Fabrication of cell-enclosing capsules with hollow-core via enzymatic functions

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Introduction

Encapsulation of mammalian cells in spherical vehicles has been studied as a basic research tool, a device for producing biological agents, and a device for delivery of cells producing therapeutic products in vivo since 1964. For these applications, a wide variety of spherical vehicles have been developed over the past decade. These vehicles can be classified into two categories from a structural point of view: (1) vehicles with a liquid core, designated "capsules"; and (2) vehicles with a solid core, designated "particles". Cells enclosed in the latter vehicles are surrounded by a solid gel, and their proliferation is therefore inhibited by microscopic stresses arising from the surrounding gel. On the other hand, cells enclosed in the former vehicles are not subject to such microscopic stresses. Thus, the former vehicle gives a more preferable environment for cellular growth. From the view point of cellular growth, size of cell-enclosing vehicles is also an important issue for supplying sufficient amount of oxygen and nutrients. We have previously developed a method for the preparation of cell-enclosing particles of less than 100 μm in diameter with a narrow distribution in size via jetting of cell-suspending polymer solution in water-immiscible coflowing fluid (Sakai 2004). In this paper, we report a technique producing cell-enclosing microcapsules having the liquid core of ca.100-150 μm in diameter using enzymatic functions, crosslinking and degradation. The sizes of liquid cores were 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 (Kim 1998). We used cell-enclosing alginate particles of ca.100-150 μm as template of the liquid cores. The alginate particles were prepared through peroxidase-catalyzed reaction in water-immiscible fluid via jetting process. Then, we coated the particles with a thin agarose gel layer via the same process. Finally, we degraded the alginate particles using alginate lyase. We determined the effects of each process on cell viability and growth. In addition, we studied the effect of cell-adhesiveness of microcapsule membrane on them.

Materials and Methods

Feline kidney cells (CRFK cell line) was suspended at 1.5×10^7 cells/ml in 1.5% (w/v) alginate with phenol moieties (Alg-Ph) dissolved in calcium-free Krebs Ringer Hepes-buffered solution (KRH, pH7.4) containing horseradish peroxidase at 1.7 unit/ml. The Alg-Ph was synthesized through the conjugation reaction of alginate and tyramine using water-soluble carbodiimide (Sakai 2007a). It contained 2.8 phenols per 100 repeat units of uronic acid. 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 Alg-Ph particles were collected in KRH via centrifugation and rinsed several times with the same buffer. The particles were then resuspended in KRH containing 4.0% (w/v) unmodified agarose or the agarose conjugated with gelatin (Aga-Ge) maintained at 38°C. The volume ratio of the particles to agarose solution was 1:20. The Aga-Ge was synthesized through the conjugation reaction of agarose and gelatin using 1,1-carbinyldiimidazole in dimethyl sulfoxide (Sakai 2007b). It contained 25% (w/w) gelatin. The resultant suspension was extruded into a coflowing stream of liquid paraffin containing lecithin

(3.0% (w/w)) 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 ungelated agarose solution containing alginate particles was collected in a plastic tube. After 10 min of cooling in an ice bath for gelation of the agarose solutions, the particles were collected in KRH via centrifugation and rinsed several times with medium. Next, the collected particles were incubated in medium containing alginate lyase (0.2 mg/ml) for 1 h to liquefy the enclosed alginate particles by enzymatic degradation. To evaluate the cytotoxicity of the encapsulation process in Alg-Ph particles, viability of the cells enclosed in Alg-Ph particles was measured by trypan blue exclusion using a hemocytometer after degrading the particles using alginate lyase. Growth profiles of enclosed cells were estimated by increase in the amount of water-soluble formazan dye, which was derived from a tetrazolium salt dissolved in medium containing suspended cell-enclosing capsules, using a colorimetric assay kit.

Results and Discussion

Encapsulation in Alg-Ph particles via enzymatic crosslinking

At first we attempted to develop cell-enclosing Alg-Ph particles as a template of hollow core by extruding cell-suspending Alg-Ph solution into coflowing stream of liquid paraffin containing H_2O_2. Diameter of the resultant particles could be controlled by changing flow rates of ambient liquid paraffin and Alg-Ph solution. Figure 2 shows cell-enclosing Alg-Ph particles of ca.100 μm in diameter. The high sphericity shows that the sufficient degree of enzymatic gelation necessary for fixing the final shape of gel does not occur instantaneously. The viability of the cells after 30 min of encapsulation was 91.8 ± 3.2 %. This value was almost the same as that measured for the cells enclosed in non-gelated droplets of sodium alginate solution. In addition, the recovered cells adhered and spread on a tissue culture dish in the same way as cells seeded on the basis of general subculture protocol. From these results we could not find any harmful of the HRP-catalyzed encapsulation via jetting process despite the existence of H_2O_2 in the reaction system.

Production of agarose capsules with hollow core via enzymatic degradation

For developing a thin agarose layer on Alg-Ph particles we injected agarose aqueous solution

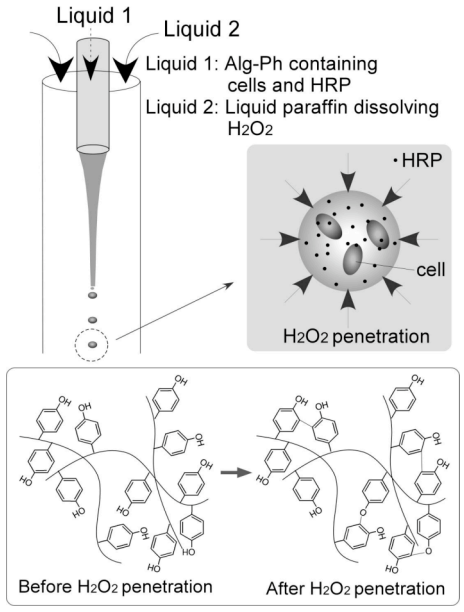

Figure 1: Schematic illustration of breakup of cell enclosing droplets via jetting and gelation via HRP reaction

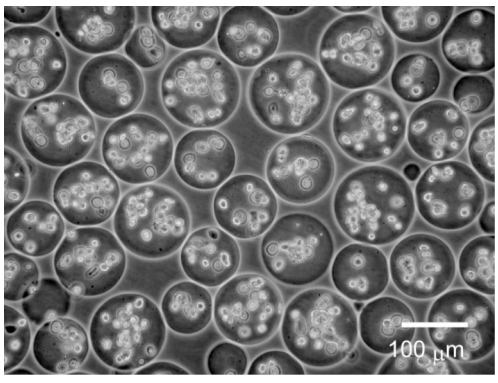

Figure 2: CRFK cell-enclosing Alg-Ph particles gelated via HRP-catalyzed oxidative reaction.

containing the particles into coflowing liquid paraffin fluid. Figure 3 shows the generation of agarose particles with obviously different sizes at the flow rate of agarose solution and liquid paraffin of 2.9 cm/sec and 18.1 cm/sec, respectively. The result is explained by the droplet breakup adopted under interfacial tension force from the stretched jet of the agarose aqueous solution having both regions that were thinner than the smaller agarose particles and the regions that were thicker than the alginate particles. The non-overlapping size distribution (Figure 3b) between the agarose particles with and without enclosing Alg-Ph particles suggested that these particles should be easy to separate using a filter with an appropriate pore size. Such a simple separation technique for removing vehicles that do not contain cells is attractive for the application toward cell therapy because there is a limit to the volume that can be implanted in cell-enclosing capsules. In addition, the size of the hollow cores was easily defined by the diameter of the alginate particles as shown in Figure 4. The ability to control the size hollow cores is attractive for controlling the size of resultant cellular clusters for efficient supply of oxygen and nutrients.

Growth of cells in capsules

To determine the feasibility of the hollow core structure prepared by degrading using alginate lyase, we enclosed CRFK cells. In addition, we studied the effect of capsule membrane having cell-adhesiveness on the growth of the enclosed cells. Immediately after encapsulation, cells existed individually in the hollow cores of microcapsules (Figure 5). Within several days of encapsulation, the cells in capsules began to self-aggregate in the hollow cores. Such a formation of cellular aggregates was not observed in Alg-Ph particles. Comparing the change in the sizes of cellular aggregates, those in Aga-Ge microcapsules increased faster than those in agarose microcapsules. After 21 days of encapsulation, the aggregates in Aga-Ge capsules almost completely filled the hollow cores (Figure 5). The time required for the aggregates completely filled the hollow-cores of

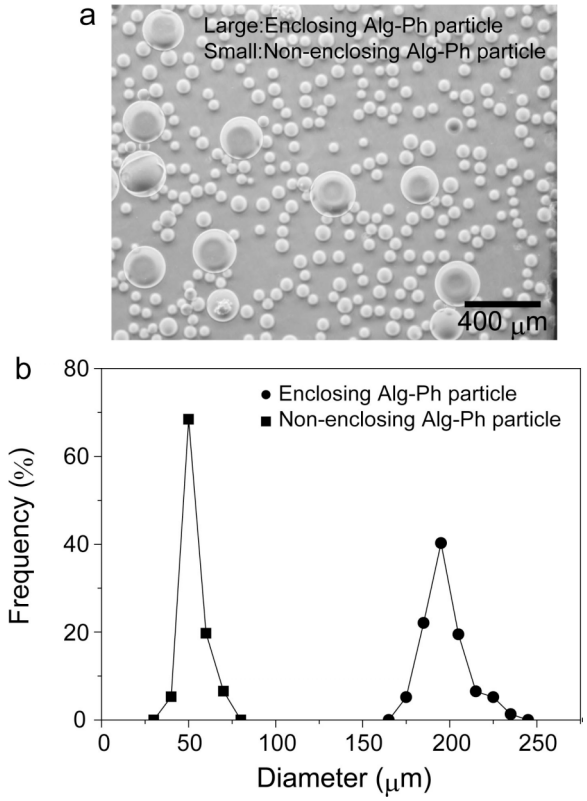

Figure 3: a) Micrograph of Alg-Ph particle enclosing and non-enclosing agarose particles. b) Size distribution of these agarose microcapsules (Sakai 2008).

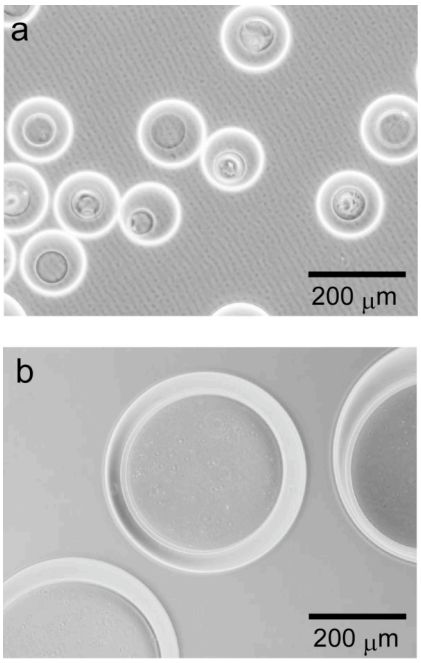

Figure 4: Agarose microcapsules with hollow cores templated by alginate particles of (a) 58 ± 7 μm and (b) 299 ± 11 μm in diameters. Hollow cores were prepared by degrading alginate gel using alginate lyase (Sakai 2008).

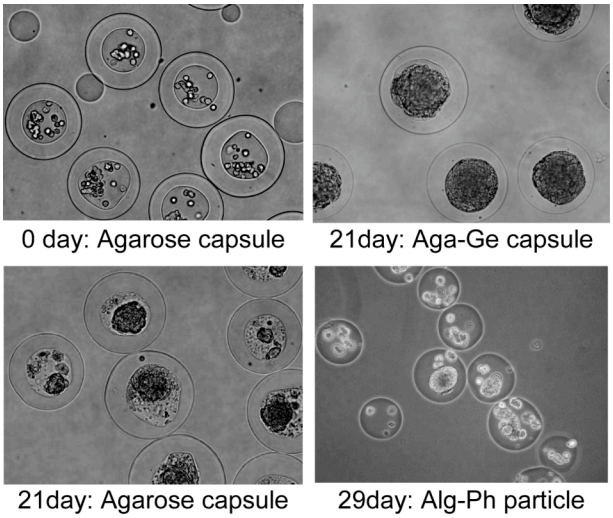

Figure 5: Morphologies of cells enclosed in each vehicles at each culture period.

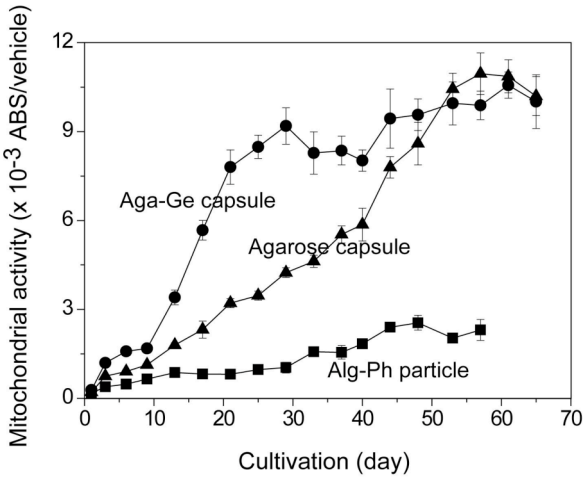

Figure 6: Time-course of mitochondrial activities of enclosed cells in each vehicle. Bars represent S.D.

agarose capsules was about 50 days. The cells in Alg-Ph particles formed cellular clumps, but did not form the clumps that large enough to fill the particles even after 60 days of encapsulation. The difference in the degree of growth in each vehicle was also recognized from the data for the mitochondrial activities (Figure 6). It is obvious that the higher growth rates and higher cell densities achieved in agarose and Aga-Ge capsules than those in Alg-Ph particles resulted from hollow core structure. The faster growth of the cells in Aga-Ge capsules than those in agarose capsules can be explained from the difference of cell-adhesiveness of microcapsule membrane.

Conclusions

We have developed a novel process for the preparation of agarose microcapsules with a spherical hollow core via jetting process in coflowing liquid paraffin. The spherical cores were templated by Alg-Ph particles obtained via peroxidase-catalyzed crosslinking reaction. Hollow cores were prepared by degrading the alginate particles using alginate lyase. The cells in the capsules with hollow core grew faster than those in Alg-Ph particles with solid core. In addition, we could enhance cellular growth in capsules by developing the capsules with the capsule membrane having cell-adhesiveness.

References

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