure 2. Structure of the two-layer (TL) and multilayer bioreactors (ML) used for comparison: (A) cross-sectional view of the TL; (B) photo of the TL; (C) cross-sectional view of the ML.

The resulting bioreactor is shown in Figure 1D. The total height of the ten-stacked layers was about 6 mm.

Two other microfluidic bioreactors are introduced to carry out side-by-side experiments with the present one. One is a simple bioreactor composed of two PDMS layers to compare the performance in terms of the volume of the culture. This corresponds to a single culture chamber incorporated in the present bioreactor as shown in Figure 2A and B. The other one is composed of eight PDMS layers as illustrated in Figure 2C, which has the same number of culture chambers as the present one but without the oxygen chamber. This is introduced to know the effectiveness of the oxygen chamber. To simplify the description of these bioreactors, we denote the present ten-layer bioreactor as MLO (Multi-Layer bioreactor with an Oxygen chamber), the two-layer bioreactor as TL (Two-Layer bioreactor), and the eight-layer bioreactor as ML (Multi-Layer bioreactor), respectively. All of the culture experiments and the measurements of the cell's activities described in the following are carried out with these three bioreactors to acquire comparative data.

Fabrication of Bioreactors. Because the material of the bioreactor is a kind of silicone elastomer, the microfluidic structure is fabricated through replica molding processes with a mold master, which contains the negative pattern of the structures. At first, a negative master is made with a SU-8 photoresist (*22*) using a conventional photolithography process. CHF_3 plasma is applied to the negative master to deposit a fluorocarbon layer onto the obtained SU-8 master for easy release of the PDMS layers (*23*). Then, the liquid-state PDMS is poured on the master, and after baking for solidification, the PDMS layer is peeled off from the master. Inlets and outlets of each PDMS layer are drilled with 1 mm diameter. Then, the PDMS layers are aligned and stacked onto each other after being treated by O_2 plasma. This alignment can be done under optical microscopy using a small drop of methanol deposited on the PDMS surface, and independently for each culture chamber (*24*). As the inlets-outlets between each single culture chamber have a 1 mm diameter, this allowed easier alignment between two cell culture chambers. Finally, silicone tubes are glued to the inlet on the top layer and to the outlet on the bottom layer in order to have the connections to the perfusion circuit.

Cells and Culture Medium. Hep G2 cells, which are human hepatocarcinoma cells (*25*), obtained from the Japanese Collection of Research Bioresources (JCRB) are used for all the culture experiments. The culture medium was Dulbecco's modified Minimum Essential Medium (DMEM; Nissui Pharm. Co., Ltd.; Tokyo, Japan) supplemented with 10% fetal bovine serum (Filtron; Altona, Australia), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Dojindo, Kumamoto, Japan), 100 U penicillin/mL (Wako, Osaka, Japan), 100 μg streptomycin/mL (Wako, Osaka, Japan) and 0.25 μg amphotericin B/mL (Sigma Aldrich, Missour, USA).

Experiments. A perfusion system composed of a culture medium tank serially connected to a peristaltic pump, a glass bubble trap, and the bioreactor is used, as shown in Figure 3. The connections between the bioreactor and the perfusion system are done with silicone tubes. First of all, the whole system is sterilized by autoclave. Then, all inner surfaces of the microfluidic network of the bioreactors are precoated with 0.03% Type I collagen (Nitta gelatin Co. Ltd., Osaka, Japan) and kept at rest in an incubator for 0.5 h. The bioreactor is then washed with an excess amount of Dulbecco's Phosphate-Buffered Saline (PBS). Then, the cells are inoculated into the culture chambers in order to avoid attachment of the

Table 1. Hep G2 Cell Cultures in Tissue-Treated-Dishes, TL, MLO, and ML[a]

			multilayers	
	dish	two layers	with O_2 chamber	without O_2 chamber
growth (times[b])	21	20	20	2.5
cell number ($\times 10^6$ cells)	4	2	10	1
cell density ($\times 10^7$ cells/cm^3)		3.3	4.2	0.4
albumin production (pg/day/cell)	1.25 ± 0.3	2.5 ± 0.5	3 ± 0.6	2 ± 0.1
glucose consumption (ng/day/cell)	0.4 ± 0.1	0.25 ± 0.08	0.33 ± 0.05	0.4 ± 0.1

[a] Data represent mean of three experiments with SD. [b] Ratio between inoculated and collected count cell.

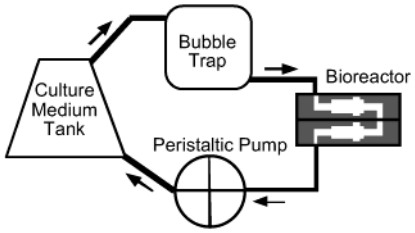

Figure 3. Schematic diagram of the perfusion system.

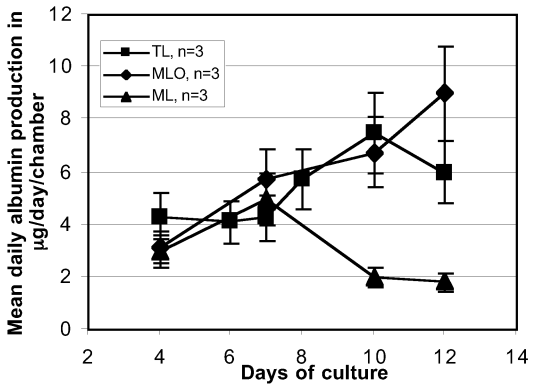

Figure 4. Comparison of daily albumin secretion in the three types of bioreactors during perfusion cultures of Hep G2 cells ($n = 3$, corresponding to the number of independent tests).

cells to other parts of the system. After one night at rest, the culture medium perfusion is started with a flow rate at 5−10 μL/min. The entire system for the culture is put in an incubator under 95% humidity and 5% partial pressure of CO_2. The culture medium is replaced by fresh medium once every 2 or 3 days during the experiment, to supply sufficient amounts of glucose to the cells.

Measurements of Liver-Specific Functions. The glucose concentration in the culture medium was measured by the glucose oxidase method using a commercially available machine (Glucose Analyser 2, Beckman Instruments Inc., Galway Ireland) to monitor the cell growth. The albumin production, which characterizes the specific functional activity of liver cells, was also measured by sampling the culture medium. The measurement was done by a sandwich-type enzyme-linked immunosorbent assay (ELISA). For ELISA measurements, anti-human albumin goat antibody as well as anti-human albumin goat antibody conjugated with horseradish peroxidase were purchased from Cappel Lab (Ohio, USA). Standard human albumin was purchased from Sigma Aldrich (Missouri, USA) (*26*). To count the cells, they were detached by conventional trypsine-EDTA solution introduced in the bioreactors and then collected. Then, they were stained by trypan blue dye and counted under an optical microscope.

Results and Discussions

Cell Number and Density in Bioreactors. To achieve a large-scale culture in microfluidic environments, we propose a multilayer PDMS bioreactor with a microfluidic network and an oxygen chamber (MLO). As a result of the effect of the oxygen chamber, the cell number in MLO was improved by a factor of 5 compared to the two-layer bioreactors (TL), as shown in Table 1, which summarizes the present data (morphological observation of the cells in the TL are described in ref 27). In addition, the cell number in the MLO was found to be 10 times larger than in the ML (see also Table 1). This comparison shows that as a result of the oxygen supply, the number of cells involved in the culture was enhanced. The values of the cell density were about 3 to 4 $\times 10^7$ cells/cm^3 in the TL and the MLO, respectively. This is consistent with other macroscale bioreactors, in which cell densities of about 3×10^7 cells/cm^3 were obtained (*26*, *28*), or silicon micro bioreactors, in which we estimated according to the reported data about 4×10^7 cells/cm^3 for about 2.4×10^5 cells (*14*, *15*). Those results show that the cell density comparable to the macroscale bioreactors could be achieved in microfluidic environments mimicking the situation in vivo.

Activity of Cells. Albumin productions at the end of the culture were about 3±0.6 pg/cell/day and 2.5±0.5 pg/cell/day in the MLO and TL, respectively (Table 1). Cell functions themselves were not so different between both bioreactors, but a larger number of cells could be kept under stable conditions in the MLO. Mainly, the MLO composed of four culture chambers act as four independent TL. Figure 4 shows the time course of albumin production normalized by a single culture chamber composed of two PDMS layers. Albumin secretion is increasing in all experiments in the first week. In the second week, the ML shows deterioration in the production, while the TL seems to reach a confluent situation shown by saturation. On the other hand, the production is continuously increasing in the case of MLO even in the second week. Since in all experiments the culture medium was periodically changed once every 2 or 3 days, the difference among these results could not originate from glucose shortage. This result also clearly shows the importance of the oxygen supply. ML has shown a cell activity of about 2 pg/cell/day but the cell number and density were very low compared to those of the MLO.

Glucose consumption of the Hep G2 cells was measured to get the information on the growth of the cells. Figure 5 shows daily glucose consumption of the bioreactors. The saturated values of the consumption in the second week were 0.5 and 0.65 mg/chamber/day for the MLO and the TL, respectively, whereas for the ML, it went down to 0.1 mg/day on the eighth day. The trend in the glucose consumption is consistent with the one in albumin production.

With the help of the oxygen chamber, we could increase the cell number without reducing the activity per one cell. That leads to higher performance of the MLO in total. The present stacked bioreactor with oxygen chambers

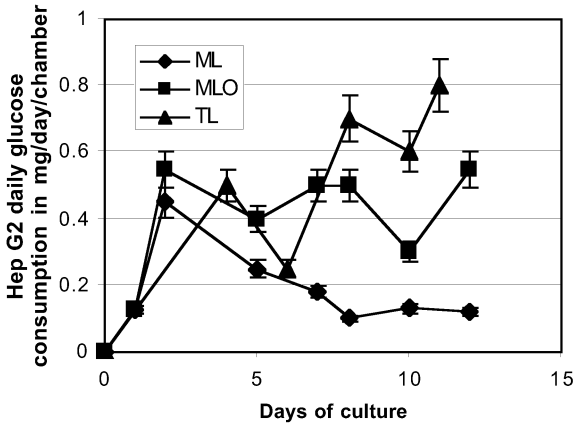

Figure 5. Comparison of daily glucose consumption in the three types of bioreactors during perfusion culture of Hep G2 cells.

should be an advantageous method to perform such large-scale culture.

Shear Stress and Oxygenation in the MLO. In our bioreactors, we used a flow rate around 5−10 μL/min, and we estimated a shear stress during the perfusion culture in the range of 0.03 (for a channel height of 600 μm) to 0.25 (for a channel height of 300 μm) dynes/cm^2. In response to this low flow rate, which limits the oxygen supply to the cells, an external oxygen supply system is necessary when long-term and high-density cultures are desired. To solve this problem, we could use the PDMS to utilize its high permeability. On the basis of eq 1, we found that the oxygen supply by the medium to the cells, at such flow rate, could only contribute to feed only about 1×10^5 cells. In addition, on the basis of the same equation, for 2×10^6 cells in the TL and 10^7 cells in the MLO as counted, we estimated that a flow rate value of 120 and 600 μL/min, respectively, should be used. Our previous work showed that at such flow rate mechanical detachment of the cells occurred (*21*). The PDMS permeability could therefore be used as an optional way to supply the oxygen to the cells.

$$Q\Delta Ccm = X_{\text{onecell}}N_{\text{cell}} \qquad (1)$$

in which Q is the flow rate (10 μL/min), X_{onecell} is the oxygen consumption for one cell (1×10^{-16} mol O_2/cell sec (*29*)), N_{cell} is the number of cells, and ΔCcm *is* the variation of the concentration of the dissolved oxygen between the inlet and outlet of the bioreactors (2×10^{-7} mol O^2/cm^3-CM).

Conclusions

A large number of cells were successfully cultured in a microfluidic environment, namely, up to 10^7 cells, with the multilayer bioreactor containing an oxygen supply system. Cell density was also found in same order of magnitude as macroscale bioreactors. With the help of the oxygen chamber, oxygen was supplied to the cells instead of applying higher flow rate of the culture medium. This helped avoid cells' detachment, and therefore cell culture could be successfully done over a period as long as 12 days. As a direct result, the cell number involved in the bioreactor was increased without reducing the activity per one cell, which led to the increased total performance of the bioreactor.

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