Video Article

Alginate Encapsulation of Pluripotent Stem Cells Using a Co-axial Nozzle

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Abstract

Pluripotent stem cells (PS cells) are the focus of intense research due to their role in regenerative medicine and drug screening. However, the development of a mass culture system would be required for using PS cells in these applications. Suspension culture is one promising culture method for the mass production of PS cells, although some issues such as controlling aggregation and limiting shear stress from the culture medium are still unsolved. In order to solve these problems, we developed a method of calcium alginate (Alg-Ca) encapsulation using a co-axial nozzle. This method can control the size of the capsules easily by co-flowing N_2 gas. The controllable capsule diameter must be larger than 500 µm because too high a flow rate of N_2 gas causes the breakdown of droplets and thus heterogeneous-sized capsules. Moreover, a low concentration of Alg-Na and $CaCl_2$ causes non-spherical capsules. Although an Alg-Ca capsule without a coating of Alg-PLL easily dissolves enabling the collection of cells, they can also potentially leak out from capsules lacking an Alg-PLL coating. Indeed, an alginate-PLL coating can prevent cellular leakage but is also hard to break. This technology can be used to research the stem cell niche as well as the mass production of PS cells because encapsulation can modify the micro-environment surrounding cells including the extracellular matrix and the concentration of secreted factors.

Video Link

The video component of this article can be found at https://www.jove.com/video/52835/

Introduction

Induced pluripotent stem cells (iPS cells) are currently the source of intense research due to their role in regenerative medicine. However, huge amounts of cells are required for tissue regeneration. For instance approximately one billion pancreatic cells required for a type 1 diabetic patient[1]. However, conventional dish culture is only able to obtain 1 × 10^5 cells/cm^2, thus requiring 1 m^2 of culture area to obtain enough stem cell-derived pancreatic cells to treat a type 1 diabetic patient. The development of a system for the mass-culture of pluripotent stem cells, such as microcarrier[2] and suspension culture is therefore required for regenerative medicine. Suspension culture represents a promising method of mass culture but controlling the aggregation of cells is challenging in direct suspension cultures of human iPS cells[3]. Indeed, suspended cells are exposed to shear stress, which causes cell damage[3] or differentiation[4].

Research into hydrogel-based encapsulation has been conducted to solve problems associated with suspension culture. In hydrogel capsules, cells are protected from the flow of the medium. Previous reports have documented the use of various types of hydrogel, including agarose[5], PEG[6], and alginate (Alg), for cellular encapsulation. Alg-Ca hydrogel is one of the most useful hydrogels for cell encapsulation because Alg −Ca hydrogel is formed immediately after dropping alginate solution into a $CaCl_2$ solution and is also readily digested by enzymes or chelating reagents.

Here, we have established a stable alginate encapsulation process for iPS cells using a co-axial nozzle. By using N_2 gas flow for forming droplets, it is possible to encapsulate cells into uniform capsules without the need for other reagents such as oil. In this method, the flow rate of N_2 and concentration of both $CaCl_2$ and alginate are the major operating conditions affecting the size, shape, and uniformity of capsules. This report demonstrates the optimization of these operating conditions through the use of a hi-speed camera and a microscope.

Protocol

1. Preparing Materials

1. Prepare 10 mM HEPES buffer. Adjust the pH to 7.0 at RT and add NaCl to 0.9%.
2. Prepare 5% alginate solution and 10 mM EDTA solution by mixing HEPES-buffered saline prepared in 1.1. Adjust the pH to 7.0 at RT.
3. Autoclave the reagents (1.1, 1.2) for 20 min at 121 °C.
4. Prepare gelatin-coated 60 mm dishes layered with 1.2 − 2.0 × 10^6 mouse embryonic fibroblast (MEF) cells, which are treated as feeder cells by incubation within DMEM containing 10% ES-qualified FBS and 10 µg/ml of mitomycin C for 90 - 120 min.

5. Maintain mouse induced pluripotent stem (iPS) cells on the dish with the feeder cells in the presence of 5 ml DMEM high glucose containing 20% of ES-qualified FBS, 50 µM of 2-mercaptoethanol, non-essential amino acid, and antibiotics (100 Units/ml of Penicillin G Sodium, 100 µg/ml of streptomycin sulfate, and 250 ng/ml of amphotericin B).
6. Seed 4 × 10^5 iPS cells on a gelatin-coated 60 mm dish and incubate them at 37 °C and 5% CO_2. Change the culture medium every day during incubation. After 3 – 4 days of culture, trypsinize cells for 1 min with 500 µl of 0.05% Trypsin containing 0.02% EDTA at 37 °C and 5% CO_2. After that, detach cells by tapping the culture dish ten times.
7. Add 2.5 ml of DMEM with 10% of ES-qualified FBS and antibiotics (following dissociation mouse iPS cells into single cells by pipetting with 1,000 µl micropipette three times) centrifuge cell suspension at 160 × g for 3 min and remove supernatant and count the cells. Following cell counting, reseed the collected cells on a gelatin-coated dish and incubate for 30 min to isolate the iPS cells from the MEF cells. After counting of the cells (usually 1 – 3 × 10^6 cells/dish can be collected), reseed 4 × 10^5 iPS cells on the dish.

2. Cell Encapsulation into Alginate Hydrogel Capsules

1. Collect the cells by the same procedure described in 1.5 and 1.6. Following centrifugation at 160 × g for 3 min, wash the iPS cell pellet with HEPES-buffered saline thrice. After re-suspending the cells in HEPES-buffered saline, filter the cell suspension through a 40 µm cell strainer in order to remove large aggregates, which could otherwise clog the nozzle.
2. Mix 2 ml of cell suspension within HEPES-buffered saline and 3 ml of 5% Alg-Na solution. Finally 5 ml of cell suspension within 3% Alg-Na solution is obtained. Cell density is desirably more than 10^6 cells/ml.
3. After collecting the cell suspension into a 5 ml syringe, install a co-axial nozzle (**Figure 1A, B**) on the syringe and set them on syringe pump. Emit N_2 gas flow (lower than 1L/min) through the outer needle of the co-axial nozzle and expel the cell suspension through the inner nozzle.
4. Collect droplets into 250 ml of 0.5% $CaCl_2$ solution and wait 10 – 20 min for gelation while stirring at 60-90 rpm.

3. Treatment of Alginate Capsules (optional)

1. Incubate Alg-Ca capsules in 0.05 % (w/v) poly-l-lysine (PLL) solution for 5 min at 37 °C and 5% CO_2.
2. After washing the capsules with HEPES-buffered saline, incubate them in DMEM containing 10% FBS in order to neutralize the electrical charge on their surface. Utilize them as coated capsules (**Figure 1C**).
3. Incubate the capsules in HEPES-buffered saline containing 10 mM EDTA for 5 min. The EDTA-treated capsules are utilized as hollow capsules (**Figure 1C**).

4. Culture and Collect Cells in Alginate Capsules

1. Incubate the encapsulated cells at 75 rpm with shaking at 37 °C and 5% CO_2 for 10 days.
2. Collect and incubate the capsules in 10 mM EDTA at 37 °C and 5% CO_2 for 10 min. Collect cells from the alginate capsules without PLL treatment by centrifugation at 1,000 × g for 3 min.
3. If alginate capsules are treated with PLL, break the Alg-PLL membrane by pipetting up and down around ten times with a needle attached to a syringe and centrifuge it at 1,000 - 5,000 × g for 5 min. Needle diameter should be lower than capsule size and 25 G (260 µm) needles are used in this experiment (600 µm)
4. After centrifugation, collect cell pellet strictly from broken Alg-PLL membranes if you want to collect mRNA samples from cells. Remaining Alg-PLL membranes possibly prevent mRNA purification.

Representative Results

At protocol 2.5, the expelled alginate solution forms a spherical shape immediately after expulsion (**Figure 2A – H**). If the suspension is expelled with a N_2 flow rate lower than 1L/min, the size of the droplets is uniform (**Figure 2I**). However, if the N_2 flow is higher than 1L/min, the droplet breaks down (**Figure 2G**, white arrowed) and the size of the droplets becomes heterogeneous (**Figure 2J**). Given this, it is difficult to prepare droplets smaller than 500 µm using this method.

In gelling process (protocol 2.4), Alg-Na droplets formed spherical shape Alg-Ca capsules in $CaCl_2$ solution if concentration of Alg-Na (more than 3%) is enough (**Figure 3E-H,K,I**). However low concentration of Alg-Na (lower than 3%) causes non-spherical and non-smooth shape of capsules (**Figure 3A-D, I**), which have a problem in coating process. Even if Alg-Na concentration is not enough, spherical capsules can be formed by increasing $CaCl_2$ concentration (**Figure 3J**). However, around 50 mM of $CaCl_2$ is appropriate for encapsulation because too high concentration of $CaCl_2$ decreases cellular growth and viability (Data not shown). After dropping into $CaCl_2$ solution, optionally you can wait for a few minutes for gelling but we recommend picking up capsules from $CaCl_2$ solution because too long incubation in $CaCl_2$ sometimes affects cellular growth. Sometimes you obtain non-spherical shaped capsules even if the concentrations are enough. This is probably because washing cellular pellet is not enough before resuspending cells in Alg-Na solution. Residual reagents such as serum possibly prevent gelling.

Encapsulated cells can grow in the capsules but cellular leakage (arrowed in **Figure 4A**) can occur in capsules without Alg-PLL coating (**Figure 4A**). In the case of mouse iPS cells, the cells form disk-shaped aggregates in each Alg-Ca capsule (**Figure 4B**), whereas the cells clump together and form single spherical aggregates in each hollow capsule (**Figure 4C**). Initial cell density does not affect the size of aggregates in capsules but low cell density (10^5 cells/ml-gel) causes the failure to grow up (data not shown). Thus, 10^6 cells/ml-gel is desirable to encapsulate.

In collecting cells from capsules, Alg-Ca hydrogel can be dissolved with alginate lyase or chelating reagents, although the Alg-PLL coating is difficult to dissolve with enzymes or chemicals. Alg-PLL should therefore be physically broken with pipetting, for example. Broken membranes can be dissociated from the cells by centrifugation.

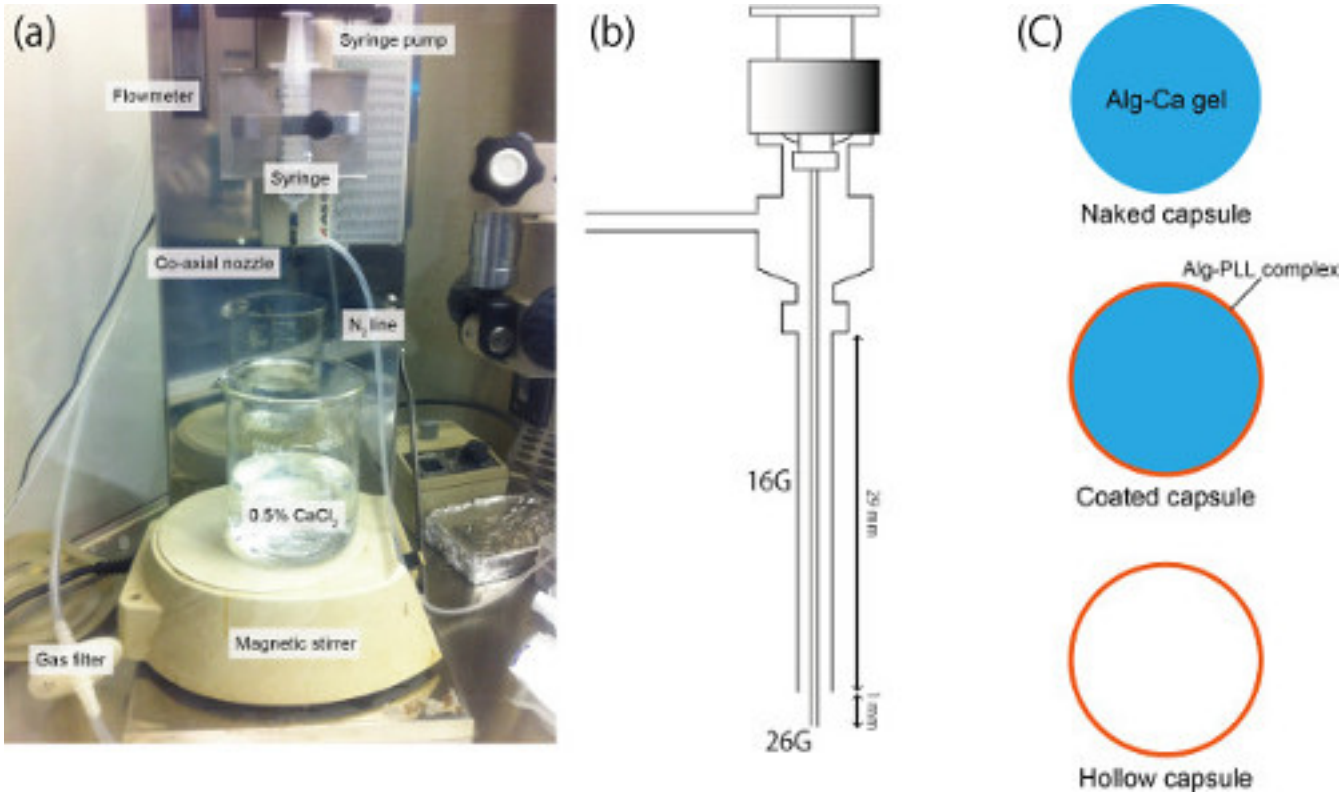

Figure 1. Images of Encapsulation System and Capsules. (**A**) An image of the encapsulation system for Alg-Ca encapsulation on a clean bench. (**B**) Schematic image of a co-axial nozzle for encapsulation. (**C**) The three different types of capsules obtained in this report. Please click here to view a larger version of this figure.

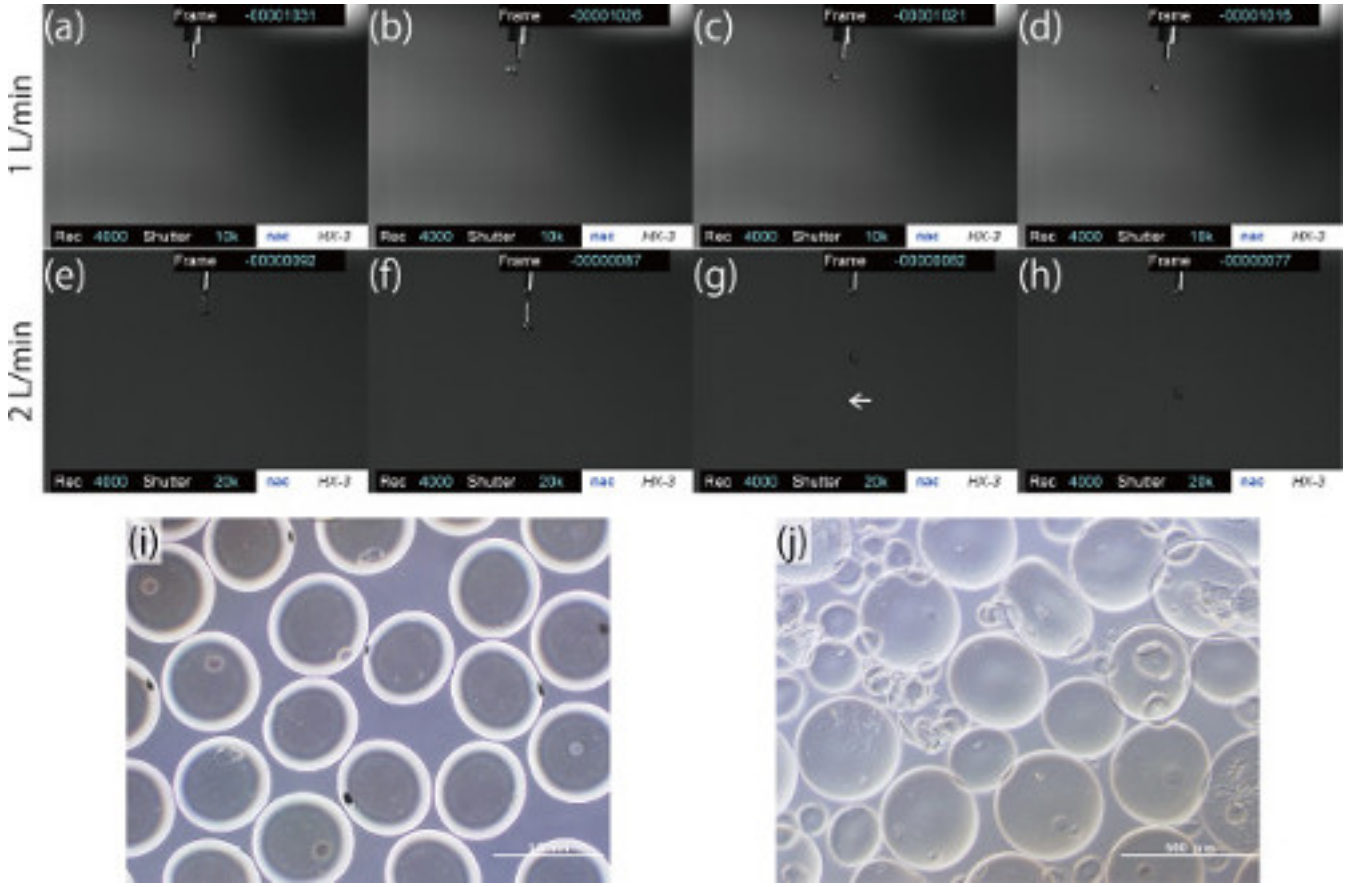

Figure 2. Effect of N_2 Flow on the Morphology of the Capsules. Continuous images (**A – H**) and appearance (**I, J**) of droplet formation of 3% Alg-Na solution from a co-axial nozzle in N_2 flow at 1 L/min (**A - D, I**) and 2 L/min (**E - H, J**). Continuous images were captured by hi-speed camera. Hydrogel capsules are formed in 0.5% $CaCl_2$. Please click here to view a larger version of this figure.

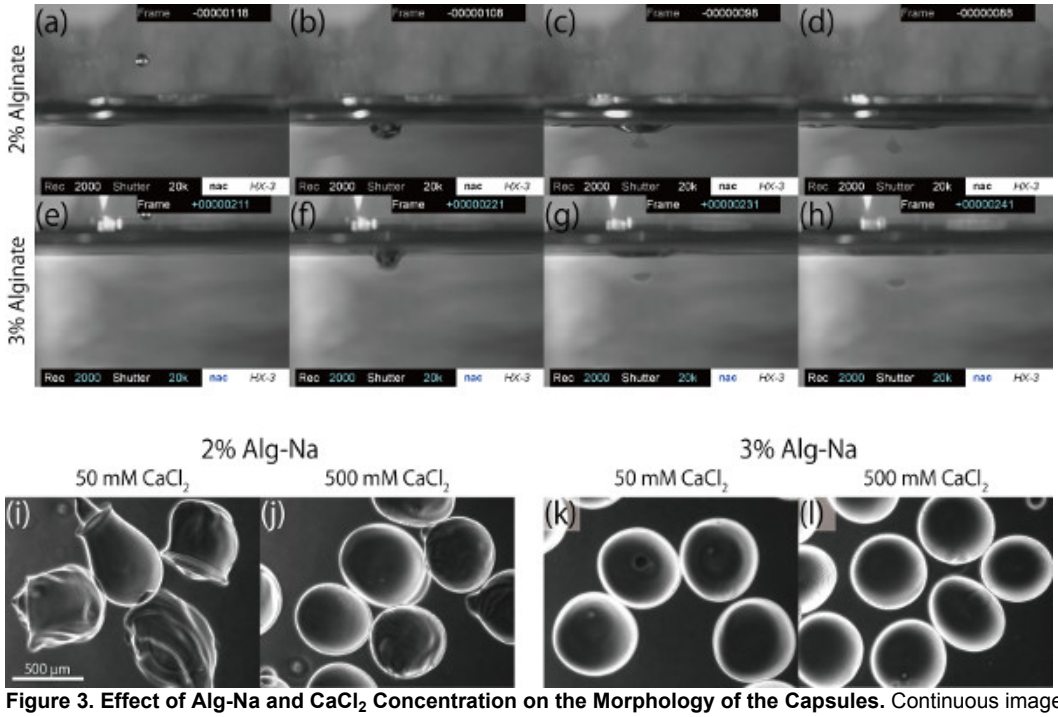

Figure 3. Effect of Alg-Na and $CaCl_2$ Concentration on the Morphology of the Capsules. Continuous images (**A – H**) and appearance (**I - L**) of Alg-Na droplets gelling in $CaCl_2$ solution. In continuous images, droplets having different concentration of Alg-Na, 2% (**A - D**) and 3% (**E - H**), are gelling in 50 mM $CaCl_2$ solution. Continuous images are captured by hi-speed camera. Appearance images show the morphological difference of Alg-Ca capsules formed by different concentrations of Alg-Na, 2% (**L, J**) and 3% (**K, L**), and $CaCl_2$, 50 mM (**L, K**) and 500 mM (**J, L**). Please click here to view a larger version of this figure.

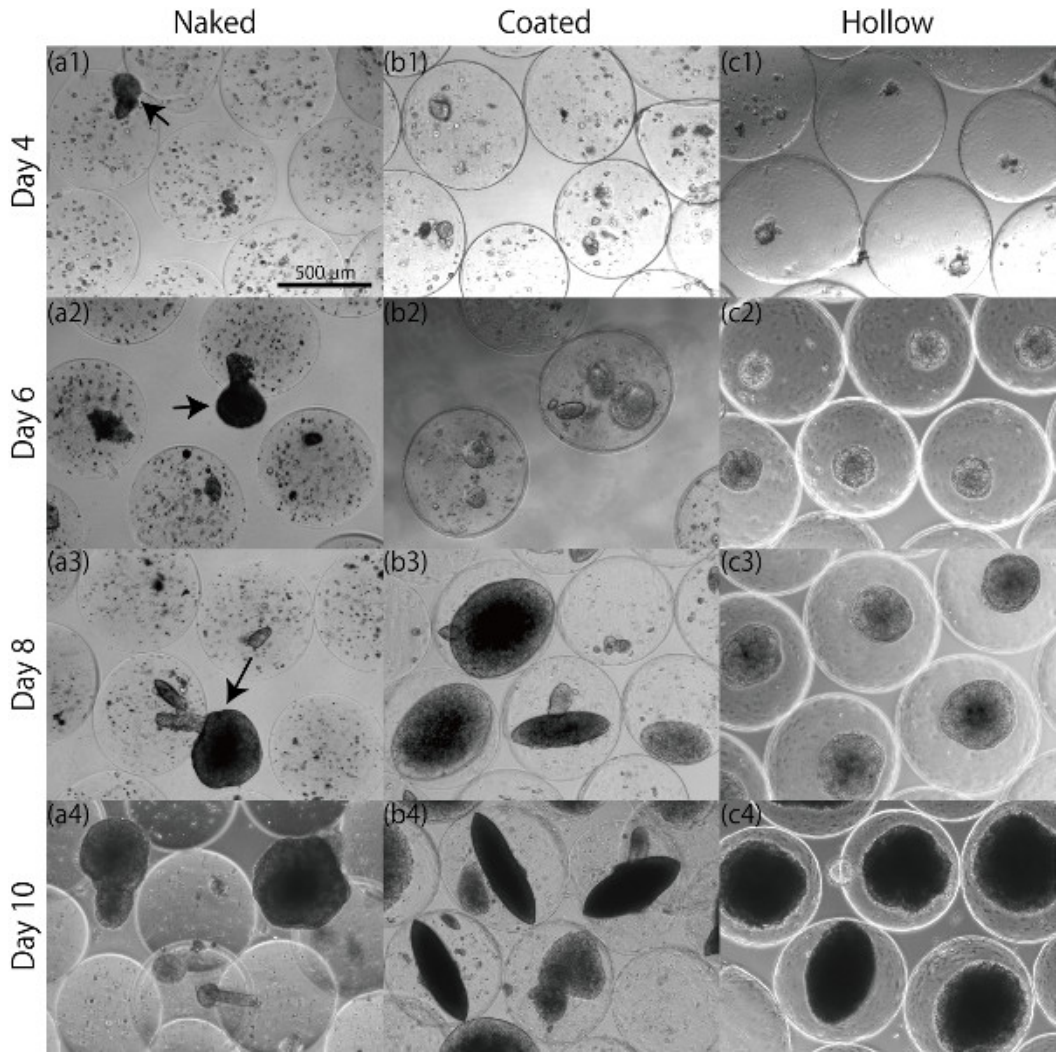

Figure 4. Morphology of Encapsulated Mouse iPS Cell Aggregates in the Capsules. Micrograph images of iPS cell-aggregates cultured for 4, 6, 8 and 10 days (1 - 4 respectively) in three different types of capsules: (**A**) naked, (**B**) coated, and (**C**) hollow capsules. Please click here to view a larger version of this figure.

Discussion

Encapsulation culture can be compared with direct suspension cultures. Suspension culture is a simpler method to obtain large quantities of pluripotent stem cells than encapsulation methods. However, controlling the aggregation of cells in suspension culture is still challenging. In encapsulation method, cellular aggregation is limited in capsules and can therefore be well controlled. A previous publication showed that encapsulated cells formed aggregates of uniform size, whereas large cell clumps appeared in a free-suspension culture[7]. Furthermore, an earlier report demonstrated that strong agitation causes cellular death in direct suspension cultures of PS cells; thus necessitating a limited agitation rate. In contrast, encapsulation is able to protect cells from shear stress even under suspension conditions. The encapsulation method therefore allows a level of flexibility in the operating conditions when growing cells in suspension.

Encapsulation is a useful tool for both stem cell research as well as modification of the mass production process. The encapsulation of stem cells is useful in researching the stem cell niche, including the extracellular matrix and growth factors. Some researchers have demonstrated that the encapsulation of mouse ES cells into VEGF-conjugated agarose hydrogels promoted the differentiation of cells into blood progenitor cells[8].

This article shows how to encapsulate mouse iPS cells into size-controlled Alg-Ca capsules easily by using co-axial nozzle and N_2 flow. Although there are some encapsulation process with various tools such as peristaltic pump[9] and electrical voltage[10], the encapsulation by using N_2 flow is a simpler and safer method than other methods. Furthermore, this method is able to form smaller (approximately larger than 600 µm) capsules than the method with peristaltic pump (approximately larger than 3 mm). Large capsules take longer time for gelling completely and long time incubation in $CaCl_2$ possibly affects cellular characteristics. Furthermore, large capsules also possibly have a problem of mass transfer of nutrient or waste such as glucose and oxygen. Therefore the size of capsules formed by N_2 flow (600 -1,000 µm) is ideal size to cell culture inside.

This method is not suitable to prepare small capsules and control the size precisely. Because of the breakdown of the droplets, this method is not suitable to prepare capsules smaller than 500 µm. Furthermore, only visual observation can determine the size of capsules, thus it is difficult to

control the size of capsules by this method. In this case, use of microfluidic devices possibly can solve these problems[11]. However, sometimes clogging occurs in microdevice because Alg-Na gels immediately after meeting $CaCl_2$ solution.

Although alginate is not suitable for chemical modification because of its high viscosity, it is able to form hybrid capsules with hydrogel, which, like PEG, is also difficult to use for encapsulation. A previous publication showed that the encapsulation of mouse iPS cells in a RGD peptide-conjugated PEG-Alg hybrid hydrogel capsule improved cellular growth[12]. Without chemical modification, the encapsulation of cells into just hydrogel capsules would be expected to modify the stem cell niche by retaining secreted factors. Indeed, previous research demonstrated that the secreted growth factors, retained in a microbioreactor, were effective in controlling stem cell fate[13,14].

The collection of cells from capsules is still an unresolved issue regarding the application of the encapsulation method to the mass production of pluripotent stem cells. A previous report showed that that PLL coating is necessary to keep mouse iPS cells inside[7]. If Alg−Ca hydrogel capsules are well coated with a polycation such as PLL (protocol 3.1 – 3.2), it will be difficult to break down the capsules by either enzyme or chelating reagents. In this case, a mechanical method such as pipetting by the needle is required (protocol 4.3). The collected cells can be isolated from broken membranes at centrifugation greater than 5,000 × g, although centrifugation might possibly affect the cell viability due to the high G-force. Thus, a technique of gently removing debris from cells as well as breaking the capsules is important in developing encapsulation technology. Although some further development is required to apply this method to the mass production of cells, encapsulation is a promising technique for both mass production and stem cell research.

Disclosures

The authors have nothing to disclose.

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