**A POROUS PERFUSION BIOREACTOR THAT POSSESS MICROCHANNELS: IT'S FABRICATION BY SELECTIVE LASER SINTERING AND PRELIMINARY EVALUATION OF CULTURE HUMAN HEPATOMA Hep G2 CELLS**

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## Abstract

For engineering implantable liver tissues, we designed a new scaffold with a three-dimensional (3D) branching and joining flow channel network (inner diameter is 1 mm) described by accumulated unit tetrahedrons (edge length is 4 mm). For its fabrication, biodegradable polycaprolactone (PCL) and 80% (w/w) of NaCl salt particles as a porogen were completely mixed and applied to the selective laser sintering (SLS) process, a rapid prototyping technique. We successfully obtained a scaffold that had a high porosity of 89% with a pore size of 100-200 μm and a 3D network flow channels whose inner diameter is 800 μm. Results of X ray CT confirmed the sintered scaffolds had completely interconnected flow channels as designed. A preliminary perfusion culture over 9 days demonstrated that such mirochannels was necessary to guide the cells to grow and function.

## 1. Introduction

There has been a growing interest in developing perfusion bioreactor for vital organs (such as liver) (1, 2, 3,4), because the flow system mimics the hepatic blood circulation and enhances mass transfer of nutrients, gases (such as $O_2$), and metabolites to maintain long-term tissue functions. One challenge in reactor design is to ensure a homogeneous distribution of flow and mass transfer. Recently, we proposed a new design of a small model scaffold in which a 3D branching and joining microchannel network was arranged as unit tetrahedral edges except the horizontal ones to distribute the flow of culture medium(5).

Precise 3D microfabrication process is necessary to realize the design. Various types of fabrication technologies have been developed to construct a porous bioreactor/scaffold: three-dimensional printing (3DP) (6), stereolithography (7), selective laser sintering (SLS) (8), deposition/layering (5), and silicon micromachining (9) for tissue engineering application. Among these technologies, SLS is a well-known rapid prototyping (RP) technology, through which 3-D structures are created layer-by-layer, by heating and sintering powdered material by means of the heat generated from a $CO_2$ laser (10, 11). SLS has recently been proposed as a promising technique over traditional manual-based techniques (8) mainly because it is applicable almost all materials and because it does not necessitates toxic binding materials such as organic solvents. We therefore examined the feasibility of a SLS for the fabrication of the designed scaffolds for hepatic tissue engineering.

Polycaprolactone (PCL), a type of linear biodegradable polyester, has acquired much attention over the last years for their low-cost and potential application in tissue engineering (12,13,14). PCL scaffolds with a controlled architecture have been fabricated successfully for bone tissue engineering (15). However, the porosity was small (ranging from 37.5-55.0%) and may not suitable for hepatic tissue engineering because hepatocytes should be packed at much higher cell densities while supplied

with plenty amounts of oxygen and nutriments. In addition, its highly crystalline property may reduce the adhesion efficiency of hepatocytes (16).

In this paper, we first describe a SLS technique for the fabrication of a highly porous scaffold (porosity 89%) with a customer-defined microchannel network structure. Then, we perform a perfusion culture of human hepatoma Hep G2 cells for evaluating its biocompatibility. Effective cell inoculation and initial attachment using novel Avidin-Bivion binding-based cell adhesion (17) is also described.

## 2. Materials and methods

### *2.1. Design of a porous perfusion bioreactor*

The basic concept of design was described in our previous report (5). Figure 1 represents the new design, in which we changed the dimension of unit tetrahedron from 2 mm to a 4 mm length edge (smaller length is not suitable for the present SLS machine). The whole model consists of 2 symmetric cones, in which tetrahedrons were accumulated in 6 layers, and all the edges except the horizontal ones were designed to organize a flow channel network. To distribute the media flow, the diameter of layers are designed as follows: 2.0 mm at the1st and 2nd layers, 1.5 mm at the 3rd and 4th layers, and 1.0 mm at the 5th and 6th layers (Figure 1). The maximum diameter and height of a bioreactor is 30 mm and 46 mm, respectively, and the whole volume are 10 $cm^3$.

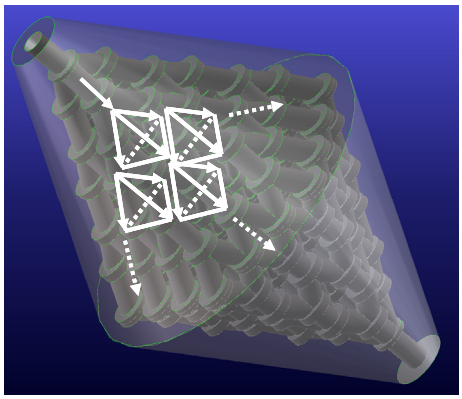

**Figure 1. A model bioreactor for perfusion culture based on 4-mm-length-edge regular tetrahedrons.**

### *2.2. Fabrication of a perfusion scaffold*

PCL (M.W. 50, 000 Da) powders with a mean size of 50 μm were selected as a raw polymer material. To increase the porosity, 80% weight of NaCl salt particles was added. The salt was seized and the particles with a mean size of 100~150 μm were used. Nano silica was added (0.4% (w/w)) to prevent the slight static electrification and moisture of PCL particles. Then, we carefully mixed the combined particles manually before sintering.

The scaffolds were fabricated by a commercial SLS machine (SEMplice, ASPECT, Japan). All parameters of SLS were set at their default values except the laser power and part bed temperature. Excess and unsintered powders were removed by blowing compressed air.

The sintered-scaffold was put into distilled water to leach out the NaCl salt. The concentration of NaCl was determined using a conductivity meter (CM-6A, TOA Elec. Ltd, Japan). After 8 h, the concentration was lower than 0.002 M. The scaffolds were dried at room temperature for 24 h. Then we sealed the scaffolds with transparent silicon glue (Baskooku, Semedain, Japan) (5 mm thick) before perfusion culture. The microstructure of the sintered scaffolds was evaluated by X ray CT (SMX-225CT, Simatu, Japan).

### *2.3. Biochemical evaluation of the sintered scaffolds*

Hepatoma Hep G2 cells were purchased from the Japanese Cancer Research Bank (JCRB). The culture medium composed of Dulbecco's modified Minimum EssentialMedium (DMEM), supplied with 10% fetal bovine serum (FBS), 20 mM hydroxyethyplpiperazine-N'-2-ehanesulfonic acid (HEPES), 1% nonessential amino acid solution (NEAA), and antibiotics.

Under the perfusion condition, cells were cultured in PCL scaffolds with and without a 3D channel network. Each whole perfusion circuit (Figure. 2) consisted of a reservoir bottle (500 mL) with a gas-permeable silicon membrane cap, a peristaltic pump (SJ-1220; Atto Biointrustment, Japan), a glass bubble trap, and the PCL scaffold-based bioreactor. The bioreactor was held vertically and the culture medium was introduced from its bottom inlet. Cells were seeded into the scaffold using a new efficient avidin-biotin adhesion method as developed in our laboratory (17). Briefly, the cells at their confluence were treated with EZ-Link Sulfo-NHS-LC-Biotin (0.5 mM in phosphate buffered saline (PBS)) in a $CO_2$ incubator. After 30 min, the cells were washed with PBS, recovered with trypsin/EDTA. Prior to use, avidin (Sigama-Aldrich; 0.5 mg/ml in PBS) was adsorbed into the PCL porous scaffolds for 2 h.

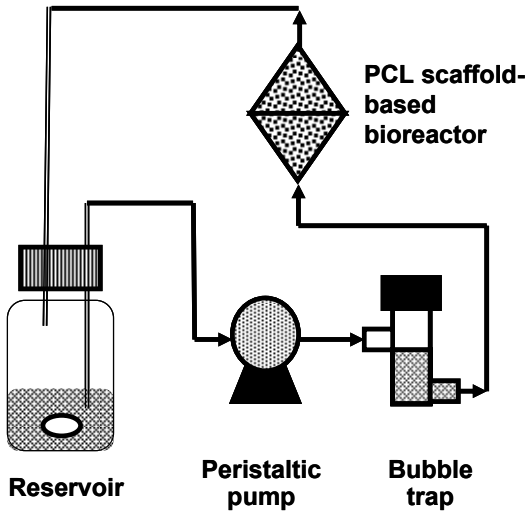


**Figure 2. Scheme of the perfusion circuit system**

A cell suspension ($4.0\times10^7$ cells in 20 mL culture medium) was introduced to the avidin-treated scaffold using two syringes that were repeatedly depressed and released for inoculation. Then, gentle perfusion at a flow rate (2 mL/min) was started. After 2h, the flow rate was increased to 10 mL/min. The culture medium was replenished first on Day 1 and thereafter at 2-day intervals until Day 9. The volume of culture medium in the reservoir was kept at 100 mL. A conventional monolayer culture and a small scale of shaking culture (1.5 mm thick, Φ10 mm, 0.1 $cm^3$) were also performed as control groups. The cells were inoculated at $4\times10^5$ cells per piece PCL disk-scaffold and at $5\times10^4$ cells/$cm^2$ in monolayer plates. The PCL culture was continuously shaken using a rotational shaker at a rotational speed of 60 rpm. All cultures were performed at 37°C in a $CO_2$ incubator (95% air/5% $CO_2$).

The concentration of glucose was measured by a glucose analyzer (Glucose Analyze 2; Beckman Instruments, Galway, Ireland). The concentration of human albumin produced in the culture medium was measured by a sandwich-type enzyme-linked immunosorbent assay (ELISA).

## 3. Results and discussion

### *3.1. Scaffold fabrication*

In order to determine the range of suitable processing parameters, the scan speed (3433 mm/s), sweep spacing (0.1 mm), and powder layer thickness (0.2 mm) were kept constant; the part bed temperature (40-50℃) and laser power (3.5-12.5 W) was investigated (8). The PCL/NaCl particles could not be sintered at 3.5 W under 40℃ part bed temperature. The specimen sintered at 4.9 W and 5.8 W was fragile and the structure was wrapped/destroyed while removing the salt at the next step, which suggested that the particles was not completely sintered at such temperature. To overcome this problem, we raised the temperature up to 50℃ and laser powder up to7.8 W, 9.8 W and 12.5 W. The remnant particles were not easy to remove and microchannels were embolized at high powder (9.8 and 12.5 W). For the successful sintering of porous structure, the optimal parameter were 7.8 W for the laser power, 3433 mm/s for the scan speed and part bed temperature of 50℃.

The microstructure of the sintered scaffolds was evaluated by X ray CT. Results of X ray CT demonstrated the fabricated scaffolds had a very homogenous distribution with interconnected microchannels which matched to the designed data.

A number of scaffold parameters including porosity and pore size play a significant role in tissue engineering application (18). Wiliams *et al.* reported the fabrication of PCL scaffold with a porosity ranging from 37.5 – 55.0% using SLS for bone regeneration (15). However, a higher porosity is necessary for hepatic tissue engineering. In this study, we successfully fabricated a highly porous scaffold (89% porosity) by adding salt as a porogen.

The pore size was about 100-200 μm, which was smaller than that reported in our previous works while 3D culturing fetal hepatocytes (19, 20, 21). The scaffold with higher pore size could be obtained by using salt particles with higher mean size.

SLS provides some advantages over other fabrication techniques. The organic reagents were not used during the whole SLS process. In addition, the fabrication process is efficient and speedy that we could make 6 scaffolds at one batch within 1 h.

*3.2. Perfusion culture*

To evaluate the importance of the arrangement of a 3D flow channel network, we performed two perfusion cultures using scaffolds with and without such a 3D network as well as monolayer and shaking cultures. The glucose consumption and albumin production up to 9 days are shown in Figure 3. Significant differences in both functions were observed between those two perfusion cultures, with and without such a 3D channel network: the rate of glucose consumption generally increased and albumin production was well maintained with an increase of culture time in the scaffold possessing such channels, but without such channels both functions were suppressed at significantly lower levels throughout the culture. The microchannels (inner diameter: 800 μm) arranged into the middle scale of bioreactors (volume: 10 $cm^3$) may promote transports of nutrients, oxygen and waste under perfusion conditions. Our results indicate the important of design and fabrication of such a 3D channel network when engineering a large liver tissue equivalent in vitro.

The two functions in perfusion culture even with such microchannels were still lower than that in shaking and monolayer culture (Figure 3). One of the reasons is that the oxygen supply may be a limiting factor in the present SLS-

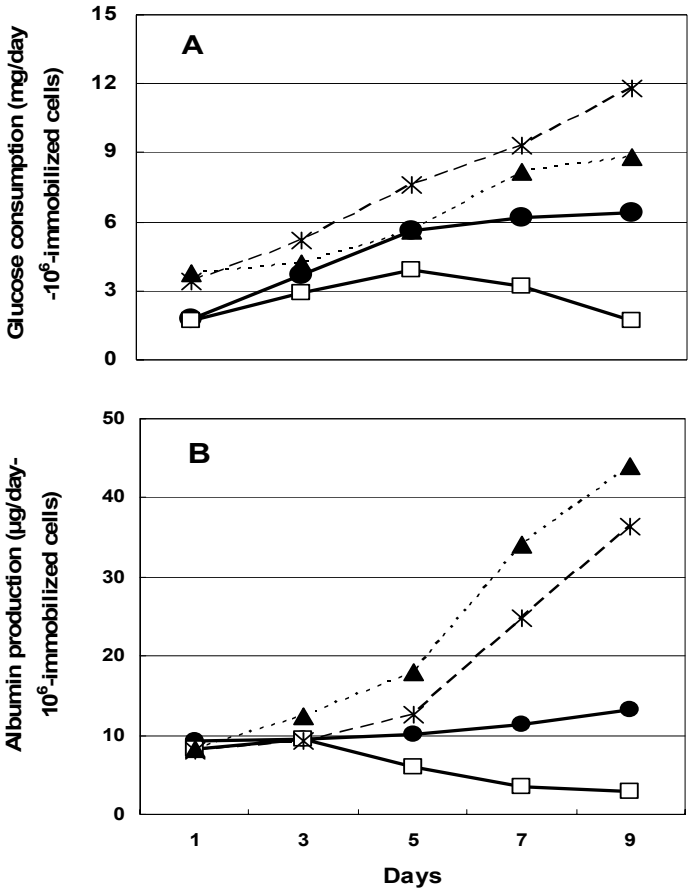


**Figure 3. Glucose consumption (A) and albumin production (B) of Hep G2 cells cultured in monolayers (∗), in shaking culture of PCL disks (▲), and in the PCL-based perfusion bioreactors with (●) and without microchannels (□).**

fabricated bioreactors that had a limited dimension of scaffold (the edge length of tetrahedrons: 4 mm; the inner diameter of microchannels: 800 μm). According to our previous estimation, even at a low finally attained cell density, the edge length of a tetrahedron, based on a simple steady-state oxygen diffusion-consumption model that describes oxygen concentration gradient from the microchannel to the center of the unit tetrahedrons, should be less than 1080 μm, much smaller than that in our present scaffolds (5). The cells in the center of the unit tetrahedron may be suffered from inadequate supply of oxygen. Much finer fabrication is expected to improve the cellular activities.

We chose biodegradable PCL as a model polymer for its ease of fabrication and low cost. PCL is widely used in hard tissue engineering such as bone (15). However, it's highly crystalline property reduces its application in soft tissue engineering (16). In this study, we enhanced cellular adhesion by a novel Avidin-Bivion binding system (17). As shown in Figure 3, the cells grow well in the 3D porous scaffold with microchannels and the finally-attainted cell density was beyond 1/2 of the shaking culture. In contrast, the functions of the cells and the finally-attainted cell density dramatically decreased without using this adhesion technique (data not shown). Although the mechanism of the Avidin-Bivion binding needs to be declared in the future experiments, this preliminary demonstration may show the potential of PCL both in hard and soft tissue engineering.

## 4. Conclusions

A middle scale bioreactor (volume: 10 $cm^3$) with designed microchannels was fabricated successfully by SLS technique. Results of X ray CT confirmed the sintered scaffolds had completely interconnected flow channels as designed. A preliminary perfusion culture demonstrated such microchannels were necessary to guide the cells to grow and function.

## References


1. Borenstein, J.; Terai, H.; King, K.; Weinberg, E.; Kaazempur-Mofrad, M.; Vacanti, J. Microfabrication technology for vascularized tissue engingeering. Biomed Micro. **4,** 167-175; 2002.
2. Chen, J.; Yu, S.; Hsu, B.; Fu, S.; Liu, H. Loofa sponge as a scaffold for the culture of human hepatocyte cell line, Biotechnol. Prog. **19,** 522-527, 2003.
3. Miyoshi, H.; Yanagi, K.; Fukuda, H.; Ohshima, N. Long-term continuous culture of hepatocytes in a packed-bed reactor utiling porous resin, Biotechnol. Bioeng. **43,** 635-644, 1994.
4. Powers, M.J.; Domansky, K.; Kaazempur-Mofrad, M.R.; Kalezi, A.; Capitano, A.; Upadhyaya, A.; Kurzawski, P.; Wack, K.E.; Stolz, D.B.; Kamm, R.; Griffith, L.G. A microfabricated array bioreactor for perfused 3D liver culture. Biotechnol. Boeing. **78,** 257-269; 2002.
5. Sakai, Y.; Osuka, M.; Hanada, S.; Nishiyama, Y.; Konishi, Y.; Yamashita, A. A novel poly-L-lactic acid scaffold that possesses a macroporous structure and a branching/jointing three-dimensional flow channel network: its fabrication and application to perfusion culture of human hepatoma Hep G2 cells. Mat. Sci. Eng. C., **24,** 379-386; 2004.
6. Kim, S.S; Utsunomiya, H.; Koski, J.A.; Wu, B.M.; Cima, M.J.; Sohn, J.; Mukai, K.; Griffth, L.G.; Vacanti. J.P. Survival and function of hepatocytes on a novel three-dimensional synthetic biodegradable polymer scaffold with an intrinsic network of channels, Ann. Surg., **228,** 8-13, 1998.
7. Dhariwala, B., Hunt, E., Boland. T. Rapid prototyping of tissue-engineering constructs, using photopolymerizable hydrogels and stereolithography. Tissue Eng., **1**0, 1316-22, 2004.
8. Tan, K.H.; Chua, C.K.; Leong, K.F.; Cheah, C.M.; Gui, W.S.; Tan, W.S.; Wiria, F.E. Selective laser sintering of biocompatible polymers for applications in tissue engineering, Bio-Medical Materials and Engineering, **15,** 113-124, 2005.

9. Kaihara, S.; Borenstein, J.T.; R. koka, S. Lalan, E.R. Ochoa, M. Ravens, H. Pien, B. Cunningham, Vancanti. J.P. Silicon micromachining to tissue engineer branched vascular channels for liver fabrication. Tissue Eng., **6,** 105-117, 2000.
10. Tsang, V. L.; Bhatia, S.N. Three-dimensional tissue fabrication, Advanced Drug Delivery Reviews, **56,** 1635-1647, 2004.
11. K.F. Leong, C.M. Cheah, C.K. Chua, Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs, Biomaterials, **24,** 2363-2378, 2003.
12. Zein,I.; Hutmacher,D.W.; Tan, K.C.; Teoh, S.H. Fused deposition modeling of novel scaffold architectures for tissue engineering applications. Biomaterials, **22,** 1169-1185, 2002
13. Causa,A.; Netti, P.; Ambroso, L.; Ciapetti,G.; Baldini, N.; Pagani, S.; Martini, D.; Giunti, A. Poly-e –caprolactone/hydroxyapatite composites for bone regeneration: in vitro characterization and human osteoblast response. J Biomed mater Res., **76**A, 151-162, 2006.
14. Kweon, H.Y.; Yoo, M.K.; Park, I.K.; Kim, T.H.; Lee, H.C.; Lee, H.S.; Oh, J.S.; Akaike, T; Cho, C.S. A novel degradable polycaprolactone networks for tissue engineering. Biomaterials, **24,** 801-808, 2003.
15. Williams, J.M.; Adewunmi, A.; Schek, R.M.; Flanagan, C.L.; Krebsbach, P.; Feinberg, S.E.; Hollister, S.J.; Das, S. Bone tissue engineering using polycaprolactone scaffolds fabricated via selective laser sintering. Biamaterials, **26,** 4817-4827, 2005.
16. Carlisle, E.C., Mariappan, M.R., Nelwon, K.D., Thomes, B.E, Timmons, R.B., Constantinescu, A., Eberhart, R.C., Bankey, P.E. Enhancing hepatocyte adhesion by pulsed plasma deposition and polyethylene glycol coupling. Tissue Eng., **6,** 45-51, 2000.
17. Kojima N, Matso T, Saki Y, Rapid hepatic cell attachment onto biodegradable polymer surfaces without toxicity using an avidin-biotin binding system. Biomaterials, **27,** 4904-4910, 2006.
18. Hollister, S.J. Porous scaffold design for tissue engineering. Nat Mater., **4**, 518-24, 2005.
19. Hanada, S.; Kayano H.; Jiang, J.; Kojima, N.; Miyajima, A.; Sakoda, A.; Sakai, Y.. Enhanced in vitro maturation of subcultivated fetal human hepatocytes in three-dimensional culture using poly-L-lactic acid scaffolds in the presence of oncostatin M. Int. J. Artif. Organs. **26,** 943-951, 2003.
20. Jiang, J.; Sakai, Y.; Kojima, N.; Guo, L.; Naruse, K.; Makuuchi, M.; Miyajima, A.; Yan, W.Q.; Sakai, Y. Efficacy of engineered liver tissue based on poly-L-lactic acid scaffolds and fetal mouse liver cells cultured with oncostatin M, nicotinamide and dimethysulfoxide. Tissue Eng. **10,** 1577-1586, 2004.
21. Huang, H.; Hanada, S.; Kojima, N.; Sakai, Y. Enhanced functional maturation of fetal porcine hepatocytes in three-dimensional poly-L-lactic acid scaffolds: a culture condition suitable for engineered liver tissues in large-scale animal studies. Cell Transplant. In press.