

FABRICATION OF MICROCHANNEL NETWORK IN LIVER TISSUE SPHEROIDS

Nobuhiko Kojima^{1,2*}, Shoji Takeuchi^{1,2} and Yasuyuki Sakai^{1,2}

¹ Institute of Industrial Science (IIS), University of Tokyo and

² Life BEANS Center, BEANS Project

ABSTRACT

This paper reports a method to fabricate liver tissue spheroids with microchannel network in which 1 μm diameter particles can pass through. We made alginate hydrogel beads that have 20 μm in diameter, and formed heterogeneous spheroids consisting of about 1,000 of hepatic cells and same number of the beads. One day culture of the hetero-spheroids resulted in network-like organization of the hepatic cells. The alginate beads were then digested by alginate lyase treatment for 10 min. The connectivity of porous spheroids was confirmed using 1 μm fluorescence beads or a micro device. These data suggest that bottom-up approach to make microchannel is effective in engineering of multi-cellular spheroids.

KEYWORDS

Liver tissue spheroids, Microchannel, Alginate gel beads, Tissue engineering

INTRODUCTION

Liver tissue spheroids are useful tool to study cell biology in three-dimensional environment as well as to validate new drugs in pharmaceutical companies. They have smooth surfaces wrapped by extracellular matrices (ECMs) and cell-packed body, showing they are not just aggregates of cells but well organized tissue. However, the dense structures prevent effective exchange of gas and nutrients supplied from environment. To improve the traffic of them, we tried to make microchannel network by embedding of alginate beads in the spheroids and enzymatic removal of them.

EXPERIMENTAL

Cell culture

The human hepatoma cell line Hep G2 was from the Japanese Center Research Bank (JCRB). The culture medium was Dulbecco's modified minimum essential medium (DMEM; Sigma-Aldrich, St. Louis, MO, US) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, US), 25 mM hydroxyethylpiperazine-N' 2-ethanesulfonic acid (HEPES; Dojindo, Kumamoto, Japan), 100 units/ml of penicillin (Wako Pure Chemicals, Osaka, Japan), 100 $\mu\text{g}/\text{ml}$ of streptomycin (Wako), and 0.25 $\mu\text{g}/\text{ml}$ of amphotericin B (Sigma). Cells were cultured in a CO_2 incubator (5% CO_2 , 37°C). These cells were stained with PKH26 (Sigma) or PKH67 (Sigma), as necessary. Cells were observed by confocal laser microscopy (LSM 7 DUO, Carl Zeiss, Germany) or fluorescence microscopy (Axio Observer. A1, Carl Zeiss).

Alginate gel beads

To prepare alginate beads in diameter about 20 μm , we discharged 1.5% sodium alginate solution (Sigma) to 5% calcium chloride (Sigma) solution by an inkjet system (WaveBuilder and PulseInjector, Cluster Technology, Osaka, Japan). The beads were incubated in a FITC-conjugated poly-L-lysine (Sigma) solution to be labeled with fluorescence as necessary.

Assembly of cells and beads to form hetero-spheroids

We established a system to gather 100 nm to 100 μm particles using 3% methylcellulose (MC; M0512; Sigma) medium [1]. The MC medium was pored into a petri dish using a positive-displacement pipette (Microman; Gilson, Middleton, WI, US) because it has a high viscosity. Hep G2 cell or the alginate bead suspended culture medium was adjusted as 2×10^6 cells/ml, or 2×10^6 beads/ml, respectively. We mixed same volume of them, and injected 1 μl of the mixture into the MC medium. In other words, 1,000 cells and 1,000 beads were put into the MC medium. Injected cells and beads were gathered within 10 min and incubated for 1 to 2 days to form hetero-spheroids. To pick the spheroids up from the 3% MC medium, we added 5 U/ml of cellulase solution (Onozuka RS; Yakult Pharmaceutical Industry, Tokyo, Japan) to the MC medium and incubate for 30 min to digest high molecular structures of MC

Visualization of actin filament and nucleus

Hetero-spheroid cultured for 24h were picked up from the 3% MC medium using cellulase solution and fixed with 4% Paraformaldehyde (Sigma). The spheroids were then permeabilized with 0.025% saponin (Sigma) solution. Rhodamine-conjugated phalloidin (Life technologies, Carlsbad, CA, US) and Hoechst 33342 (Dojindo) were added and incubated for 2 to 4 hours and washed with phosphate buffered saline (PBS; Life technologies).

Digestion of alginate beads and labeling of surface cells with 1 μm fluorescence beads

To hollowing the hetero-spheroids, we incubated them in 200 $\mu\text{g}/\text{ml}$ of alginate lyase (Sigma) solution for 10 min. Spheroids with or without microchannel were incubated in PBS suspending concentrated 1 μm fluorescence beads (Polysciences, Warrington, PA, USA) for 15 min at room temperature. Labeled spheroids were observed with a confocal

microscope.

Fabrication of micro devices

A silicon wafer was surface-treated using a mixture of 30% hydrogen peroxide and sulfuric acid at a volume ratio of 1:2, and fluorinated acid. Negative photo-resist SU-8 (MicroChem, Newton, MA, US) was then spin-coated for ten seconds at 1,000 rpm, heated to 65 °C for 60 minutes and to 95 °C for 60 minutes, then cooled to room temperature. The SU-8-coated wafer was irradiated by ultraviolet light at 300 W/cm² through a photo-mask for 80 seconds, and heated 95 °C for 60 minutes. Followed by infiltration filtered in 1-methoxy-2 propylacetone for 60 minutes, SU-8 was removed from the non irradiated part of the wafer, and a cast plate was obtained by coating the entire wafer with trifluorocarbon. A mixed solution of PDMS polymer: cross linker at a volume ratio 10:1 was poured on the cast plate, which was heated to 75 °C for 60 minutes to yield a solid PDMS. After the solid PDMS was peeled off the cast plate and the PDMS was surface treated with O₂ plasma for 10 seconds followed by permanent bonding to the glass coverslips. A silicon tube with an internal diameter of 1 mm was installed on the devices to make inlet and outlet.

RESULTS AND DISCUSSION

Figure 1 shows alginate gel beads. When we used an inkjet head it had 25 µm nozzle diameter, adequate size (20 µm in diameter) beads were able to be made. To visualize under fluorescence or confocal laser microscope, the beads were incubated with FITC-conjugated poly-L-lysine for 10 min. Figure 2 represents the procedure for fabricating spheroids with microchannels. Injection of suspended cells/beads into 3% MC medium resulted in rapid aggregation of them because of the swelling property of the MC medium [1]. The heterogenic aggregates were cultured for 1 to 2 days to organize cell-to-cell adhesion. Spheroids with beads were removed from the MC medium and treated with alginate lyase to make microchannels. Spheroids with or without microchannels were stained for actin fibers and nuclei as shown in Figure 3. In normal spheroid were fully stuffed with cells. On the other hand, the spheroid with beads had network-like spaces connecting hole body of the spheroids. We tried to observe whether a flow was able to pass through the microchannels. We soaked the spheroids in a solution suspending 1 µm polystyrene beads that can stain cells by accumulation on the cells. Figure 4 shows only one or two layers of the cells in the normal spheroid were stained. In contrast, almost all area of the spheroids with microchannels was stained, indicating that the polystyrene beads reached to the center of the spheroid via microchannel network. As shown in Figure 5, we made simple micro devices that can trap and perfuse the spheroids. After trapping of the spheroids, we applied a buffer containing FITC-conjugated albumin. Figure 5-C showed that the buffer passed through the inside of the liver tissue spheroids.

CONCLUSION

These results showed that it is possible to make the liver tissue spheroids with microchannel network and indicated that these engineered spheroids may improve the supply and removal of gas and nutrients.

ACKNOWLEDGEMENTS

This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO), and by the JSPS Grant-in-Aid for Young Scientists (B) Grant Number 24710142.

REFERENCES

- [1] N. Kojima, S. Takeuchi, and Y. Sakai, "Rapid aggregation of heterogeneous cells and multiple-sized microspheres in methylcellulose medium" *Biomaterials*, 33, 4508-4514, (2012).
- [2] N. Kojima, S. Takeuchi, and Y. Sakai, "Fabrication of multicellular heterospheroids by a dispenser robot system" *Biofabrication* 2011 in Toyama (2011).
- [3] K. Pataky, T. Braschler, A. Negro, P. Renaud, M.P. Lutolf, and J. Brugger, "Microdrop Printing of Hydrogel Bioinks into 3D Tissue-Like Geometries" *Advanced Materials*, 24, 391 (2012).

CONTACT

*Nobuhiko Kojima, tel: +81-3-5452-6545; nobuhiko@iis.u-tokyo.ac.jp

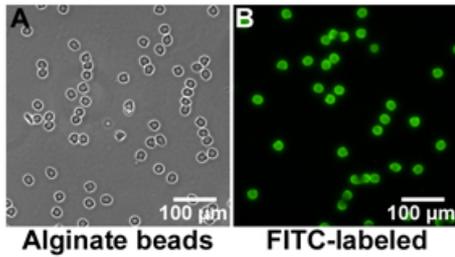


Figure 1: Alginate gel beads in diameter about 20 μm (taken with a fluorescence microscope)

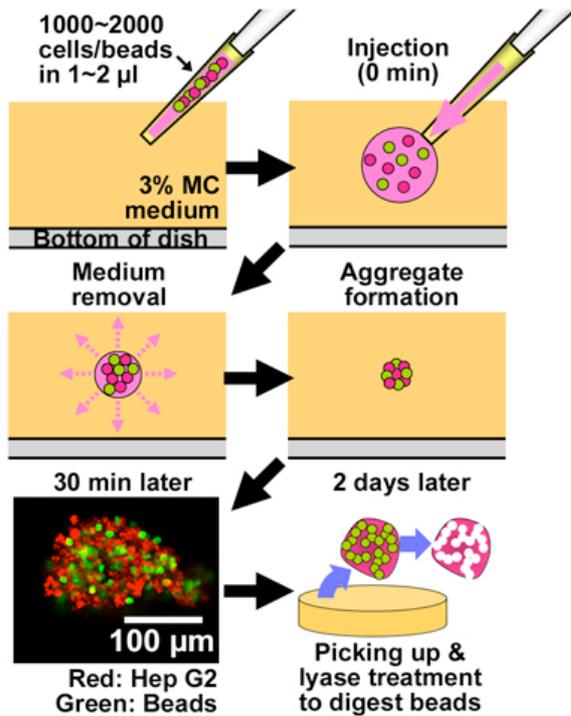


Figure 2: Procedure to fabricate spheroids with microchannel network. The MC medium can absorb normal culture medium by swelling. Therefore, suspended cells/beads were gathered rapidly. Alginate beads can be enzymatically digested by alginate lyase. (Taken with a confocal microscope)

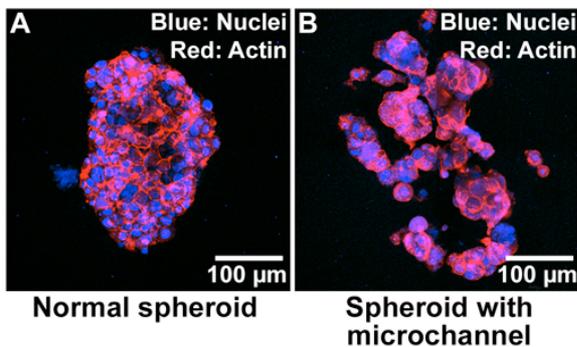


Figure 3: Effect of alginate gel beads on organization of liver tissue spheroids. The area where the alginate beads existed was hollowed. (Projection images taken with a confocal microscope)

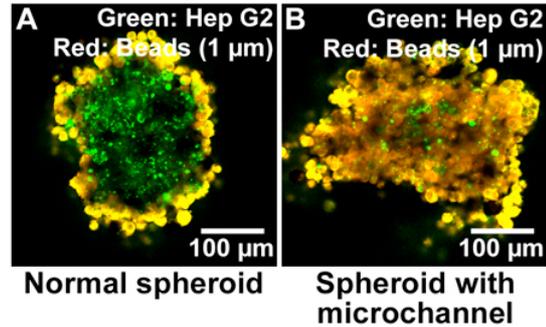


Figure 4: Microchannel-dependent diffusion of 1 μm polystyrene beads. Spheroid with microchannels was able to take in the 1 μm beads even in the center of the body, whereas only one or two surface layers in normal spheroid contacted the beads. (Taken with a confocal microscope)

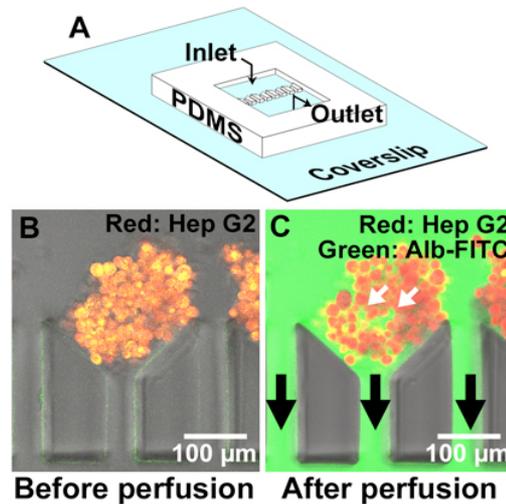


Figure 5: Perfusion test of liver tissue spheroid with microchannels. Albumin-FITC containing buffer was introduced from inlet. Microchannels inside of the spheroid were filled or flowed by the buffer. (Taken with a confocal microscope)