Development of microcapsules for producing spherical tissues of < 150 μm in size

S. Ito, S. Sakai*, Y. Ogushi, I. Hashimoto and K. Kawakami
Department of Chemical Engineering, Faculty of Engineering, Kyushu University, Fukuoka, Japan (sakai@chem-eng.kyushu-u.ac.jp).

Introduction

Cell-enclosing spherical microcapsules have been studied as a basic research tool, therapeutic device for tissue transplantation, and production of useful proteins (Uludag 2000). We have previously developed a method for preparing cell-enclosing microcapsules with solid core of less than 100 μm in diameter (Sakai 2004). The microcapsules are much smaller than conventional cell-enclosing microcapsules of 500-1,000 μm in diameter. We prepared such small capsules via a droplets breakup technology in a laminar flow of water-immiscible liquid. In addition, we developed a technique producing cell-enclosing microcapsules with hollow core of ca.100-150 μm in diameter using the small microcapsules with solid core as template of the liquid cores (Sakai 2008). The size of liquid cores was less than the maximum allowable capsule size of 260 μm in diameter for a tightly packed cell mass that does not limit the oxygen supply resulting in necrotic region formation inside spherical tissues (Kim 1998). Previously (Sakai 2008), we enclosed mouse embryonic stem cells in the microcapsules with 20-50 μm of agarose gel membrane and with hollow core of 100-150 μm in diameter. The cells grew and formed embryoid body-like spherical tissues having the same size with the hollow cores (Sakai 2008). In some applications such as tissue engineering, it is required to collect the spherical tissues from microcapsules without damaging them. However, in this point, agarose gel is not a suitable material for microcapsule membrane. For removing agarose gel membrane, we have to heat them more than 60°C. In addition, in general, agarase, an enzyme of digesting agarose molecules, can not liquefy agarose gel under the condition mild for mammalian cells. In this paper, we attempted to develop microcapsules with hollow cores of < 150 μm in diameter and degradable membrane under mild condition for mammalian cells for collecting spherical tissues formed in the vehicles.

Materials and Methods

Feline kidney cells (CRFK cell line) was suspended at 1.5×10^7 cells/ml in 2.5% (w/v) carboxymethylcellulose (CMC) with phenol moieties (CMC-Ph) dissolved in Krebs Ringer Hepes-buffered solution (KRH, pH7.4) containing horseradish peroxidase (HRP) at 10 units/ml. The suspension was extruded from a 26-gauge needle into a co-flowing immiscible stream of liquid paraffin containing H_2O_2 at 0.82 mmol/l and lecithin at 3.0% (w/w). Liquid paraffin-suspended particles that became partially gelated during the flow were collected in a plastic tube and incubated for a further 10 min to allow enzymatic gelation (Figure 1). The resultant CMC-Ph particles were collected in calcium-free KRH (CF-KRH) via centrifugation and rinsed several times with the same buffer. The particles were then resuspended in CF-KRH containing 2.0% (w/v) alginate with phenol moieties (Alg-Ph) and HRP at 10 units/ml. The resultant suspension was extruded into a coflowing stream of liquid paraffin containing lecithin (3.0% (w/w)) and H_2O_2 from a 26-gauge needle under a flow rate that resulted in capsules with a single core and membranes of ca.20 μm in thickness. The resultant emulsion of Alg-Ph solution containing CMC-Ph particles was collected in a plastic tube. After 10 min of allowing standing for a further enzymatic gelation, the particles were collected in KRH via centrifugation and rinsed several times with medium. Next, the collected particles were incubated in medium containing cellulase (10 units/ml) for 4 h to liquefy the enclosed CMC-Ph particles by enzymatic degradation. After 2 weeks of culture, Alg-Ph microcapsules were soaked in

the medium containing alginate lyase at 0.2 mg/ml for collecting spherical tissues formed in the vehicles. The CMC-Ph and Alg-Ph used in this study were synthesized through the condensation reaction of carboxyl groups of CMC and Alg with amino group of tyramine using water soluble carbodiimide (Ogushi 2007, Sakai 2007a). Each polysaccharide derivatives contained 13 and 8 phenol moieties per 100 repeat units of uronic acid.

Results and Discussion

CMC-Ph particles

In this study, we used CMC-Ph and Alg-Ph as materials for obtaining cell-enclosing microcapsules with hollow-core of less than 150 μm in diameter. CMC gel and Alg gel are enzymatically degradable using cellulase and alginate lyase, respectively, under mild condition for cells. These enzymes are not proteolytic enzymes, thus, it is expected that the cell-to-cell connection in spherical tissues is preserved after the enzymatic degradation processes. We used CMC-Ph particles of about 150 μm in diameter as a template of hollow core and Alg-Ph gel as a membrane of microcapsules because mechanical strength of Alg-Ph gel can be enhanced via subsequent crosslinks formation using divalent cations such as Ca^{2+} and Sr^{2+} (Sakai 2007a).

At first, we attempted to develop cell-enclosing CMC-Ph particles by extruding cell-suspending CMC-Ph solution into coflowing stream of liquid paraffin containing H_2O_2 based on the previous study for cell encapsulation in Alg-Ph particles (Sakai 2007b). Diameter of the resultant particles could be controlled by changing flow rate of ambient liquid paraffin under the fixed flow rat of CMC-Ph of 0.05 ml/min (Figure 2); a similar result to that previously reported for agarose (Sakai et al., 2005) and Alg-Ph (Sakai 2007b). The resultant particles were spherical in shape as shown in Figure 3. This sphericity means that the sufficient degree of enzymatic gelation necessary for fixing the final shape of CMC-Ph gel does not occur instantaneously. In addition, the resultant particles were degradable using cellulase. The viability of the cells recovered via the enzymatic degradation was 87.0 ± 4.5%. These results clearly demonstrate the possibility of applying CMC-Ph for obtaining the template of spherical hollow-core of microcapsules.

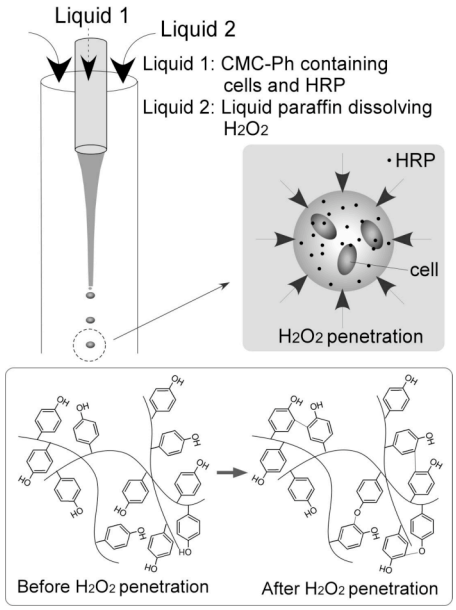

Figure 1: Schematic illustration of breakup of cell enclosing droplets via jetting and gelation via HRP reaction

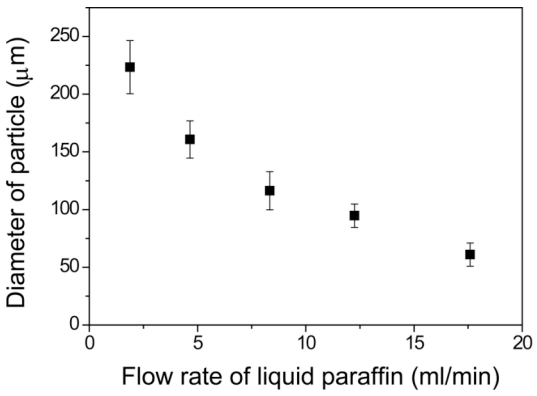

Figure 2: CMC-Ph particle diameter as a function of the velocity of liquid paraffin.

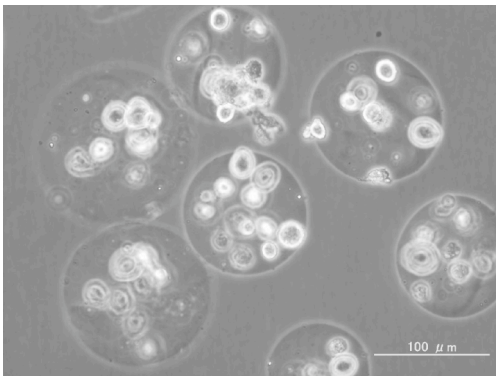

Figure 3: Photograph of cell-enclosing CMC-Ph particles. Bar: 100 μm.

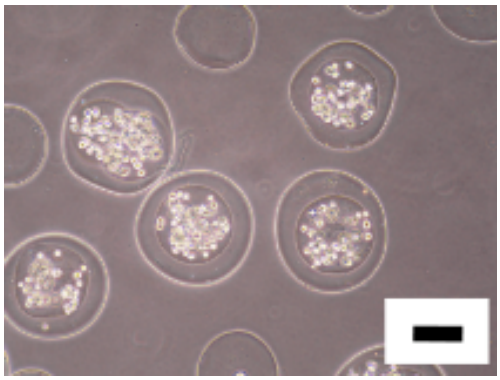

Figure 4: Photograph of Cell-enclosing Alg-Ph microcapsules after degradation of CMC-Ph particles. Bar: 100 μm.

Alg-Ph microcapsules with hollow core

The microcapsules with hollow-core and with a thin Alg-Ph gel layer were developed by injecting Alg-Ph solution suspending cell-enclosing CMC-Ph particles into coflowing liquid paraffin dissolving H_2O_2. The resultant particles had a thin Alg-Ph gel layer with dozens micrometer in thickness. By soaking the resultant particles in the medium containing cellulase, we could obtain Alg-Ph microcapsules with hollow-core templated by CMC-Ph particle (Figure 4). The enclosed cells formed some clusters in individual microcapsules within several days of culture and continued to grow. After 2 weeks of incubation, the spherical tissue in microcapsules almost completely filled the hollow core. No obvious adverse effect specific for Alg-Ph microcapsules was detected compared with agarose microcapsules (Sakai 2008).

After formation of spherical tissues in Alg-Ph microcapsules, we soaked them into the medium containing alginate lyase. The Alg-Ph gel membrane disappeared within 1 min (Figure 5). Thus, we could collect only spherical tissues from the suspension of microcapsules. For determining the effect of enzymatic degradation, we observed transition of morphological change of the spherical tissues. The surfaces of the tissues were smooth just after degrading Alg-Ph membrane. The surface of the tissues became slightly lumpy at 40 min after degradation. At this point, some spherical tissues resisted movement with the medium resulting from mild perturbation of the medium. After 180 min of incubation, the cells of the collected

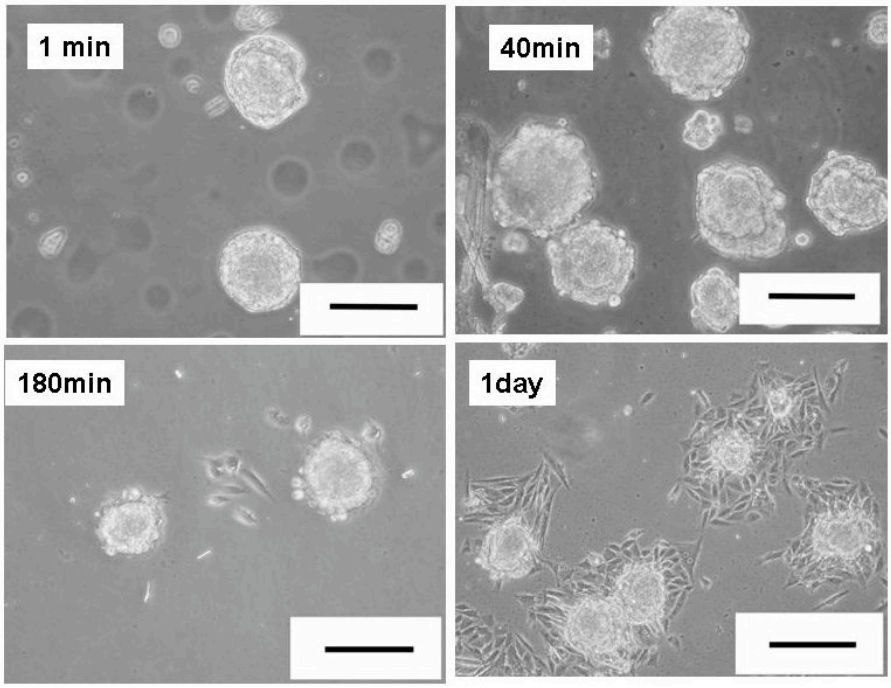

Figure 5: Transition of morphology of spherical tissues after soaking in the medium containing alginate lyase. Bars: 200 μm.

spherical tissues adhered and spread on the tissue culture dish. In addition, at 1 day of the cells grew on tissue culture dish. These results demonstrate the cells in the collected spherical tissues kept alive, i.e., it means the harmless of the spherical tissues collecting process using alginate lyase, including exposure of cells to the digestion of Alg-Ph.

Conclusions

We developed microcapsules with hollow cores of < 150 μm in diameter and with enzymatically degradable membrane. The microcapsules were prepared by coating cell-enclosing CMC-Ph gel particles of < 150 μm with a thin Alg-Ph gel layer and subsequently degrading the CMC-Ph gel using cellulase. Both the CMC-Ph and Alg-Ph gels were obtained via HRP-catalyzed crosslinking reaction between Ph moieties in water-immiscible liquid dissolving H_2O_2. From the microcapsules, we could collect the tissues having almost the same size with the hollow core by degrading the Alg-Ph gel using alginate lyase after 2 weeks of cultivation. From these results, we concluded that the Alg-Ph microcapsules are hopeful vehicles for producing spherical tissues with the size smaller than maximum allowable tissues size without necrotic region formation.

References

H. Uludag et al. (2000) *Technology of mammalian cell encapsulation*. Advanced Drug Delivivery Reviews 42(1) 29-64.

S. Sakai et al. (2004) *Preparation of mammalian cell-enclosing subsieve-sized capsules (<100 μm) in a coflowing stream*. Biotechnology and Bioengineering 86(2) 168-173.

S. Sakai et al. (2008) *Production of cell-enclosing hollow-core agarose microcapsules via jetting in water-immiscible liquid paraffin and formation of embryoid body-like spherical tissues from mouse ES cells enclosed within these microcapsules*. Biotechnology and Bioengineering 99(1) 235-243.

S.K. Kim et al. (1998) *Calculations on O_2 transfer in capsules with animal cells for the determination of maximum capsule size without O_2 limitation*. Biotechnology Letters 20(6) 549-552.

Y. Ogushi et al. (2007) *Synthesis of enzymatically-gellable carboxymethylcellulose for biomedical applications*. Journal of Bioscience and Bioengineering 104(1) 30-33.

S. Sakai et al. (2007a) *Synthesis and characterization of both ionically and enzymatically crosslinkable alginate*. Acta Biomaterialia 3(4) 495-501.

S. Sakai et al. (2007b) *Peroxidase-catalyzed cell-encapsulation in subsieve-size capsules of alginate with phenol moieties in water-immiscible fluid dissolving H_2O_2*. Biomacromolecules 8(8) 2622-2626.

S. Sakai et al. (2005) *Development of mammalian cell-enclosing subsieve-size agarose capsules (<100 μm) for cell therapy*. Biomaterials 26(23) 4786-4792.