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Enzymatically fabricated and degradable microcapsules for production of multicellular spheroids with well-defined diameters of less than 150 μm

Shinji Sakai [a,*], Sho Ito [a], Yuko Ogushi [a], Ichiro Hashimoto [a], Natsuko Hosoda [b], Yoshinori Sawae [b], Koei Kawakami [a]

[a] Department of Chemical Engineering, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan
[b] Department of Intelligent Machinery and Systems, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

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ABSTRACT

Microcapsules with a single, spherical hollow core less than 150 μm in diameter were developed to obtain multicellular spheroids with well-defined sizes of less than 150 μm in diameter. An aqueous solution of phenolic hydroxyl derivative of carboxymethylcellulose (CMC-Ph) containing human hepatoma cell line (HepG2) cells and horse radish peroxidase (HRP) was injected into a coflowing stream of liquid paraffin, containing H_2O_2, resulting in cell-enclosing CMC-Ph microparticles, 135 μm in diameter, via a peroxidase-catalyzed crosslinking reaction. The CMC-Ph microparticles were then coated with a phenolic hydroxyl derivative of alginate (Alg-Ph) gel membrane several dozen micrometers in thickness, crosslinked via the same enzymatic reaction process, followed by further crosslinking between the carboxyl groups of alginate by Sr^{2+}. A hollow core structure was achieved by immersing the resultant microcapsules in a medium containing cellulase, which degrades the enclosed CMC-Ph microparticles. The HepG2 cells in the microcapsules then grew and completely filled the hollow core. Multicellular spheroids the same size as the CMC-Ph microparticles, with living cells at their outer surface, were collected within 1 min by soaking them in a medium containing alginate lyase to degrade the Alg-Ph gel microcapsule membrane.

1. Introduction

Three-dimensional, multicellular spheroids are increasingly recognized as valuable advanced tools for investigating cell behavior and intercellular interactions [1,2] and for evaluating the efficacy of therapeutic interventions [3,4] because they appear to mimic the morphology and physiology of the cells in living tissues and organs better than conventional, two-dimensional, monolayer cultures [5,6]. In particular, the usefulness of multicellular spheroids is gaining increased recognition in anticancer drug [3,4] and radiotherapy research [7,8]. More recently, multicellular spheroids, generated from embryonic stem cells, have become widely known as 'embryoid bodies' and play important roles in the field of regenerative medicine for obtaining a variety of differentiated cell types [9–12].

Various techniques have been developed for obtaining multicellular spheroids. One well-known technique employs cultivation on dishes with non-adherent surfaces [13]. A drawback of this technique is that the resultant spheroids usually show a heterogeneous size distribution. Uniformity of spheroid size is important for obtaining reproducible results and, in drug assays, it is required for proper statistical analysis. During embryoid body production, the proliferation and differentiation potential of cells are strongly correlated with size [14,15]. Hanging-drop culture and culture using microfabricated microwells with low-adherent surfaces are both effective for obtaining homogeneous spheroids but large-scale production is difficult. Techniques using spinner flasks and roller bottles are useful for large-scale spheroids production [16,17] and these systems are also attractive from the view point of oxygenation and nutrition, compared with stationary culture systems [3,18]. However, one concern when using such techniques is that the spheroids can be exposed to rather strong shear forces, which may affect cell physiology [18].

In the present study, we aimed to develop a technique for producing multicellular spheroids with a well-defined size of about 150 μm in diameter. The maximum allowable size for spheroids to avoid limitation of the oxygen supply, which results in the formation of necrotic regions inside the tissues, is reported to be about 200 μm in diameter [18,19]. Our methodology involves the use of hollow core microcapsules as templates for spheroids and

* Corresponding author. Tel./fax: +81 92 802 2768.
E-mail address: sakai@chem-eng.kyushu-u.ac.jp (S. Sakai).

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subsequent collection of the generated tissues by degradation of the microcapsule membranes. Due to the existence of the microcapsule membrane, the enclosed cells are expected to be protected from shear forces if they are cultured using rotating vessels during mass production. Mammalian cell-enclosing microcapsules have been studied for more than 40 years as research tools, devices for the production of useful biomolecules and as therapeutic devices for cell therapies [20–24]. A common feature of these previous studies is that there was no necessity to extract the enclosed cells from the microcapsules.

We recently developed a technique for producing microcapsules with hollow cores of about 100 μm in diameter and agarose membranes of about 20 μm in thickness [25]. Both mature and embryonic stem cells grew in the hollow cores and formed spheroids with a size almost identical to that of the hollow cores templated by cell-enclosing alginate microparticles, prepared from the alginate possessing phenol (Alg-Ph) via a peroxidase-catalyzed reaction consuming H_2O_2 [26]. One drawback of the microcapsule was that the agarose gel membrane was not degradable under the mild conditions required for mammalian cells, even using agarase. In the present study (using the methodology shown in Fig. 1), we prepared cell-enclosing microparticles for use as hollow core templates from carboxymethylcellulose, derivatized with phenol (CMC-Ph). Subsequently, we coated the CMC-Ph microparticles with an Alg-Ph gel. We then created a hollow core structure by degrading the internal CMC-Ph particles using cellulase. After the formation of spherical tissues, we collected them by degrading the Alg-Ph gel membrane using alginate lyase. Cellulase and alginate lyase are not proteolytic enzymes and they act under mild pH and temperature conditions that are compatible with mammalian cells.

2. Materials and methods

2.1. Materials

Sodium alginate with a high content of guluronic acid and a molecular weight of 70,000 was purchased from Kimica (I-1G, Tokyo, Japan). Sodium carboxymethylcellulose with a viscosity of 400–800 cps at 2% aqueous solution at 25 °C, tyramine hydrochloride and alginate lyase from *Flavobacterium* sp. were purchased from Sigma (St Louis, MO, USA). Alg-Ph and CMC-Ph were synthesized from the sodium alginate and the sodium carboxymethylcellulose by previously reported methods, through conjugation with tyramine using aqueous-phase carbodiimide activation chemistry [27,28]. The Ph contents in Alg-Ph and CMC-Ph were 3 and 13 per 100 repeat units of uronic acid, respectively. Cellulase from *Trichoderma viride* (Onozuka RS, 2.0 units/mg) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Horse radish peroxidase (HRP) (170 units/mg) and soybean lecithin were obtained from Wako Chemicals (Osaka, Japan). Human hepatoma cell line, HepG2, cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum, 75 μg/l penicillin, and 50 μg/l streptomycin under a humidified atmosphere at 37 °C in 5% CO_2/95% air.

2.2. Mechanical properties

CMC-Ph and Alg-Ph were dissolved in calcium-free Krebs Ringer Hepes buffer solution (CF-KRH, pH7.0) at 1.0% (w/v). To these solutions, HRP was dissolved at 1.0 unit/ml. These solutions were placed in a dialysis tube (MWCO 10,000, 10 mm in diameter) and submerged in CF-KRH containing H_2O_2 at 15 mM for 24 h at 25 °C to obtain cylindrical gels. The resultant cylindrical gels were cut into 10 mm lengths. For treatment with Ca^{2+} and Sr^{2+}, the cylindrical gels were submerged for 6 h in 25 mM $CaCl_2$ or $SrCl_2$ solution buffered with 10 mM Hepes (pH 7.4). The resultant cylindrical gels were positioned on a flat glass plate. Compression-resistance stress profiles for the specimens were measured using a Table-Top Materials Tester (EZ-Test-500N, Shimadzu, Kyoto, Japan) at a compression rate of 10 mm/min.

2.3. Encapsulation of cells in Alg-Ph microcapsules

CMC-Ph microparticles of about 150 μm diameter, with a narrow size distribution, were prepared as templates for the hollow core microcapsules, based on a previously described method [29]. Briefly, KRH containing 2.5% (w/v) CMC-Ph, 10 units/ml of HRP and 3.0×10^7 cells/ml of HepG2 cells was extruded from a 26-gauge needle at 0.1 ml/min into a coflowing immiscible stream of liquid paraffin, containing H_2O_2 and lecithin (3.0% (w/w)) and flowing at 2.7 ml/min. A coaxial droplet generator, designed in our laboratory, was used for this process [30]. The CMC-Ph microparticles, suspended in the liquid paraffin, became partially gelled in the flow and were collected in a 50 ml plastic tube. After 9 min of standing to allow further enzymatic gelation, KRH was added to the tube, followed by centrifugation at 1000 rpm for 1 min to collect the microparticles. After several rinses with CF-KRH, the CMC-Ph microparticles were suspended in 2.0% (w/v) Alg-Ph in CF-KRH containing 10 units/ml of HRP. The content of the CMC-Ph particles was 0.1 ml of CMC-Ph gel per ml of Alg-Ph solution. The suspension was extruded from a 26-gauge needle at 0.08 ml/min into a coflowing stream of the liquid paraffin, containing lecithin and H_2O_2 and flowing at 5.5 ml/min. The resultant emulsion of partially gelled Alg-Ph droplets containing CMC-Ph microparticles was collected in a 50 ml plastic tube. After 9 min of standing to allow further enzymatic gelation, KRH was added to the tube and then centrifuged at 1000 rpm for 1 min. The resultant Alg-Ph particles were soaked in 25 mM $SrCl_2$ solution for 1 min to create crosslinks between the carboxyl groups of Alg-Ph. Alg-Ph particles containing CMC-Ph microparticles with a diameter of 200 μm were collected by filtering through a nylon mesh filter with 150 μm pores. After several rinses with medium, the collected Alg-Ph particles containing CMC-Ph microparticles were incubated in a medium containing cellulase at 1.0 mg/ml to liquefy the enclosed CMC-Ph microparticles by enzymatic degradation. After 3 h of immersion in the cellulase medium, the medium was exchanged with a medium containing cellulase at 0.1 mg/ml for 12 h. The medium was then exchanged to fresh medium containing no cellulase.

Viability of the cells enclosed in CMC-Ph microparticles and Alg-Ph microcapsules were determined by trypan blue exclusion, using a hemocytometer after degrading these vehicles using cellulase and alginate lyase, respectively.

2.4. Culture of enclosed cells

The growth profile of the enclosed cells was estimated by the increase in the amount of a water-soluble formazan dye, which was derived from a tetrazolium salt dissolved in medium containing suspended cell-enclosing microcapsules, using a colorimetric assay kit (Cell Counting Kit-8, Dojindo, Kumamoto, Japan). Conversion to the water-soluble formazan dye indicates the presence of dehydrogenase within intact mitochondria in viable cells and therefore reveals the physiological state of the enclosed cells. Briefly, 400 μl of aliquots of medium containing suspended cell-enclosing microcapsules were poured into the wells of a 24-well cell culture dish and 40 μl of the reagent from the assay kit was added to each well. After 4 h of incubation at 37 °C, the absorbance at 450 nm was measured using a spectrophotometer. Distribution of living cells in the spheroids was determined from a cross-section of the cell-enclosing microcapsules stained with hematoxylin and eosin after 27 days of encapsulation. To obtain the cross-section, microcapsules were embedded in gelatin gel by suspending them in a 10% gelatin aqueous solution followed by cooling for gelation. The gelatin gel was fixed using neutral buffered formalin, decalcified, embedded in paraffin and sectioned.

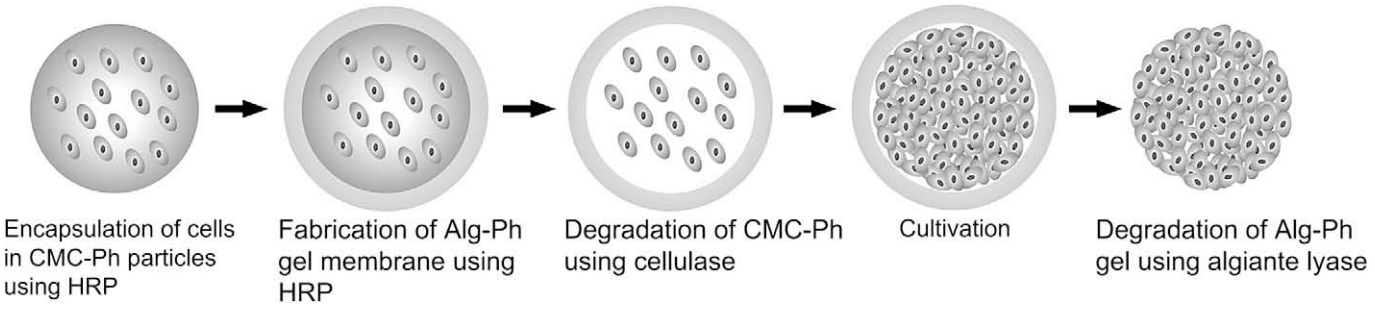

Fig. 1. Schematic illustration of the production of spherical tissues via enzymatic crosslinking and degradation reactions.