Video Article

Mammalian Cell Encapsulation in Alginate Beads Using a Simple Stirred Vessel

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Abstract

Cell encapsulation in alginate beads has been used for immobilized cell culture *in vitro* as well as for immunoisolation *in vivo*. Pancreatic islet encapsulation has been studied extensively as a means to increase islet survival in allogeneic or xenogeneic transplants. Alginate encapsulation is commonly achieved by nozzle extrusion and external gelation. Using this method, cell-containing alginate droplets formed at the tip of nozzles fall into a solution containing divalent cations that cause ionotropic alginate gelation as they diffuse into the droplets. The requirement for droplet formation at the nozzle tip limits the volumetric throughput and alginate concentration that can be achieved. This video describes a scalable emulsification method to encapsulate mammalian cells in 0.5% to 10% alginate with 70% to 90% cell survival. By this alternative method, alginate droplets containing cells and calcium carbonate are emulsified in mineral oil, followed by a decrease in pH leading to internal calcium release and ionotropic alginate gelation. The current method allows the production of alginate beads within 20 min of emulsification. The equipment required for the encapsulation step consists in simple stirred vessels available to most laboratories.

Video Link

The video component of this article can be found at https://www.jove.com/video/55280/

Introduction

Mammalian cell encapsulation has been broadly studied as a means to protect transplanted cells from immune rejection[1] or to provide a three-dimensional support for immobilized cell culture[2,3,4]. Pancreatic islet encapsulation in alginate beads has been used to reverse diabetes in allogeneic[5,6] or xenogeneic[7,8,9,10,11,12] rodents. Preclinical and clinical trials of encapsulated pancreatic islet transplantation to treat type 1 diabetes are ongoing[13,14,15]. For transplantation applications or larger-scale *in vitro* immobilized cell production, nozzle-based bead generators are generally used. Typically, a mixture of alginate and cells is pumped through a nozzle to form droplets that fall into an agitated solution containing divalent cations, resulting in the external gelation of the droplets. Coaxial gas flow[16,17], nozzle vibration[18], electrostatic repulsion[19] or rotating wires[20] facilitate droplet formation at the nozzle tip.

The main drawbacks of conventional bead generators are their limited throughput and the limited range of solution viscosities that will result in adequate bead formation[21]. At high flow rates, the fluid exiting the nozzle breaks up into droplets smaller than the nozzle diameter, decreasing size control. Multi-nozzle bead generators can be used to increase the throughput, but the uniform distribution of flow among the nozzles and the use of solutions >0.2 Pas is problematic[22]. Lastly, all of the nozzle-based devices are expected to impart some damage to islets, since the diameter of the nozzles used is between 100 µm and 500 µm, while ~15% of human islets can be larger than 200 µm[23].

In this video, we describe an alternative method to encapsulate mammalian cells by forming droplets in a single emulsification step instead of drop-by-drop. Since bead production is performed in a simple stirred vessel, the method is suitable for small (~1 mL) to large-scale (10^3 L range) bead production with low equipment costs[24]. This method allows the production of beads with high sphericity using a broad range of alginate viscosities with short (*e.g.* 20 min) bead generation times. This method was originally developed by Poncelet *et al.*[25,26] and used to immobilize DNA[27], proteins[28] including insulin[29], and bacteria[30]. We have recently adapted these methods to the encapsulation of mammalian cells using pancreatic beta cell lines[31,32] and primary pancreatic tissue[32].

The principle of the method is to generate a water-in-oil emulsion consisting of alginate droplets in mineral oil, followed by internal gelation of the alginate droplets (**Figure 1**). First the encapsulant (*e.g.,* cells) is dispersed in an alginate solution containing a fine grain calcium salt with low solubility at the initial process pH. The alginate mixture is then added to an agitated organic phase to create an emulsion, usually in the presence of a surfactant. In the case of mammalian cell encapsulation, components present in serum can act as surfactants. Next, the pH is reduced in order to solubilize the calcium salt by adding an oil-soluble acid that partitions into the aqueous phase. Acetic acid, with a mineral oil/water partition coefficient <0.005[33], should be pre-dissolved in oil, then added to the emulsion where it is mixed in the oil phase and rapidly partitions

into the aqueous phase[34]. **Figure 2** illustrates the chemical reactions and diffusion that take place during the acidification and internal gelation step. Finally, the encapsulated cells are recovered by phase inversion, phase separation accelerated by centrifugation, repeated washing steps and filtration. These steps can then be followed by bead and cell sampling for quality control analyses, *in vitro* cell culture and/or transplantation of the encapsulated cells.

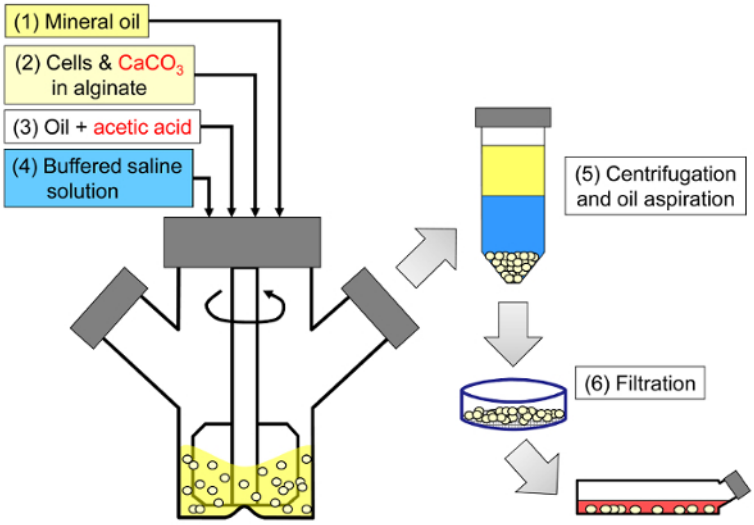

Figure 1: Schematic of the emulsification-based process to encapsulate mammalian cells. Beads are first produced by emulsifying an alginate, cell and $CaCO_3$ mixture in mineral oil (steps 1 and 2 in the schematic), triggering internal gelation by adding acetic acid (step 3). The gelled beads are then separated from the oil by adding an aqueous buffer to trigger phase inversion (step 4), followed by centrifugation and oil aspiration (step 5), and then filtration (step 6). Finally, the beads collected on the filter are transferred into cell culture medium for *in vitro* culture or for transplantation. Please click here to view a larger version of this figure.

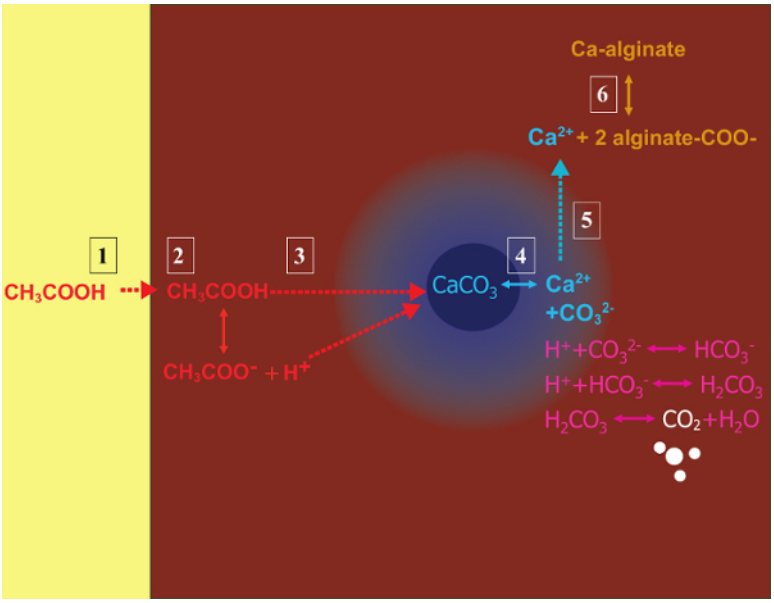

Figure 2: Reactions and diffusion steps occurring during internal gelation. (1) Acetic acid is added to the organic phase and is transported to the alginate droplets by convection. (2) The acetic acid partitions into the aqueous phase. (3) In the presence of water, the acid dissociates and diffuses to reach the $CaCO_3$ grains depicted in dark blue. (4) The H^+ ions are exchanged with the Ca^{2+} ions in $CaCO_3$, releasing Ca^{2+} ions. (5) The calcium ions diffuse until they encounter unreacted alginate, leading to the ionotropic cross-linking of the alginate chains. Please click here to view a larger version of this figure.

Contrary to conventional nozzle-based cell encapsulators, a broad bead size distribution is expected from this process due to the mechanism of droplet formation in stirred emulsification. For a subset of applications, this bead size distribution may be problematic. For example, a larger fraction of cells may be exposed at the bead surface in smaller beads. If nutrient (*e.g.* oxygen) limitations are a concern, these limitations may be exacerbated in larger beads. An advantage of the stirred emulsification method is that the average bead size can readily be adjusted by changing the agitation rate during the emulsification step. The broad bead size distribution can also be exploited to study the effect of bead size on encapsulated cell performance.

Mammalian cell encapsulation by emulsification and internal gelation is an interesting alternative for laboratories that are not equipped with a bead generator. Furthermore, this method gives users the option of reducing the processing time, or generating beads at very low or very high alginate concentrations.

The protocol outlined below describes how to encapsulate cells in 10.5 mL of 5% alginate solution prepared in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. The alginate consists of a 50:50 mixture of transplantation-grade LVM (low viscosity high mannuronic acid content) and MVG (medium viscosity high guluronic acid content) alginate. Calcium carbonate at a final concentration of 24 mM is used as the physical cross-linking agent. Light mineral oil constitutes the organic phase, while acetic acid is used to acidify the emulsion and trigger internal gelation. However, the alginate type and composition, as well as the process buffer selected depend on the desired application[32]. A variety of alginate types (see Table of materials) have been used to produce beads with this protocol.

Protocol

1. Prepare the Alginate Solution, the $CaCO_3$ Suspension and the Acidified Oil

1. **Prepare the process buffer and medium.**
    1. Prepare the typical process buffer used to generate high-concentration alginate beads using 10 mM HEPES, 170 mM NaCl. Adjust the pH to 7.4.
    2. Prepare the typical culture medium using Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 6 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin.

2. **Prepare the alginate stock solution at 1.17 fold the final desired concentration in the beads.**
    1. First, weigh the appropriate amount of alginate powder. For example, to obtain a final concentration of 5% alginate with 50:50 LVM and MVG alginate, weigh out a total of 1.17 g sodium alginate by combining 0.583 g LVM alginate powder and 0.583 g MVG alginate powder.
    2. Place 20 mL process buffer into an autoclavable glass jar on a magnetic stir plate and agitate at ~200 rpm. Progressively add the sodium alginate powder to the solution.
    3. Leave the solution stirring overnight at a low speed. If necessary, fasten the flask to the stir plate.
    4. If the solution is incompletely dissolved, fasten the bottle onto a rotary mixer and continue mixing at 37 °C for an additional 24 h.
    5. Sterilize the alginate solution by autoclaving the solution for 30 min in a vessel that is less than half full. Allow the temperature to decrease below 60 °C before retrieving the solution.
    6. If necessary, remove particles by filtering the alginate solution shortly after autoclaving before it reaches room temperature, while the viscosity remains sufficiently low.

3. **Prepare the calcium carbonate suspension at 21-fold the final desired concentration for internal gelation.**
    1. Weigh out the $CaCO_3$ powder. For example, add 1 g $CaCO_3$ to 20 mL process buffer to obtain 24 mM $CaCO_3$ as the final gelling agent concentration.
    2. Autoclave the $CaCO_3$ suspension for 30 min.
    NOTE: The $CaCO_3$ concentration will change over time due to the bicarbonate - CO_2 equilibrium. Avoid using the same stock $CaCO_3$ suspension for more than 10 encapsulation procedures.

4. Autoclave the stirred vessel used for the emulsification process. Prior to use, remove any traces of condensed water remaining in the spinner flask.
5. Immediately before the emulsification process, prepare the acidified oil. Dissolve 44 µL acetic acid per 11 mL mineral oil placed in a 50 mL conical tube.
NOTE: A common mistake is incomplete dissolution of the acetic acid. Avoid pipetting small amounts of acetic acid and ensure that the acetic acid is completely dissolved in oil by repeated vortexing.
CAUTION: acetic acid is a toxic and volatile acid. Handle this reagent under a fume hood and keep the acetic acid/oil solution in a closed container until the emulsification step. Refer to the MSDS information on this reagent for further information.
6. Allow all the solutions to reach room temperature before proceeding to cell encapsulation.

2. Alginate Bead Generation by Emulsification and Internal Gelation

1. Place 10 mL light mineral oil in the spinner flask and start agitation at a low rotation speed (*e.g.* 250 rpm for the spinner flask used in this video).
2. **If the cells used for the process are from adherent cultures, apply trypsin to suspend the cells. End the reaction by adding FBS-containing medium or trypsin inhibitor and collect a sample for cell enumeration.**
    1. Determine the cell concentration and viability after Trypan blue staining using a hemocytometer or an automated cell counter, as previously described[32].

3. Centrifuge the cells for 7 min at 300 x g and wash them once in the desired medium for cell immobilization. Ensure that this medium contains emulsifiers such as either FBS or bovine serum albumin (BSA). For example, use DMEM containing 10% FBS.
4. Re-suspend the cell pellet in the same medium to obtain 10.5-fold the desired final concentration in the beads.
5. Add 1.1 mL of the 10.5-fold concentrated cell stock to 9.9 mL of the alginate solution. Then, add 550 µL of $CaCO_3$ suspension and mix by stirring with a sterile spatula to ensure an even distribution of the $CaCO_3$.
6. Immediately transfer 10.5 mL of the alginate, cell and $CaCO_3$ mixture into the agitating oil using a syringe.
NOTE: For highly viscous solutions, aspirate and eject the mixture very slowly to avoid air bubbles. In some cases, it is preferable to stop the agitation while adding the mixture to the flask to avoid alginate entrainment onto the impeller shaft.

7. Immediately after adding the alginate, cell and $CaCO_3$ mixture to the oil, increase the agitation rate.
   NOTE: For the 5% LVM:MVG alginate used here, a rotation speed of 1025 rpm was applied to generate beads of approximately 900 µm diameter suitable for *in vitro* culture[35]. Higher rotation speeds and lower alginate concentrations will lead to lower average bead diameters, as described in previous publications[25,32]. Slight changes in impeller and vessel geometry, as well as alginate properties can greatly affect the average bead diameter. For each change in vessel configuration or alginate lot, a standard curve relating the surface area moment average bead diameter to the rotation speed should be generated[32].
8. Start the timer and emulsify the alginate for 12 min.
9. Add 10 mL of the oil and acetic acid solution to acidify the emulsion, dissolve the $CaCO_3$ and hence physically cross-link the alginate into gelled beads. Allow 8 min for this acidification step.

3. Bead Recovery

1. Reduce the agitation rate to 400 rpm. Add 40 mL of process buffer mixed with 10% medium to increase the pH and to cause phase inversion.
2. Stop the agitation 1 min later and transfer the mixture to 50 mL centrifuge tubes. Rinse the spinner flask with an additional 20 mL medium and add this to the tube. Aspirate as much aqueous phase as possible from the spinner flask before aspirating the oil phase to minimize bead contact with the oil phase.
   NOTE: Use a large-bore pipette (*e.g.* 25 mL pipette) at this step and from this point on to avoid damaging the beads. For smaller volumes, the bead suspension can also be manipulated with cut pipette tips. To obtain such tips, cut the end of the pipette tip with scissors while wearing eye protection.
3. Centrifuge the tubes for 3 min at 630 x g to accelerate bead settling and phase separation.
4. Remove the oil and excess aqueous solution by aspirating with a Pasteur pipette.
5. Wash the beads at least once with medium. Filter the bead suspension on 40 µm nylon cell strainers, and aspirate excess liquid associated with the beads from below the strainer. Transfer the beads into a known volume of medium using a sterile spatula.
   NOTE: Smaller or larger pore size filters can be used at this step, depending on the target minimum bead diameter. However, gravity filtration is recommended rather than pressure-driven filtration through sub-micron pore size filters to avoid damaging the beads.
6. Measure the bead volume by volume displacement (volume after adding the beads, minus the volume prior to adding the beads) and top up the medium to obtain the desired bead:total volume ratio, which is typically 1:5, or 1 mL beads in 4 mL medium. From this point on, always use large bore pipettes to avoid damaging the beads.
7. Transfer the encapsulated cells into T-flasks for *in vitro* culture or transplantation experiments.

4. Quality Control and Applications

NOTE: In order to ensure encapsulated cell and bead quality, the bead size distribution and cell survival after the process should be quantified. Reversing the gel to recover the cells from within the beads for further analysis is commonly performed.

1. **To assess the bead size distribution, stain the beads with toluidine blue-O.**
   1. Place 0.5 mL beads into 4 mL process buffer containing 10% complete medium.
   2. Add 500 µL of a 1 g/L toluidine blue solution prepared in process buffer.
   3. Incubate for 60 min in a conical tube on a rotary shaker at 50 rpm.
   4. Add 5 mL process buffer containing 10% complete medium and immediately transfer the beads and solution into a Petri dish and acquire images on a low-magnification microscope or using a hand-held digital camera. If needed, place the Petri dish on a light box before acquiring images with a hand-held camera to enhance the contrast and avoid shadows.
   5. Perform image analysis to quantify the bead size distribution as previously described[32], for example using image analysis freeware[36].

2. **To assess cell survival qualitatively, stain the cells using ethidium homodimer to identify dead cells and calcein AM to identify live cells.**
   1. Add 1 volume of beads to 4 volumes of process buffer containing 10% complete medium.
   2. Add the appropriate amount of calcein AM and ethidium homodimer stock solutions to obtain 4 µM and 2 µM concentrations, respectively. Generate single stain controls by adding only one of the reagents to some bead samples.
   3. Incubate the beads on ice for 20 min.
   4. Place the solution between a slide and coverslip using a spacer such as an o-ring to avoid compressing the beads.
   5. Proceed to fluorescence microscopy. Use the single stain controls to assess fluorescence bleed-through. Select appropriate fluorescence filters based on the excitation/emission wavelengths associated with calcein AM (494/517 nm) and ethidium homodimer bound to DNA (528/617 nm).

3. **To recover the cells from the beads for further analysis, degel the alginate using citrate or another chelating solution.**
   1. Prepare a degelling solution containing 55 mM citrate, 10 mM HEPES and 95 mM NaCl at pH 7.4.
   2. Mix this solution with 10% medium, and then add 1 mL alginate beads to 9 mL of the mixture.
   3. Degel the beads on ice with 75 rpm agitation for 20 min.
      NOTE: The cells can now be used for analysis or removed from the degelled alginate solution by centrifugation. For example, cell viability after degelling can be quantified by Trypan Blue staining. Alternatively, the cells can be centrifuged and washed, followed by mRNA, DNA and/or protein sampling and analysis.

Representative Results

At the end of the emulsification and internal gelation process, a bead volume similar to the initial alginate and cell mixture volume should be recovered. The beads should be highly spherical with few defects (**Figure 3**). The beads should be sufficiently strong to withstand pipetting through large-bore pipettes. At high alginate concentrations, encapsulated oil or air droplets may be observed in the beads. This likely occurs due to the entrapment of oil in alginate droplets during the emulsification step (oil/water/oil double emulsion). In our hands, these beads represented a small fraction (<5%) of the bead volume and most of these beads were removed while aspirating the oil during the bead recovery step, due to their lower density.

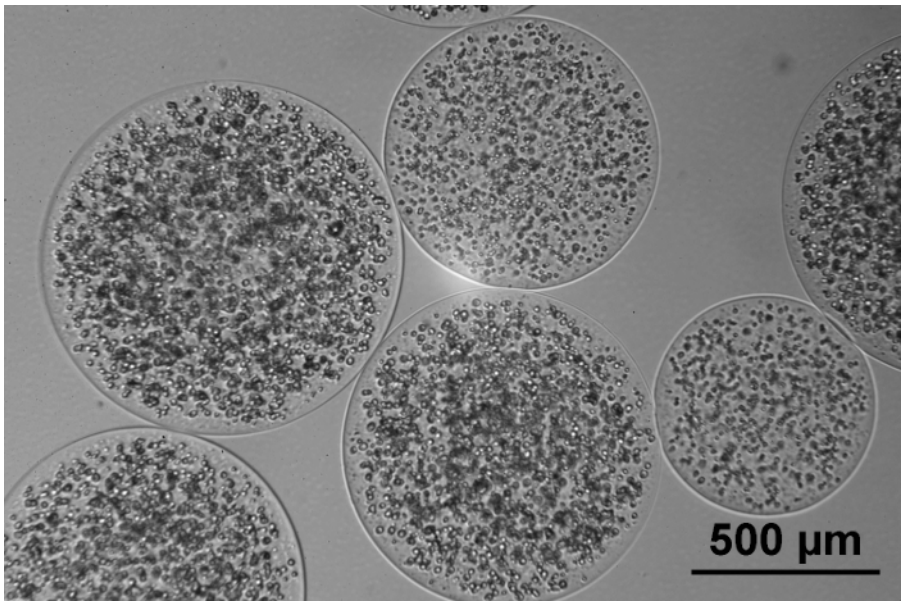

Figure 3: Representative 5% alginate beads containing encapsulated βTC3 cells obtained by the emulsification and internal gelation process. Phase contrast image of 5% alginate beads containing 5 x 10^6 βTC3 cells/mL beads. Please click here to view a larger version of this figure.

The size of the beads obtained will not be uniform - this is an intrinsic feature of stirred emulsions (**Figure 4A**). During emulsification in turbulent flow, pressure fluctuations act to disrupt droplets[37,38], counteracting the surface tension and the viscous forces in the dispersed phase[39]. Viscous forces are non-negligible if the viscosity of the dispersed phase is much higher than that of the continuous phase. Assuming that the maximum local energy dissipation rate is proportional to the average energy dissipation rate in the vessel, the maximum stable droplet diameter d_{max} (if >> than the Kolmogorov scale) can be calculated as follows[39,40,41,42]

$$\frac{\rho_C \varepsilon^{2/3} d_{max}^{5/3}}{\sigma} = C\left(1 + \frac{1}{2^{3/2}} \frac{\mu_d (\varepsilon d_{max})^{1/3}}{\sigma}\right)$$

where ρ_c is the density of the continuous phase, ε is the average energy dissipation rate in the vessel, σ is the interfacial tension between the two fluids, μ_d is the viscosity of the dispersed phase and C is a proportionality constant. When surface tension forces predominate over viscous forces, the right-hand side simplifies to C, whereas if the viscous forces predominate it simplifies to:

$$\frac{1}{2^{3/2}} \frac{\mu_d (\varepsilon d_{max})^{1/3}}{\sigma}.$$

In stirred vessels, the energy input under turbulent flow scales is proportional to the power input per unit volume, *i.e.* to $N_i^3 D_i^2$, where N_i is the agitation rate and D_i is the impeller diameter. Therefore, combined with Equation 1 and given a constant D_i, if surface tension forces predominate the d_{max} is predicted to be proportional to $N_i^{-1.2}$ (**Figure 4B**), while it is proportional to $N_i^{-0.75}$ if viscous forces within the beads predominate as the main retentive force. **Figures 4B** and **4C** suggest that interfacial effects determine the droplet size for low alginate concentrations, while viscous forces become more dominant at higher alginate concentration (*e.g.* 5% for alginate #3 described in the Table of materials). Since it can be impractical to determine d_{max} experimentally, high quantile values (*e.g.* the 95th quantile) of the diameter or the surface area moment mean diameter (d_{32}) have been used instead, as they generally are proportional to d_{max}[43].

Before encapsulating cells, it is recommended that a curve of d_{32} as a function of N_i be generated (**Figure 4B**). This curve will help select the adequate N_i to obtain a desired average bead size. A new standard curve will also likely be required for different batches of oil or alginate, or if the alginate concentration is modified. Depending on the vessel geometry and on the alginate concentrations, a relatively wide range of average bead diameters should be achievable. For example, d_{32} values between 200 µm and > 1 mm can be obtained using 2% (**Figure 2C** in Hoesli *et al.*[32]) to 5% alginate (**Figure 4**) using the protocol described above.

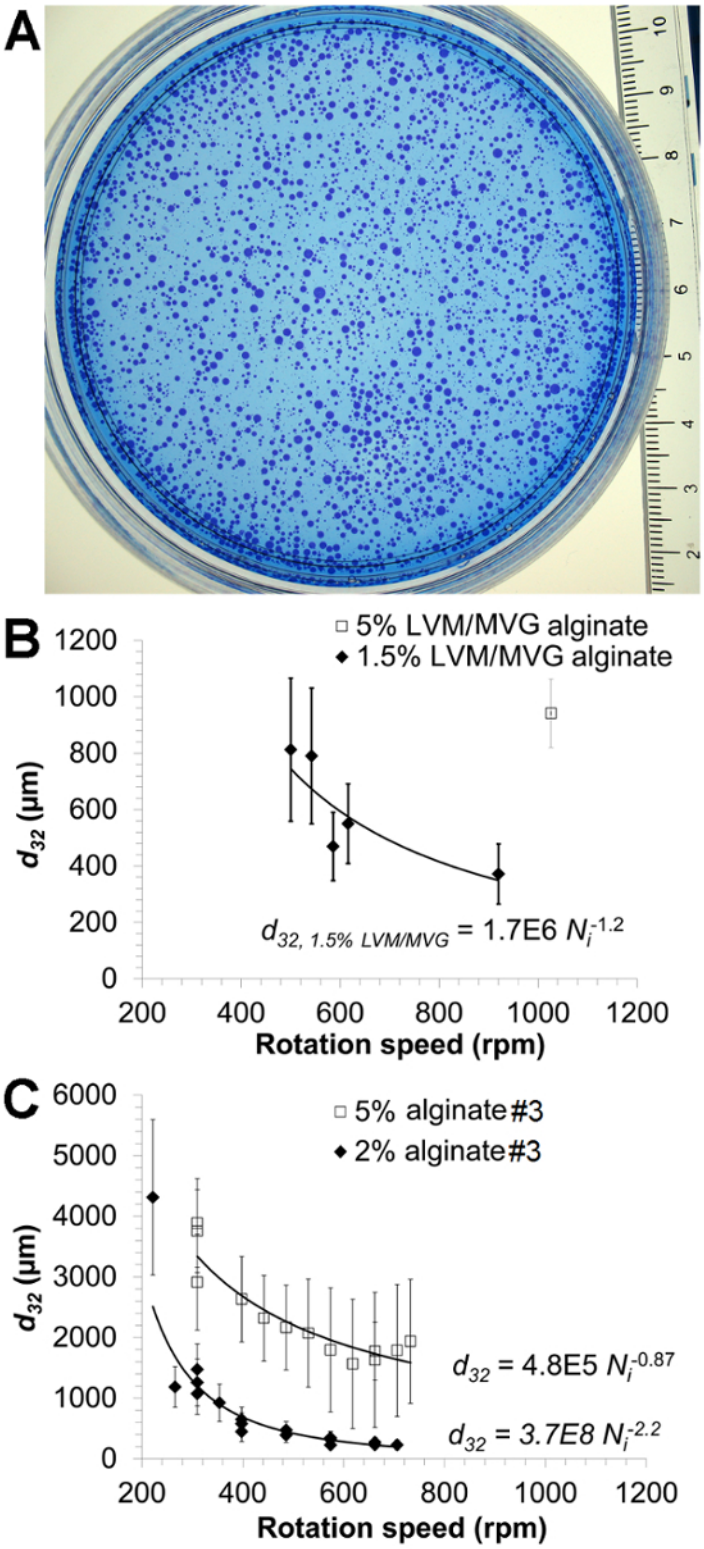

Figure 4: Bead size distribution. (**A**) Toluidine blue-O stained 5% alginate beads. (**B**) d_{32} as a function of the agitation rate (N_i) for 1.5% and 5% alginate beads using a 50:50 mixture of alginates #1 and #2 (see Table of materials). A single point is shown for the 5% alginate beads. (**C**) d_{32} as a function of N_i for 2% and 5% alginate #3 (see Table of materials). The error bars represent the standard deviation of bead sizes within a sample (N >150 beads). Please click here to view a larger version of this figure.

The cell survival from the emulsification and internal gelation process mainly depends on the extent and duration of the pH drop experienced by the cells. Using the process described in this video, 76 ± 2% βTC3 cell survival was measured[31]. When encapsulating dispersed cells at 10^5 cells/mL alginate to 10^7 cells/mL alginate seeding densities, all beads should contain cells. When encapsulating cell clusters such as pancreatic cells, a subset of beads can be expected to be empty. **Figure 5** shows typical live cell/ dead cell staining results obtained using this process. Higher cell survival can be achieved by increasing the process buffer capacity and by decreasing the duration of the acidification step. For example, 90 ± 2% MIN6 cell survival was obtained by using 60 mM MOPS (3-(N-morpholino)propanesulfonic acid) as the process buffer and by decreasing the overall process time to 4 min[32]. However, the beads generated were significantly stronger with the 10 mM HEPES process buffer than with the 60 mM MOPS process buffer due to the increased amounts of Ca^{2+} released at lower pH[31]. Both the short MOPS process and the longer HEPES process should generate beads that are sufficiently stable for >2 weeks *in vitro* culture or transplantation. However, beads may swell or degel entirely in solutions with low calcium or with high chelator concentrations (for example, calcium-free phosphate-buffered saline solution).

For encapsulated MIN6 cells grown *in vitro*, visible cell aggregates form after ~5-7 days of *in vitro* immobilized cell expansion and ~150 µm diameter spheroids can be harvested after 2 weeks. The growth rate of alginate-immobilized cells is expected to be lower than in non-immobilized cultures, particularly for gels with higher alginate concentration or higher guluronic acid content.

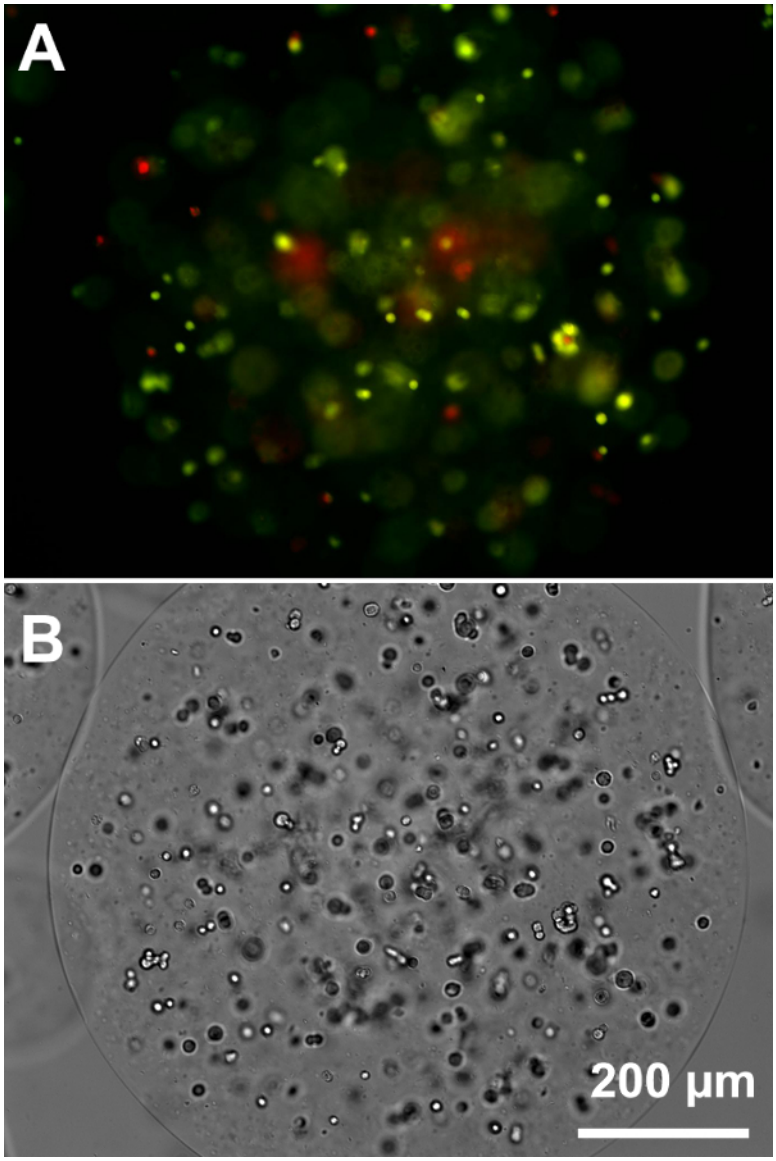

Figure 5: Encapsulated cells immediately after the process. (**A**) Live cell (green, calcein AM) and dead cell (red, ethidium homodimer) staining of encapsulated cells. (**B**) Phase contrast image of the same bead. The MIN6 cells were encapsulated in 2% alginate beads using the 12 min emulsification, 8 min acidification and a 10 mM HEPES buffer as described in this protocol. Please click here to view a larger version of this figure.

Discussion

Various steps (depicted in **Figure 2**) during the internal gelation reaction can limit the overall kinetics. For calcium carbonate grains larger than ~2.5 µm, the rate of carbonate dissolution has been shown to be rate-limiting[26,44]. The acidification step that leads to internal calcium release has also been shown to be the critical process variable affecting cell survival[32]. The conditions that lead to internal gelation are therefore crucial both for bead quality as well as cell survival. Depending on the final application for the beads, different optimal process conditions may be chosen. For example, a higher pH drop (*e.g.* using 10 mM HEPES to buffer the alginate solution) may be desirable for transplantation applications where the long-term stability of the beads is crucial. On the other hand, a lower pH drop (*e.g.* using 60 mM MOPS to buffer the alginate solution) may be preferable for *in vitro* applications.

In addition to adjusting the level and duration of acidification, other process modifications can be envisioned. For example, other stirred vessel configurations, alginate concentrations, process buffers, organic phases and acidifying agents can be used. As discussed previously, the average bead size can easily be adjusted by changing the agitation rate during the emulsification step. In addition, longer emulsification times can lead to smaller bead sizes, but can cause decreased cell viability. The publications by Poncelet *et al.*[25,26,45] and Hoesli *et al.*[31,32] have tested and discussed some of these options.

Slight changes in the protocol described above can have a profound impact on bead generation, bead quality and cell survival. **Table 1** provides a troubleshooting guide to understand and solve problems that may occur.

Problem	Typical cause	Suggested solutions
The impeller stops rotating during alginate or acid oil addition	The magnetic field is insufficient for adequate mixing or the impeller shaft is misaligned	Ensure that the spinner flask is well-centered on the magnetic plate and taped onto the plate in an optimal position prior to adding the alginate mixture. Test very high agitation rates and then decreased to the desired agitation rate prior to adding the alginate mixture.
		Ensure that the impeller shaft is well-aligned and stable. For the Bellco spinner flasks, ensure that the impeller is firmly held in place by bolts on both sides of the spinner flask cap. Ensure that the impeller movement is limited to the radial direction (the impeller should not wobble on the shaft).
No beads obtained	The alginate was improperly emulsified	Ensure that alginate microdroplets are visible during the emulsification. If not, verify that the oil phase density and the vessel geometry[23] are similar to those described in this protocol. A dense oil phase or vessel geometry that does not provide sufficient local energy dissipation may not allow proper alginate emulsification.
No beads obtained	The internal gelation did not occur	A common error is incomplete dissolution of the acetic acid in the oil phase. Ensure that the acid oil mixture is sufficiently vortexed and no acetic acid is observed at the bottom of the tube.
		If the impeller does not reach the oil, more oil can be added prior to adding the alginate. However, the amount of acetic acid should be adjusted according to the following equation: $V_{ac}^2 = V_{ac}^1 \frac{\Phi_1(D_{ow}+\Phi_2)}{\Phi_2(D_{ow}+\Phi_1)}$ Where $\Phi 1$ is the oil/water phase ratio prior to modifications (e.g. 0.525 for 10.5 mL alginate in 10 mL oil + 10 mL acid oil), $\Phi 2$ is the oil/water phase ratio after modifications to the protocol, and Dow is the acetic acid distribution coefficient between the two phases. This distribution coefficient can be measured experimentally or approximated by values taken from the literature. For instance, the distribution coefficient of acetic acid between heptane and water[46] is 0.011.
No beads obtained, or beads are mechanically unstable	The alginate solution zero-shear viscosity is below 0.004 Pa·s or above 112 Pa·s	Due to the high batch-to-batch variability of alginate lots, it is recommended to measure the viscosity of different concentrations of alginate solutions. The combination of a high-viscosity batch with a low-viscosity batch may be required to reach a target viscosity. For the 5% LVM and MVG mixture used here, the zero-shear viscosity was 3.3 Pa·s. It should be noted that alginates with too short guluronic acid block lengths or short overall chain lengths may not allow gel formation.
Beads are too large or too small	The agitation rate during the emulsification is too low or too high for the alginate concentration or viscosity used	Increasing turbulence by increasing the agitation rate or adding baffles will decrease the bead size. The alginate concentration, the temperature, the vessel and impeller geometry will all impact the bead size obtained.
Many broken beads are observed	Harsh bead handling, damage during the internal gelation step or insufficient bead strength.	Mechanical damage to the beads can lead to bead rupture once internal gelation is initiated. Reduce the agitation rate immediately prior to the acidification step and ensure that the beads

		are handled gently by using 25 mL pipettes or 1000 µL pipettes with cut tips. If damaged beads are observed even with careful handling and reduced agitation during acidification, the bead strength may be inadequate. This could be due to insufficient guluronic acid content or incomplete gelling (e.g. insufficient acidification).
Low cell viability	Initial process pH too high or final pH too low	Mammalian cell process survival can be increased by limiting the pH drop and the acidification time[23]. However, these modifications may reduce the bead mechanical strength. Another potential issue may be the use of process solutions (e.g. alginate solution) with inadequately adjusted osmolalities.
Low cell viability in the smaller beads	Insufficient alginate solution buffering capacity	A greater pH drop is expected in the smaller beads than in the larger beads due to the greater surface/volume ratio of these beads. Since the acetic acid added to the emulsion is limited, higher acetic acid concentrations are predicted in smaller beads for the same $CaCO_3$ concentration. Increasing the alginate solution buffering capacity (e.g. using 60 mM MOPS instead of 10 mM HEPES) can increase cell survival in the smaller beads[32]. Small beads with high cell losses could be selectively removed by filtration or sedimentation without incurring large volumetric losses.
Incomplete $CaCO_3$ dissolution	Insufficient acidification or acidification time. Note that this issue may not be problematic if bead stability is sufficient.	Incomplete $CaCO_3$ dissolution has been observed in the larger beads following this protocol[32]. The extent of $CaCO_3$ dissolution can vary depending on the $CaCO_3$ grain size, the pH drop during the process, the duration of the pH drop, as well as the alginate concentration used. This issue is not considered problematic as long as the bead mechanical stability is sufficient for the desired application. To increase bead cross-linking, ensure that the $CaCO_3$ grain is sufficiently small (~2.5 µm). Sonicating the $CaCO_3$ suspension can help disrupt aggregated grains[26]. At very low or high alginate concentrations, adjustments in the $CaCO_3$ concentration may be required. Lastly, chelators or solutions with low levels of divalent ions will lead to the gradual loss of Ca^{2+} in the gel. If gradual loss of bead mechanical stability is observed, verify that the bead storage or culture buffer contains >2 mM Ca^{2+}.

Table 1: Troubleshooting guide. List of problems, probable causes and potential solutions.

Potential limitations of the emulsification and internal gelation process for *in vitro* culture of mammalian cells and transplantation applications include: (1) the necessity for transient exposure to low pH, (2) the need to remove residual oil, (3) the polydisperse bead size distribution, (4) the potential shear stress during alginate droplet formation and (5) the higher porosity of internally gelled beads compared to externally gelled beads[47].

High cell survival and function of MIN6 and βTC3 have been obtained after process optimization[32]. However, this process may not be suitable for more pH-sensitive cells. The complete elimination of oil from the beads may also be challenging, and Food and Drug Administration (FDA)-approved oil types are required for eventual clinical applications. The polydisperse bead size distribution may also be somewhat problematic for immunoisolation applications, where complete cell encapsulation and long-term cell survival are desirable. Whereas small bead sizes can lead to cell protrusion[48], large beads can hinder nutrient diffusion and/or cell function[35,49]. On the other hand, the emulsification process offers an interesting opportunity to investigate the effect of bead size on cell survival after transplantation by generating both small and large beads in a single process. The average bead size can readily be adjusted by modifying the agitation rate during the emulsification step (**Figure 4**). Lastly, the higher porosity of internally gelled beads may also be detrimental for immunoisolation, where molecules such as antibodies and cytokines should be excluded from the beads. However, the emulsification process also allows bead generation from very concentrated alginate solutions, thereby reducing the alginate bead pore size.

The emulsification and internal gelation process is an interesting alternative to nozzle-based encapsulators for laboratories wishing to scale up cell encapsulation or lacking access to an encapsulator. This process is a robust and simple method to immobilize cells in alginate beads using very dilute or very concentrated alginate solutions. Some of the subsequent experiments that can be performed with the immobilized cells are to determine the effect of the alginate matrix on cell fate decisions, or to determine to what extent the beads provide a barrier between transplanted cells and the host immune system. The emulsification and internal gelation method provides a new approach to encapsulate primary islets in large batches. The method also allows cell encapsulation in highly concentrated alginate beads with reduced porosity and hence reduced antibody access to the encapsulated cells. The emulsification and internal gelation method therefore provides a new means to generate clinically relevant quantities of encapsulated therapeutic cells.

Disclosures

The authors have nothing to disclose.

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