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Scalable Production and Cryostorage of Organoids Using Core-Shell Decoupled Hydrogel Capsules

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Abstract

Organoids, organ-mimicking multicellular structures derived from pluripotent stem cells or organ progenitors, have recently emerged as an important system for both studies of stem cell biology and development of potential therapeutics; however, a large-scale culture of organoids and cryopreservation for whole organoids, a prerequisite for their industrial and clinical applications, has remained a challenge. Current organoid culture systems relying on embedding the stem or progenitor cells in bulk extracellular matrix (ECM) hydrogels (e.g., Matrigel™) have limited surface area for mass transfer and are not suitable for large-scale productions. Here, we demonstrate a capsule-based, scalable organoid production and cryopreservation platform. The capsules have a core-shell structure where the core consists of Matrigel™ that supports the growth of organoids, and the alginate shell form robust spherical capsules, enabling suspension culture in stirred bioreactors. Compared with conventional, bulk ECM hydrogels, the capsules, which could be produced continuously by a two-fluidic electrostatic co-spraying method, provided better mass

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transfer through both diffusion and convection. The core-shell structure of the capsules also leads to better cell recovery after cryopreservation of organoids probably through prevention of intracellular ice formation.

Graphical abstract

Keywords

organoid; encapsulation; biomanufacturing; cryopreservation

Organoids are stem cell-derived, lab-grown multicellular structures that closely mimic the physiology and hierarchical cell organizations in their counterpart tissues or organs *in vivo*.^[1–5] Recognized recently as a major science advance^[6], the organoid technology holds enormous potential for studying stem cell biology, modeling diseases, screening drugs and engineering cell therapies.^[1,7–12] Organoids corresponding to several organs have been developed, including gut^[13–16], lung^[17,18], liver^[19,20], retina^[21,22], brain^[7,23] and kidney^[24], to name a few. For example, the culture of intestinal organoids first established by Clevers and his colleagues in a landmark paper^[13] has initiated numerous follow-up investigations. Intestinal organoids not only became a prime model for studies on adult stem cells in health and disease but also represented a promising potential source for cell therapies for intestinal diseases such as microvillus inclusion disease, ulcerative colitis, and post-endoscopic mucosal resection.^[2] Eiraku *et al.*^[21] reported the autonomous formation of the optic cup (retinal primordium) structure from mouse embryonic stem cell aggregates, which opened up new avenues for the next-generation of regenerative medicine in retina degeneration therapeutics. Lancaster *et al.*^[7] showed that cerebral organoids recapitulated features of human cortical development and could be used to model microcephaly, a brain disorder that has been difficult to study in mice. Dekkers *et al.*^[25] used patient-derived intestinal organoids to model the cystic fibrosis. These studies and many others have clearly shown the great potential of organoids as *in vitro* human tissue/organ surrogates for various important applications.^[1,2]

In almost all cases, the 3D culture in extracellular matrices such as Matrigel™ played an essential role in the formation of organoids.^[7,8,13,14,16,22] Current 3D cultures are typically accomplished by embedding the cells in bulk Matrigel™ usually deposited as sessile drops on flat surfaces. While these culture methods enabled numerous insightful studies in laboratories, they were not suited for large-scale productions. This method of culture requires manual seeding and becomes extremely labor-intensive when performed at large scales; in addition, the macroscopic Matrigel™ drops or blocks have limited surface area for mass transfer and are not suitable for suspension culture (e.g., in stirred bioreactors). The scale-up of organoid culture that is critically important for high-throughput screening and

clinical applications has remained challenging.^[28–30] Another factor that limits the rate of organoid technology development and clinical translation is the long-term storage, such as cryopreservation of the organoids.^[31] The challenge for complex tissue preservation is the potential cryoinjury from ice crystal formation during the freezing and recovery processes. Usually, a cryoprotectant (e.g., dimethyl sulfoxide (DMSO)) in combination with a slow freezing process is applied to avoid intracellular ice formation, which is the major cause to damage the cryopreserved cells. However, the recovery process usually involves devitrification that can still induce ice crystal formation.^[31–33] The ice crystal might not only hurt the cells but also disrupt cell-cell interactions among these organized tissues. To prevent devitrification during the warming process, hydrogels have been used to protect the cryopreserved cells/tissues in a rapid recovery procedure to enhance the viability of cells in cryopreservation.^[34,35]

Here, we report an efficient and scalable organoid culture and cryostorage system using hydrogel capsules. The capsules have a core-shell structure where the core consists of Matrigel™ that supports the growth of organoids (i.e., “organogenesis” in capsules), and the shell is alginate that forms robust spherical capsules, enabling suspension culture. Compared with conventional culture in bulk Matrigel™, the capsules provide better mass transfer from both diffusion and convection in a stirred bioreactor and can be continuously produced by a two-fluidic electrostatic co-spraying.^[36] Using mouse stomach and small intestinal organoids as models, we demonstrated that core-shell capsules not only enable scalable, suspension culture of organoids, but also enhanced the stem cell expansion within the organoids. In addition, these core-shell capsules also allowed a higher recovery rate from cryopreservation of organoids, probably due to the protection from the mechanical damage by the hydrogel layers during freezing or thawing processes. This new, capsule-based organoid culture and cryo-storage method will likely facilitate current organoid studies and accelerate its translation into clinical or industrial applications.

The most common method to culture organoids is to use reconstituted basement membrane matrix (Matrigel™) as a scaffold to support the organoid growth and maintain the stemness.^[7,13,18,19,21,26,27] This method is easy to implement in a small scale, for example by simply dripping a droplet of pre-gelled Matrigel™ in a dish. (Figure 1a) However, this method is not suitable for large-scale productions. In contrast, the core-shell capsules, which can be made continuously by a two-fluidic electrostatic co-spraying technique, provide a more scalable and efficient organoid culture platform (Figure 1b). During the electrospraying, the alginate shell crosslinks instantaneously when the droplets hit the Ca²⁺ solution in the grounded collector and the pre-cooled Matrigel™ core, protected by the shell, gradually gels through a temperature increase. The capsules, due to their smaller sizes, have higher surface to volume ratios than the bulk hydrogels and therefore better mass transfer even when cultured in dishes. When cultured in stirred flasks or bioreactors, the alginate shell protects the softer Matrigel™ and the cells from the shear force and importantly the mass transfer is further enhanced through convection. For example, based on a simple diffusion assay, we found that the mass diffusion reaches equilibrium in hydrogel capsules in static culture within 2 hours, while the time required to reach equilibrium in a stirred flask was merely 20 minutes. (Figure S1a) In addition, using a human breast cancer cell line, MCF10DCIS.COM, as a model, we showed the growth of the cells in the core of each

hydrogel capsule (Figure S1b, S1c) in the stirred flask, confirming the potential of this platform for scalable cell culture and production.

To examine whether the core-shell capsule system is suitable for organoid culture, we first used gastrointestinal organoids as a model. The gastric fragments, isolated from *Lgr5^{tm1(cre/ERT2)Cle/J} (Lgr5^{EGFP-Ires-CreERT2})* knock-in mouse stomach, were dispersed in Matrigel™ and then dripped in a dish (i.e. bulk culture) or encapsulated in the alginate-shell capsules. The capsules had a nearly monodisperse diameter of $1900 \pm 25 \mu\text{m}$ with the Matrigel core size of $900 \pm 40 \mu\text{m}$ [36]. After 9 days of culture, the tissue fragments grew into gastric organoids in both bulk and core-shell capsules (Figure 2a). The stem cell markers within the organoids, CD44 and LGR5-eGFP [37], were measured by immunofluorescence (Figure 2b). Interestingly, the LGR5-eGFP expression seemed to be higher and the columnar differentiation marker (CK8) expression lower in hydrogel capsules than in the bulk Matrigel™ [37,38]. These biomarker expressions suggest that the hydrogel capsules might enhance the expansion of stem cells in the organoids, probably due to the mechanical confinement effect from the alginate shell. It was previously reported that the mechanical heterogeneity in core-shell capsules composed of softer ECM core and harder alginate shell enhanced the development of ovarian follicles [39,40].

We also tested the culture of another type of organoids, mouse small intestinal organoids (crypts), in core-shell capsules and compared that to the bulk culture (Figure 2c-g and Figure S2). In both methods, the small intestinal crypts isolated from mice formed organoids after a few days of culture (Figure 2c). The organoids had lumen structures with apoptotic inner cells as shown by the caspase-3 staining (Figure 2d). The intestinal stem cells in the organoids were identified by CD44 and LGR5-eGFP. Using a quantitative real-time PCR (polymerase chain reaction), we found that the organoids grown in the capsules had similar *Cd44* but significantly higher *Lgr5* expression than those grown in bulk (Figure 2e and 2f). This observation of enhanced stem cell expansion in capsules was consistent with our observed results from the gastric organoids. In a recent study, it was found that stiff matrix in the microenvironment effectively improved intestinal stem cell expansion, and softer matrix supported the intestinal differentiation and organoid formation [41]. In our core-shell capsules, the shell hydrogel layer may provide a mechanical confinement to enhance the stem cell expansion, while the Matrigel™ core, with unaltered composition and mechanical property, supports the organoid formation. This is consistent with earlier reports that core-shell hydrogel capsules might be used to simulate the microenvironment of early-stage embryos to enhance the pluripotency [42–45]. The expression of proliferation marker, Ki67, was also higher in capsules than in bulk (Figure 2g), suggesting again that the core-shell capsules were not only suitable for organoid culture but might also improve the efficiency of organoid expansion.

Next, to demonstrate the culture of organoids in core-shell capsules was scalable, we performed repeated encapsulation, expansion, dissociation and re-encapsulation. First, small intestinal crypts were encapsulated in the capsules; the crypts grew into organoids in 6 days. Then, we used alginase to dissolve the alginate hydrogel shell and EDTA to remove alginate residues and dissociate organoids. The dissociated organoids were broken into smaller fragments through physical pipetting, and the fragments were re-encapsulated in core-shell

capsules for the next round of culture (Figure 3a). After re-encapsulation and culture, these intestinal organoids maintained their stem cell population with expression of LGR5-eGFP and CD44 (Figure 3a). Furthermore, after 4 passages, the organoids contained not only stem cells with *Lgr5* expression but also normal intestinal niches, such as Paneth cells with Lysozyme expression and intestinal goblet cells with MUC2 expression based on the fluorescence *in situ* hybridization (FISH) and immunofluorescent staining (Figure 3b). To demonstrate the scalability and feasibility of suspension culture in bioreactors, we transferred the capsules from culture dishes to a stirred flask. (Figure 3c and 3d) The small intestinal organoids grew and expanded efficiently in the stirred bioreactor and after a 6-day culture, we obtained ~15,000 organoids from a 100-ml stirred flask. In principle, we may produce a much larger or potentially any number of organoids using larger reactors. In contrast, to produce the same large number of organoids using the conventional bulk Matrigel™ culture, it would require many plates or dishes and become laborious. It is also worth mentioning that when we compared the expansion efficiency (or the increase of organoid numbers after an expansion) between cultures in bulk Matrigel™ and in capsules, we found that the efficiency from capsules was on average 1.5 times higher than the bulk culture. (Figure 3e) (The efficiency could be even higher as expansion continues due to the higher stem cell population in organoids grown from capsules.)

Lastly, we investigated whether the core-shell capsules could also be used for the cryo-storage of the organoids. Tissue/organ preservation is a challenge for tissue engineering in clinical application, especially for complex tissues or organs which constitute different cell types in an organized structure.^[31–33] The most common methods for cryopreservation involve a slow freezing process and a penetrating cryoprotectant, such as DMSO to avoid intracellular ice formation, which is the major cause of cell death during vitrification. However, during warming process, the ice crystal formation might still be induced by devitrification.^[34,35,46] In current methods^[47–49], organoids are frozen in medium with high concentrations of serum and a cryoprotectant, and the cooling temperature is controlled at a rate of 1°C per minute. These steps can prevent ice formation inside the cells in the freezing process, but crystal formation from devitrification, which might disrupt the cell alignment within tissues or organs, cannot be avoided during the conventional cryopreservation procedure. Here, we combined core-shell hydrogel capsules and cryoprotectant together to protect organoids from both vitrification and devitrification.^[32,34,35,46] Compared with the bulk Matrigel™, the capsules had a much higher recovery of cryo-preserved organoids (Figure 4a-c and Figure S3a-c). In bulk, only small cell aggregates could grow into organoids after thawing and re-culturing in Matrigel™ (Figure 4a and Figure S3a); larger ones failed to survive (Figure 4b and Figure S3b). The recovery efficiency (i.e., the fraction of viable cells recovered after thawing) was only around 20%. In contrast, in capsules even whole organoids could survive and grow after recovery from the cryo-storage (Figure 4c and Figure S3c). The cell viability was improved to 80%. (Figure 4d and 4e), suggesting that the core-shell hydrogel structure might suppress the ice formation during cryopreservation. The recovered organoids showed the stem cell markers, LGR5-eGFP and CD44 after being cultured for a week after thawing. (Figure 4f and 4g) Although the alginate shell deformed due to the mechanical force generated during freezing or thawing process, the capsules clearly played a protective role in the cryo-storage of organoids.

In summary, we reported here a new, capsule-based platform for scalable production and cryo-storage of organoids. The key to this platform is the core-shell decoupled design of the capsules, which can be continuously produced through a two-fluidic electrospraying, where the Matrigel™ core creates a permissive environment for organoid growth while the alginate shell enables the formation of mechanically robust capsules. Compared with the conventional droplet or bulk Matrigel™ currently used for organoid culture, the new capsules provide a higher surface area for mass transfer, support enhanced expansion of the stem cell population within organoids and can be suspended in stirred bioreactors for large-scale production. The alginate shell, which could be easily dissolved to harvest the organoids, protects the organoids from shear force during suspension culture and potential mechanical damage during the cryopreservation process. Given the significance of organoid technology, our core-shell decoupled hydrogel capsules would be a useful platform for many laboratory studies and potential clinical or industrial applications.

Experimental Section

Encapsulation of cells and crypts in core-shell alginate capsules: Cell encapsulation was accomplished using two fluidic electro-co-spraying technique described previously.^[36] Briefly, the isolated cells or small intestinal crypts were dispersed in an ice-cold Matrigel™ solution, which was pumped (0.06 mL min^{-1}) into an high electric field ($\sim 5.8\text{ kV}$) as the core fluid, and 1.8% (w/v) sterile alginate (UPLVG FMC Biopolymer) dissolved in 0.9% (w/v) sodium chloride was pumped (0.3 mL min^{-1}) as the shell fluid. The two fluids were pumped simultaneously and sprayed into small core-shell droplets under the electric field; the droplets were crosslinked in a 100mM calcium chloride solution placed $\sim 1.8\text{ cm}$ under the nozzle. After encapsulation, the capsules were transferred to a culturing dish or stirred bioreactor in an incubator at 37°C with 5% CO_2 for culturing.

Gastrointestinal organoid isolation

The details of organoid isolation are described in the supporting information.

Quantification of organoid expansion

The expansion efficiency is defined by the equation

$$\text{Expansion Efficiency} = \frac{(\text{Final amount of fragments} - \text{initial amount of fragments})}{\text{initial amount of fragments}}$$

(1)

Statistical analysis

All quantification data are presented as mean $\pm$ SEM with at least three independent replicates, and the sample size “n” in each experiment is given in the legend of each figure. In statistical analysis, all the p values were calculated by one-way ANOVA using Prism 7. In

addition, all the figures were depicted from Prism 7. (N.S.: non-significant difference, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A Schematic comparison between a conventional organoid culture and the more scalable, capsule-based culture

(a) Conventional method using Matrigel™ drops placed in a dish. (b) A schematic of the capsule-based organoid culture and expansion process. The cells or cell aggregates (e.g. crypts) dispersed in Matrigel™ is first encapsulated in core-shell capsules through a two-fluidic electrospraying; the capsules may then be cultured in a dish or a stirred bioreactor. For expansion, the capsule shell (i.e. alginate) may be dissolved and the organoids dissociated and re-encapsulated.

Figure 2. Culture of Gastrointestinal organoids in bulk Matrigel™ and core-shell decoupled capsules

(a) Growth of mouse gastric organoids in bulk Matrigel™ and capsules during a 9-day culture period. (b) Whole mount staining for stem cell markers, CD44 and LGR5, and columnar differentiation marker, CK8 in bulk Matrigel™ and capsules. (c) Growth of mouse small intestinal organoids in bulk Matrigel™ and capsules during a 6-day culture period. (d) Immunofluorescent (IF) stainings from paraffin-embedded sections for apoptosis marker, caspase-3. (e, f) IF staining and qPCR analysis of stem cell markers (*Cd44* and *Lgr5*) (n=3 for bulk and capsules). (g) IF staining of the proliferation marker, Ki67 and quantitative analysis based on the staining (n=3 for both bulk and capsules).

Figure 3. Expansion of mouse small intestinal organoids within hydrogel capsules and suspension culture in a stirred flask

(a) The process of organoid expansion in hydrogel capsules including encapsulation, culturing, dissociation and re-encapsulation. Whole mount staining for LGR5-eGFP and CD44 in capsules confirms the presence of stem cells. The whole process may be repeated multiple times. (b) The stem cell biomarker, *Lgr5*, and two intestinal cell markers, Lysozyme and MUC2 identified by fluorescence *in situ* hybridization (FISH) and immunofluorescent staining, confirming that the key features of stem cell such as self-renewal and multi-differentiation capabilities remained stable in hydrogel capsules after 4 times of passaging. (c) Capsules containing growing crypts being transferred from a dish to a stirred flask for suspension culture. (d) Growing organoids in capsules in a stirred flask. (e) A quantitative comparison of expansion efficiency between cultures in bulk and in hydrogel capsules (n=4 for each group). The expansion efficiency is defined as the fold-increase over the initial number of organoids.

Figure 4. Improved cryostorage of organoids in hydrogel capsules

(a) Small fragments of organoids thawed from bulk Matrigel™ were re-suspended in growth medium/Matrigel for culturing. (b) Large fragments or structured organoids thawed from bulk Matrigel™ were re-suspended in growth medium/Matrigel for culturing. (c) Organoids thawed from capsules were re-suspended in growth medium/Matrigel for culturing. (d) Live/dead staining for re-cultured organoids from capsules with z-stack imaging. (e) A quantitative comparison of the cell viability in organoids recovered from bulk and capsules ($n=3$ for each group). (f, g) Expression of stem cell markers, LGR5-eGFP and CD44, in the organoids that were thawed from capsules and re-cultured for a week after freezing at -80°C for 6 weeks.