# Alginate microcapsule with liquid core templated by gelatin microparticle

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## INTRODUCTION

Encapsulation of mammalian cells in microcapsules has been studied since the 1960s as a basic research tool, a device for producing biological agents such as antibodies, enzymes and peptides and a device for delivery of cells. We recently revealed the potency of cell-enclosing microcapsules as a novel platform for obtaining multicellular spheroids (Sakai S, 2008). Multicellular spheroids are gaining increased recognition in anticancer drug research filed because they appear to mimic the morphology and physiology of cells in living tissues (Gaedtke L, 2007), and regenerative medicine field as embryoid bodies for obtaining a variety of differentiated cell types (Weitzer G, 2006).

The most often described spherical vehicle system is based on Ca-alginate gel core surrounded by a polycation layer such as poly-L-lysine and chitosan which at the same time is covered by an outer alginate membrane (Murua A, 2008). For giving the microenvironment suitable for growth, movement, rotation, and aggregation of enclosed cells, liquefaction of the core alginate gel crosslinked by calcium ions is accomplished by chelating the calcium ions. However, insufficient liquefaction resulting in residual Ca-alginate regions (Pajic-Lijakovic I, 2007), leaching out of just a small fraction of the total dissolved alginate molecules (Thu B, 1996) and re-gelation of the dissolved alginate by cations in the medium and body fluid (Sakai S, 2004a) considered as resulting in heterogeneous growth of the cells in the liquefied cores have been reported. Apart from the problem, the size of the spaces in microcapsules in which cells can potentially grow and form aggregate should smaller than 200 μm in diameter for suppressing necrotic region formation inside the resultant spherical tissues.

In this contribution, we report a novel technique for producing multicellular spheroids with a well defined size of about 150 μm in diameter. Compared with our previous technique using the polysaccharide derivatives containing phenol moieties crosslinkable via peroxidase-catalyzed reaction (Sakai S, 2009), the procedure of this contribution is simple and commonly-available materials are used: Microcapsules were prepared from gelatin and sodium alginate without any chemical modifications using the methodology shown in Figure 1. First, we prepared cell-enclosing gelatin microparticles for use as template of liquefied cores. Subsequently, we enclosed them in Ca-alginate microparticles using a conventional method. We then incubated the resultant microparticles under the conventional environment for cell culture at 37°C. Gel-to-sol transition of the enclosed gelatin gel around 37°C resulted in a formation of spherical non-gel space. After formation of spherical tissues, we collected them by degrading Ca-alginate gel membrane.

## MATERIALS AND METHODS

As a template for the hollow core in alginate microcapsules, gelatin microparticles were prepared on the basis of a previously described method that hardly hinders the viability of mammalian cells and results in microparticles with a narrow size distribution (Sakai S, 2004b; 2005). A coaxial droplet generator designed in our laboratory composed of two commercially available needles with



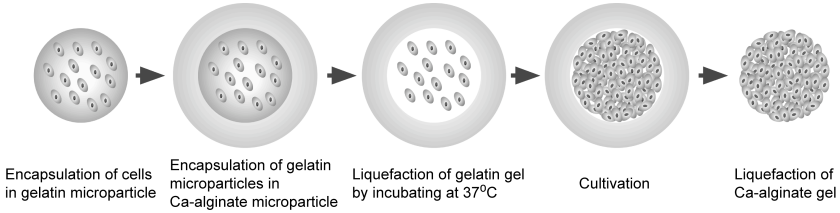


**Figure1: Schematic illustration of the production of multicellular spheroids**

different diameters was used for it. Briefly, calcium-free Krebs Ringer Hepes buffer solution (CF-KRH, pH7.4) containing 5% (w/v) gelatin and kept at 37°C was extruded from a 26-gauge needle into a coflowing immiscible stream of liquid paraffin flowed at laminar flow and containing lecithin at 3.0% (w/w). Cell-enclosing microparticles were prepared using the gelatin solution containing feline kidney cells (CRFK cell line) at $1.5 \times 10^7$ cells/ml. Liquid paraffin suspending gelatin droplets was collected in a 50-ml plastic tube. The tube was cooled at 4°C in an ice bath for 10 min. Then, CF-KRH cooled at 4°C was added and the tube was centrifuged at 1200 rpm for 1 min. The collected gelatin microparticles were washed twice with cooled CF-KRH to remove the liquid paraffin. The mean diameter of the resultant gelatin microparticles was determined based on measurements of more than 50 microparticles using an optical microscope.

Alginate microcapsules enclosing gelatin microparticles were prepared using an electrostatic droplet generator by dropping 1.1% (w/v) sodium alginate solution suspending gelatin microparticles into a 100 mM $CaCl_2$ solution. After at least 10 min of soaking in the gelling solution, the resultant microparticles were washed several times using medium. Liquefaction of the enclosed gelatin microparticles was accomplished by incubating the resultant Ca-alginate microparticles at 37°C.

After 21 days of culture, the Ca-alginate microcapsules containing multicellular spheroids were soaked in a medium containing alginate lyase at 0.2 mg/ml. The released spherical tissues were then transferred to fresh cell culture dishes for evaluating behavior of the collected cells.

## RESULTS AND DISCUSSION

Figure 2 shows cell-enclosing gelatin microparticles prepared by extruding cell-suspending gelatin solution into a coflowing liquid paraffin lamina flow. The microparticles of 168 ± 10 μm were obtained and the viability of the enclosed cells was 97.8%. We enclosed the gelatin microparticles in Ca-alginate microcapsules using a conventional electrostatic droplets production procedure. The size of the resultant Ca-alginate microcapsules was about 250 μm in diameter. According to this successful production of Ca-alginate microcapsules enclosing gelatin microparticles, we studied behavior of the enclosed gelatin microparticles in medium at 37°C using FITC-gelatin. Green fluorescence attributed to the FITC-gelatin was observed only in the center part of the microcapsules immediately after encapsulation (Figure 3). After 3days of incubation at 37°C, the area showing intense green fluorescence enlarged to surrounding Ca-alginate gel membrane. Green fluorescence was also observed in medium. These results clearly show the enclosed gelatin microparticles dissolved in the medium at 37°C and diffused into medium. It means we could successfully obtain the Ca-alginate microcapsules with liquid hollow core by using gelatin particles as its template.

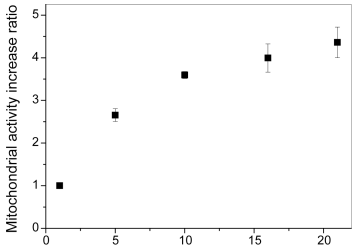


**Figure 5: Transition of mitochondrial activity increase ratio per microcapsule normalized by the value day 1**

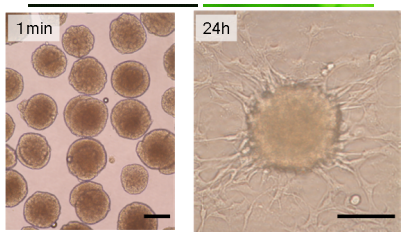


**Figure 3: Fluorescence images of Ca-alginate microcapsules enclosing FITC-gelatin microparticles. Bars: 200 μm**

**Figure 6: Spheroids just after and 24 h after degrading capsule membrane. Bar: 100 μm**

Figure 4 shows transition of morphologies of enclosed cells in Ca-alginate microcapsules. The cells existed individually before culturing at 37℃ because of the existence of gelatin gel. During several days of culturing the cells formed some clusters in the hollow cores. The cells grew and almost completely filled the core at day 10. The growth of enclosed cells was also observed from the result for transition of mitochondrial activity per microcapsule (Figure 5). No specific hindrance in cellular growth was detected for the Ca-alginate microcapsules compared with our previous microcapsules made from alginate and carboxymethylcellulose derivatives with phenol moieties via peroxidase-catalyzed crosslinking reaction (Sakai S, 2009).

Finally, extraction of spherical tissues from Ca-alginate microcapsules was attempted by immersing cell-enclosing Ca-alginate microcapsules in the medium containing alginate lyase at 0.2 mg/ml after 21 days of cultivation. The Ca-alginate microcapsule membrane disappeared within 1 min of

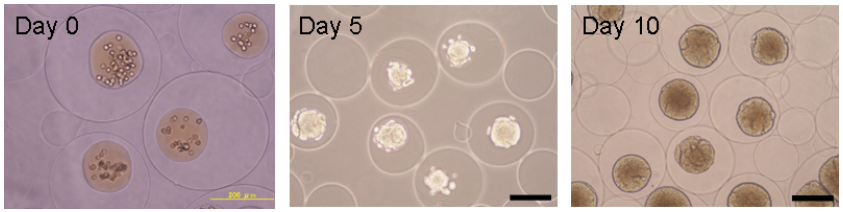


**Figure 4: Transition of morphological changes in CRFK cells encapsulated in hollow cores developed in Ca-alginate microcapsules. Bars: 200 μm**

immersion (Figure 6). Spherical tissues having about 150 μm in diameters were collected without breakup into small pieces. The collected spherical tissues transferred to a fresh cell culture dish were adhered to the surface within 2 hours of cultivation. Then, the cells contained in the tissues spread and grew. These results demonstrate the microcapsules suitable for spherical tissues production was developed from commonly-available gelatin and sodium-alginate via combining simple microparticles production procedures.

## CONCLUSIONS





We developed the microcapsule suitable for multicellular spheroids production from gelatin and sodium-alginate. The microcapsules contained spherical hollow cores templated by cell-enclosing gelatin microparticles of about 150 μm in diameter. The hollow core structure was achieved by a spontaneous gel-to-sol transition of gelatin gel at the temperature used for cell culture, 37℃. The enclosed cells grew and formed spherical tissues having the same size with the hollow cores. The spherical tissues were collected within 1 min by immersing the microcapsules in the medium containing alginate lyase. The cells in collected tissues adhered and proliferated on tissue culture dish. These results demonstrates that the efficiency of using the microcapsules for producing spherical tissues.

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