Development of microcapsules as a tool for producing multicellular spheroids

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INTRODUCTION

Three-dimensional, multicellular spheroids are increasingly recognized as valuable advanced tools for investigating cell behavior and intercellular interactions and for evaluating the efficacy of therapeutic interventions because they appear to mimic the morphology and physiology of cells in living tissues and organs better than conventional, two-dimensional, monolayer cultures (Yamauchi N, 2003). In particular, their usefulness is attracting attention in anticancer drug and radiotherapy research. 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 (Bibel M, 2004).

Various techniques have been developed for obtaining multicellular spheroids. One well-known technique employs cultivation on dishes with non-adherent surfaces. 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 in drug assays, and it is required for proper statistical analysis. During embryoid body production, the proliferation and differentiation potential of cells are strongly correlated with size (Valamehr B, 2008). 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.

In this contribution, we report 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 (Lin RZ, 2008). Our methodology involves the use of hollow core microcapsules as templates for spheroids and subsequent collection of the generated tissues by degradation of the microcapsule membranes. We developed the hollow core by degrading cell-enclosing microparticles of about 150 μm in diameter via enzymatic reaction. The microparticles were prepared based on the method reported previously via jetting of cell-suspending polymer solution in water-immiscible coflowing fluid and peroxidase-catalyzed reaction (Sakai S, 2007). 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. In this presentation the microcapsules are shown as an efficient tool to produce multicellular spheroids.

MATERIALS AND METHODS

Alginate and carboxymethylcellulose derivatives having phenol moieties (Alg-Ph and CMC-Ph) were synthesized from sodium alginate and sodium carboxymethylcellulose through conjugation with tyramine using aqueous-phase carbodiimide activation chemistry. The Ph contents in Alg-Ph and CMC-Ph were 3 and 13 per 100 repeat units of uronic acid, respectively. For obtaining cell-enclosing CMC-Ph microparticles of about 150 μm in diameter, HepG2 cells were suspended in 2.5% (w/v) CMC-Ph in

Krebs Ringer Hepes buffer solution (KRH, pH7.4) containing horse radish peroxidase (HRP, 10 units/ml) at $3.0{\times}10^7$ cells/ml. The suspension was extruded from a 26-gauge needle into a coflowing immiscible stream of liquid paraffin containing H_2O_2 at 0.82 mmol/ml and lecithin at 3.0% (w/w). 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.

RESULTS AND DISCUSSION

We first enclosed cells in CMC-Ph microparticles 135 ± 17 μm in diameter by extruding a cell-containing CMC-Ph solution at 0.1 ml/min into a coflowing liquid paraffin stream. This resulted in about 4.5×10^6 microparticles/h, containing about 40 cells/microparticles. The viability of the HepG2 cells enclosed in CMC-Ph microparticles was 95.2%. After subsequent Alg-Ph gel membrane formation via a peroxidase-catalyzed reaction, followed by degradation of the inner CMC-Ph gels, the viability of the cells remained high but slightly decreased to 90.2%. This slight reduction in viability may be explained by an insufficient oxygen supply during the enzymatic gelation process in water-immiscible liquid paraffin and potential cytotoxicity of H_2O_2. The Alg-Ph microcapsules were 229 ± 14 μm in diameter. The production rate of microcapsules containing CMC-Ph microparticles was about $3.5{\times}10^5$ microcapsules/h. In the well-known hanging-drop culture method or culture using microfabricated microwells, both of which produce well-defined multicellular spheroid sizes, the large number of substrate surface spotting or plate wells required to match this production rate are not feasible. The use of Alg-Ph microcapsules therefore has a higher potential for the large-scale production of multicellular spheroids compared with these methods.

Figure 1 shows the transition of morphological changes of enclosed cells in the Alg-Ph microcapsules with hollow core and with solid core. The cells existed individually just after encapsulation. With several days of culture, the cells self-aggregated and formed several aggregates in the cores. The cells further grew in the cores and almost completely filled the hollow core at day 5. In contrast, the cells in the microcapsules with solid core, CMC-Ph microparticles core, showed no such aggregation. The faster growth of the cells in the Alg-Ph microcapsules with hollow core was also shown by the transition of mitochondrial activities per microcapsules (Figure 2). Even after complete filling of the hollow core by cells at day 5, the mitochondrial activity per microcapsule increased with increasing culture period, reaching a 2.7-fold higher value by day 13 over that at day 5. Then, the value achieved steady during the following period to day 27. The increase of mitochondrial activity from day 5 to day 13 is explained by further cell growth resulting in a more dense structure. The plateau of mitochondrial activity per microcapsule from day 13 to day 27 shows the microcapsule membrane suppressed growth of the

Figure 1: Transition of morphological changes in HepG2 cells encapsulated in Alg-Ph microcapsules

Figure 2: Transition of mitochondrial activities of enclosed cells in microcapsules. Bars: S.D. ($n = 3$)

enclosed cells. Breakage of microcapsules resulting from the growth of enclosed cells was not observed during the 27 days of culture. This result means the Alg-Ph microcapsule membrane had sufficient mechanical strength to resist the increased stress resulting from the growth of cells.

Our reason for setting the size of the resultant spheroids as less than 150 μm in diameter was to minimize the formation of central necrotic region resulting from insufficient oxygenation and nutrition. Formation of necrotic region in the central part of spheroids, typically surrounded by a rim of viable cells about 100-300 μm thick, has been reported for spheroids beyond the maximum critical size for sufficient oxygenation and nutrition (Friedrich J, 2007). A cross-section of the cell-containing Alg-Ph microcapsules having a diameter of about 200 μm in diameter at day 27 was stained by hematoxylin and eosin. It showed the existence of living cells in the central part of the microcapsules (Figure 3). This result means that setting a hollow core size less than 150 μm in diameter was effective for obtaining spheroids without a necrotic region in the center.

We degraded the Alg-Ph gel microcapsule membrane using alginate lyase after 27 days of cultivation. The Alg-Ph microcapsule membrane completely disappeared within 1 min of soaking in the medium containing alginate lyase at 0.2 mg/ml. After the degradation, the tissues formed in the microcapsules

Figure 3: Cross-section of HepG2 cell-enclosing microcapsule after 27 days of encapsulation

Figure 4: Micrographs of spheroids just after and 24 hours after degrading microcapsule membrane

maintained a spherical shape without breakup into small pieces (Figure 4). No breakup of the spheroids was observed even after pipetting and centrifugation when replacing the alginate lyase medium with fresh medium or when transferring to a fresh cell culture dish. This indicates that there are robust connections between the cells in the spheroids and that degradation of the CMC-Ph microparticles using cellulase was successful for obtaining a hollow core structure. The diameter of the collected spheroids was 139 ± 14 μm and there was no significant difference between the size of the CMC-Ph microparticles used as a template for the hollow core and the size of the final spheroids ($p = 0.20$). This result demonstrates we can obtain multicellular spheroids with a well-defined size by controlling the size of the cell-containing CMC-Ph microparticles.

CONCLUSIONS

We have developed microcapsules for producing multicellular spheroids that are small enough to avoid the formation of a necrotic region at their centers from Alg-Ph and CMC-Ph via peroxidase-catalyzed crosslinking reaction and cellulase catalyzed degradation reaction. Enclosed cells in the resultant Alg-Ph microcapsules showed 90.1% viability and grew in the hollow core until formation of multicellular spheroids having the same size as the hollow core. The spheroids were easily collected from the microcapsules by degrading the Alg-Ph gel membrane using alginate lyase within 1 min and living cells existed at the center of the spheroids. From these results, we conclude that the Alg-Ph microcapsule is an effective tool for producing multicellular spheroids with well-defined sizes for tissue engineering and therapeutic applications.

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