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Cell Encapsulation for Biotechnological Applications

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Cell Encapsulation for Medical Applications & Bioprocessing

Introduction

For over 50 years the encapsulation of enzymes, flavors, API's, chemicals, cosmetics, food ingredients, bioactives and various other materials in beads and capsules has found a myriad of successful applications in many industries [1]. Recently the technology, in the form of cell encapsulation, has been applied to the medical sector, where it has been used to help scientists develop new treatments for many diseases [2]. A newer application has also seen the technology being applied to the bioprocessing industry. Here it has been examined on its potential to increase production yields [3] and significantly reduce the costs associated with producing recombinant proteins used for the development of new medicines.

Medical Applications

Cell encapsulation can be defined as the envelopment of cells in porous or impermeable membranes which allow a physical separation of the cells from their immediate environment, thus protecting them from unfavorable conditions.

Since the pioneering work performed in 1980 by Lim and Sun [4], it has been very much anticipated that this technology could be used as a viable means for the constant generation of artificial immuno-isolated cells for human transplantation and provide a continuous way to deliver therapeutic products to the recipient, without fear of implant rejection and the need immuno-suppressive drugs [5]. This concept for disease treatment has received enormous interest from the medical community and has been studied for the treatment of many other diseases such as Parkinson's, Alzheimer's, anemia, hemophilia B and organ failure, as well as been used to control pain and to deliver drugs effectively [3,5].

Despite the initial promise, cell encapsulation has yet to reach its full potential and several key areas need to be addressed. Nevertheless in the past two years significant breakthroughs have been made. In 2011 doctors in London cured a baby boy of a life threatening liver infection [6], by encapsulating (liver) cells and subsequently injecting them into the boy at the site of infection. The coated cells helped him to overcome the disease and make a full recovery without the need of organ transplantation. Several other companies are also hoping to make similar progress when using cell encapsulation in late stage clinical trials for tissue replacement and drug delivery [7].

Bioprocessing Applications

The high costs and low yields associated with existing bioprocessing operations make the risk for the development of new recombinant drugs very high and likely to fail, resulting in substantial loss for a company. As a possible means to overcome this problem, the last two decades have seen cell encapsulation technology being investigated as a possible way to achieve bioprocess intensification and generate higher cell density and higher productivity for mammalian cell cultures.

The use of (fragile) animal cell culture is presently the only way to produce highly glycosylated recombinant proteins required for drug production. Cells used to produce such proteins can be cultured using many types of bioreactor technologies [3]. However, the low cell densities attainable by many of these techniques result in low product concentrations, with low productivity and poor yield of high quality recombinant proteins of consistent quality. This increases complexity for subsequent isolation and purification of the bioproduct, leading to a substantial increase in overall production costs.

To reduce costs and speed up process development within the bioprocessing industry (for the production of medical important recombinant proteins), the development of new (bioprocess) intensified production techniques is required. One promising route to bioprocess intensification is to generate high density cell cultures by encapsulating cells within a permeable hydrogel membrane to effectively create micro-bioreactors systems with defined size, shape and physical/functional properties (Figure 1). The permeable membrane allows the bi-directional diffusion of

small molecules such as oxygen, nutrients, waste and the produced recombinant protein. Most importantly cell encapsulation provides an environment free of shear stress and forces, allowing the cells to freely proliferate to very high (intensified) concentrations compared to conventional suspension cultures. This leads to increased production levels, as well as a significant reduction in complexity by reducing the number of downstream processing steps needed to obtain the required product purity. All these advantages have a significant positive effect of overall process economics by greatly reducing costs.

Several types of microcapsules have been developed for encapsulating animal cell cultures including the use of polymers such as alginate, agarose, pectin, chitosan, gelatin and acrylate copolymers, of which the most successful and common are based on the alginate-poly-L-lysine-alginate encapsulation system as developed by Lim and Sun [4].

This will help many more medically important drugs to reach the market place at a quicker rate and more importantly at more affordable prices.

Production Techniques for Animal Cell Encapsulation

To successfully apply cell microencapsulation to medical research and clinical applications, a production technique which must adhere to stringent criteria is required, which includes the ability to produce small, mono-dispersed, homogenous and spherically shaped capsules, with a narrow size distribution under mild and gentle conditions, using a short production time and is repeatable [1]. One such technique, commonly referred to as Vibrating Nozzle or Prilling by Vibration (www.buchi.com), is available and can be performed on the Encapsulator B-395 Pro (Figure 3) produced by BÜCHI Labortechnik AG. This production technique has gained significant interest from manufacturers and scientific researchers for many years on account of its ability to produce the required capsules with the desired characteristics for both medical and bioprocessing applications [1]. In addition the machine is easy to set up and operate, has low operating costs and can be integrated into a GMP process. For these reasons it is one of the

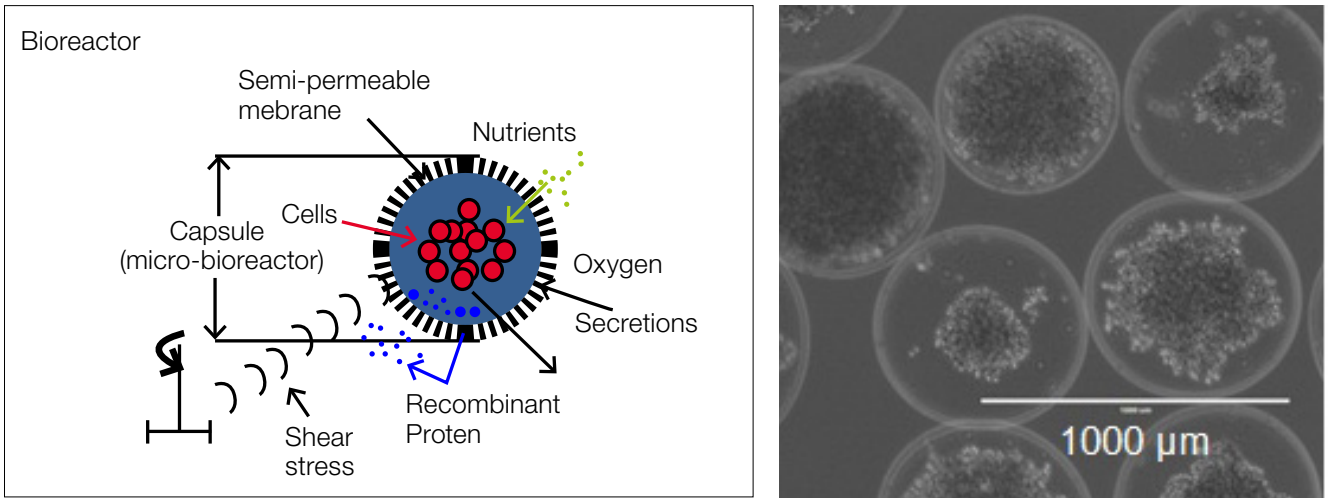


Figure 1: (i) Schematic representation of micro-capsular bioreactor system whereby cells are encapsulated within a semi-permeable membrane for protection from environmental influences. (ii) Proliferation of Chinese hamster ovary (CHO) cells to high cell densities within microcapsules of size 750 μm after 192 hr in culture [experiment was performed by BÜCHI Labortechnik AG][1].

[1] Flawil, Switzerland

most commonly employed and successful techniques to produce cell-containing microcapsules; for example a precursor model of the Encapsulator B-395 Pro was employed to encapsulate liver cells used to help the baby boy in London overcome a life threating liver disease [6].

Microencapsulation continues to be an extremely exciting tool for production of artificial tissues and high density cell cultures for both medical and bioprocessing applications and has the possibility to improve the way doctors and scientists effectively treat or develop new treatments for many high social impact diseases (Diabetes, Parkinson's etc.). While several significant challenges still face the technology, recent developments in medical applications such as those in human transplantation and employment in clinical trials show that the methodology has a bright future, not only from a scientific but also an economic point of view.

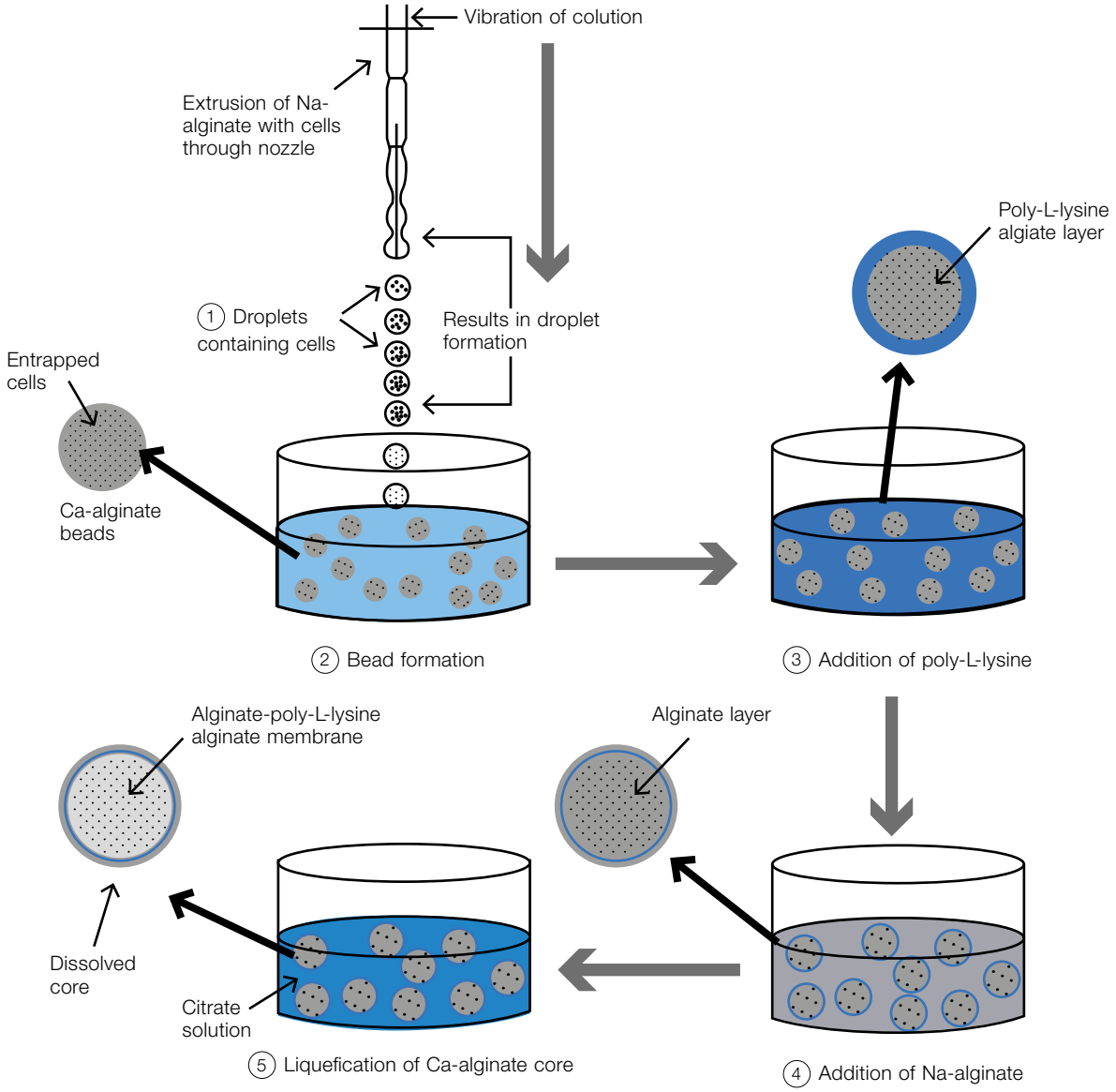


Figure 2: Schematic showing the five different steps (labelled 1-5) involved in the procedure for encapsulating cells in the alginate-poly-L-lysine-alginate membrane system.

Alginate-Poly-L-Lysine-Alginate Cell Encapsulation System

The alginate-poly-L-lysine-alginate membrane system for encapsulating (animal) cells was first described in the ground breaking work of Lim and Sun in 1980 [4], and is still the most widely used technique to encapsulate animal cells within a polymeric membrane. The scientific paper which accompanied the work has been cited over 1200 times in scientific literature.
The procedure is divided up into five main steps which are schematically shown in Figure 2.

Step 1: The first step in the procedure is the formation of homogenous Na-alginate droplets which contain the cells to be encapsulated. The cell containing droplets are usually produced by gentle production techniques such as Prilling by Vibration as performed by the BUCHI Encapsulator B-395 Pro. In this technique the alginate solution containing the cell mixture is extruded through a nozzle (of controllable size) and forms a liquid jet which is broken up into droplets using vibrational frequencies. This very gentle and simple technique enables the formation of droplets without harming the cells.

Step 2: In this step the Na-alginate droplets produced in step one are hardened (gelled) into solid homogenous beads which entrap the cells. This is usually performed by landing the droplet into a gelling solution containing a divalent cation such as $CaCl_2$. Upon landing in the hardening solution, the calcium cations immediately replace the sodium counterions and cross-link the alginate chains to form a tight hydrogel (bead[II]) structure which entraps the cells inside. The droplets are allowed to harden for a specific period of time to ensure complete gelation throughout the bead. This length of time depends mainly on the droplet size with bigger droplets requiring more time to fully harden. It is in the range of some minutes.

Step 3: The third step involves the coating of the outer surface of the Ca-alginate bead by a layer of the polymer poly-L-lysine, which forms a thin membrane around the Ca-alginate bead. This is achieved by incubating the beads in a solution of poly-L-lysine. The positively charged amino-groups of the poly-L-lysine react with free carboxyl groups of the alginate (not previously bound to calcium ions) and form a rigid outer membrane. The membrane thickness is depended on the incubation time as well as the concentration and size of the poly-L-lysine particles, with smaller particles being able to diffuse further into the Ca-alginate droplet and form a thicker membrane. After the desired membrane thickness has being achieved the beads are removed from the solution and washed with a saline buffer to stop the reaction.

Step 4: In step 4 the capsules are incubated in diluted Na-alginate solution. This results in the alginate ions combining with unreacted poly-L-lysine on the surface of the membrane, which causes the formation of a second thin membrane on the outside of the capsule structure. This treatment neutralizes unreacted positively charged poly-L-lysine ions in the membrane, thus generating a negatively charged surface (from the unreacted alginate ions) and reducing attachment of cells to the outside of the alginate-poly-L-lysine membrane. On account of its (new) make up the structure is now referred to as a capsule.

Step 5: After the addition of the outer alginate layer the capsule now consists of a rigid core consisting of tightly branched chains of Ca-alginate. This Ca-alginate can inhibit cellular growth of the cells and must be liquefied to make room for cells to grow. This is achieved by incubating the capsules in a citrate or other calcium chelating solution which causes the removal of the calcium ions from the alginate and are replaced by the chelating agent counterions (Na^+). This results in a liquidified core consisting of re-solubilized Na-alginate within the alginate-poly-L-lysine-alginate membrane (Note: The citrate step does not affect the integral structure of alginate-poly-L-lysine-alginate shell). Large amounts of the solubilized alginate remains entrapped within the core as it is too big to diffuse out through the membrane. After dissolution of the core, the capsules are removed from the chelating solution and washed in a saline solution and are then added to appropriate media to enable cell growth. Due to the porous nature of the capsule membrane, nutrients and oxygen can diffuse in and cell wastes and by-products can diffuse out. The rate of diffusion is mainly dependent on the capsule size with smaller sizes enabling greater rates.

[II] Commonly referred to as a Ca-alginate bead

Laboratory Protocol for the Encapsulation of Animal Cells in Alginate-Poly-L-Lysine-Alginate membranes

The procedure is an adaption of the encapsulation technique developed by Lim and Sun [4] and will describe the encapsulation of animal cells in alginate-poly-L-lysine-alginate microcapsules and is performed with the BUCHI Encapsulator B-395 Pro by using the Prilling by Vibration technique. The Encapsulator works by using vibrational frequencies to break up a laminar jet of liquid into equally sized droplets, which are subsequently converted into beads by a gelation technique (addition to $CaCl_2$). The setup of the BUCHI Encapsulator B-395 Pro enables the addition and removal of liquids that are required for the formation of the alginate-poly-L-lysine membrane and all steps in the process can be performed under fully sterile conditions. The very gentle process can be used to encapsulate most cell types (including animal and stem cells) due to minimal shear stress being exerted on the cells during the encapsulation process. The reproducible and scalable technique can be fully integrated into a GMP process. For more information on the operation and applications of the BUCHI Encapsulators see BUCHI Encapsulator Laboratory Guide (www.buchi.com)

Encapsulating Equipment

The BUCHI Encapsulator model B-395 Pro with glass reaction vessel (Figure 3) in place is used to encapsulate the cells. The glass reaction vessel fits around and completely encloses the bead production unit enabling complete sterile conditions to be obtained. The reaction vessel can be steam autoclaved, along with all other parts which come into contact with the cells and the solutions used to produce the beads and capsules. For alginate polymer solution it is highly recommended to use purified and sterile filtered Na-alginate solution (Figure 4) supplied by BUCHI.[III] The solution has been pre-treated and tested to allow the production of high quality beads and capsules for the encapsulation of many different types of cells.

Figure 3: The standard set up of the BUCHI Encapsulator model B-395 Pro with sterile reaction vessel in place.

Figure 4: BUCHI sterile filtered 1.8 % (w/v) Na-alginate solution for cell encapsulation. Details: Isotonicity: 300 – 330 mOsm. pH value: 7.0 – 7.4. Viscosity: 90 – 160 mPas.

[III] For more details or order information contact your local BUCHI office or distributor

Solutions:

The following solutions at specified volumes are required for making 12 mL of alginate-poly-L-lysine-alginate microcapsules containing animal cells at a concentration of 10^6 cells/mL. A short description explaining why each solution is used is also given. All chemicals were obtained from Sigma unless stated otherwise.

1. MOPS (washing) buffer

· 10 mM MOPS (Morpholinopropanesulfomic acid)
· 0.85 % (w/v) NaCl
· Adjust pH to 7.4 at room temperature

The MOPS buffer solution is used to wash the capsules after each production step to remove unreacted components. The buffer also helps to maintain physiological conditions.

2. BUCHI Na-alginate solution

· 1.8 % (w/v) Na-alginate sterile filtered solution
· pH 7.0 - 7.4 at room temperature

The Na-alginate is mixed with the cells and is produced into Ca-alginate beads encapsulating the cells.

3. Hardening (gelling) solution

· 10 mM MOPS
· 100 mM $CaCl_2$ (dihydrate)
· pH 7.4 at room temperature

Gelling solution reacts with the Na-alginate to form Ca-alginate beads.

4. Poly-L-Lysine Solution

· 0.05 % (w/v) poly-L-lysine[IV] in hardening solution

The positively charged poly-L-lysine forms a stable permeable membrane on the outer surface of the produced Ca-alginate bead. The thickness of the poly-L-lysine membrane depends on the molecular weight and concentration of the polymer as well as the length of the reaction (incubation) time.

5. 0.03 % Na-alginate solution

· 2 mL of 1.8 % Na-alginate solution in 118 mL of
· MOPS buffer

This solution is used to form an outer alginate layer around the capsule by combining with unreacted PLL. This results in the neutralization of the PLL and generates a negatively charged surface around the capsule and reduces the attachment of cells to its surface.

6. Core-solubilization solution

· 50 mM Sodium citrate
· 0.45 % (w/v) NaCl
· 10 mM MOPS
· pH 7.4 at room temperature

The solution solubilizes the Ca-alginate core within the bead to provide room for cell growth.

Required Volumes

The following volumes are required when producing 12 mL[V] of alginate-poly-L-lysine-alginate microcapsules containing animal cells at a concentration of 10^6 cells/mL. Note: All non-sterile solutions are passed (filtered) through a 0.2 μm liquid filter membrane when being delivered to the enclosed reaction vessel during capsule production to maintain sterile conditions.

12 mL	1.8 % BUCHI sterile alginate solution
120 mL	0.03 % alginate solution (non-sterile)
250 mL	Hardening solution (non-sterile)
50 mL	Poly-L-lysine solution (non-sterile)
2000 mL	MOPS washing buffer (non-sterile)
5 mL	MOPS washing buffer[VI] (sterile filtered through 0.22 μm filter membrane)
200 mL	Solubilization solution (non-sterile)

If larger volumes of capsules are required please increase the quantity of the poly-L-lysine solution appropriately as the membrane material is consumed proportionally to the quantities of alginate beads that are present.

[IV] Poly-L-lysine hydrobromide, MW 30 – 70 kDa. Sigma order no. P2636.
[V] Recommend volume for a single run, however greater volumes can be produced in a single run.
[VI] Used to re-suspend cells – see step 4.

Procedure

1. Prepare the reaction vessel as described in section 5.4 of the BUCHI operation manual (or shown in the YouTube video[VII]) with the 300 μm nozzle in place and autoclave it as described in section 6.11 and 6.12 of the manual.

2. Prepare all solutions and lab ware. Note: Before beginning the operators are advised to establish the optimal parameters for bead production under non-sterile conditions, whereby the machine is operated in open-mode (operating without the reaction vessel). This will enable optimal parameters to be obtained much quicker and reduce cell waste. For tips on how to determine the optimal parameters for a selected nozzle size as well as some pre-determined parameters, see section 5 (Quick-start guide for the single nozzle system) of the Encapsulator Laboratory Guide.

3. Pump 200 mL of hardening solution into the autoclaved reaction vessel using the pressure bottle. The hardening solution is sterilized by pumping it through the autoclaved liquid filter before entering the reaction vessel.

4. Place the reaction vessel under a laminar air flow cabinet.

5. Prepare a culture of cells with ca. of $1x10^6$ cells/mL under a laminar flow cabinet. Centrifuge the cells, decant the supernatant and re-suspended the pellet in 2 mL sterile MOPS washing buffer. Add 10 mL of the 1.8 % sodium-alginate solution to the re-suspended cells using a sterile 25 mL pipette (Figure 5). The solution is mixed by re-aspirating it into the 25 mL pipette twice. Make sure that no or few air bubbles are introduced during re-suspending and mixing.

Take a sterile 20 mL syringe and fix on it an autoclaved 15 cm long silicone tube (inner diameter 3 mm) This tube will be used to transport the cells into the syringe, after which the tube is removed. Fill the syringe with the cell-alginate suspension and attach the syringe to the bead producing unit on the reaction vessel, after removing the luer lock blind plug (Figure 6).

Fix the reaction vessel to the Encapsulator control unit, which is placed on the bench (not in the laminar flow cabinet).

6. Place the bypass cup directly underneath the nozzle to prevent any unwanted alginate landing in the gelling solution.

7. Turn on the magnetic stirrer.

8. Start bead formation with previously established parameters. Once a stable droplet chain of alginate droplets has been established remove the bypass cup from underneath the nozzle and allow the droplets to land in the stirred gelling solution. (Note: Just before all alginate solution is pumped through the syringe, place the by-pass cup underneath the nozzle once more).

9. Allow the droplets to harden for 10 minutes in the gelling bath to form the Ca-alginate beads and then drain of the gelling solution.
Note: The beads and later the capsules should always be covered by a small amount of liquid to prevent clumping, otherwise re-suspension of the beads and capsules can be difficult and the membrane could get damaged.

10. Pump in 200 mL of MOPS buffer, wash the beads for 5 minutes and drain off.

11. Pump in 50 mL of 0.05 % poly-L-lysine solution and leave to incubate for 15 minutes.

12. Drain off the 0.05 % poly-L-lysine solution and then pump in 200 mL of MOPS buffer. Wash the beads for 5 minutes and drain off. Repeat the washing step twice.

13. Pump in 100 mL of the 0.03 % alginate solution and stir for 5 minutes to allow the formation of the outer alginate membrane and then drain off the solution.

[VII] http://www.youtube.com/watch?v=BGsvTfLk7hQ

14. Pump in 200 mL of MOPS buffer, stir for 1 min and then drain off the buffer. Repeat the washing step once.

15. Pump in 200 mL of the solubilization solution and incubate by stirring for approximately 15 minutes to allow solubilization of the alginate in the core. Appropriate solubilization time is dependent on the molecular weight and purity of the alginate and the susceptibility of the encapsulated cells to the dissolution solution.

16. Drain off the solubilization solution and pump in 200 mL of MOPS buffer. Wash the beads for 5 minutes and drain off. Repeat the washing step twice.

17. Pump in the culture medium and drain off with the capsules into the collecting flask.

18. Transfer the capsules aseptically into a flask or bioreactor to allow them to proliferate.

Note: Dissolved alginate diffuses out slowly. Depending on the alginate in use and the thickness of the capsule membrane, it can take up to 2 hr for substantial amounts to leave the capsule. To maximize removal of the core you can:

- Extend the extraction time in MOPS or in a culture medium without bivalent ions.
- Cultivate the cells in a medium containing < 50 mg/L of Ca ions.
- Cultivate the cells in a medium with a ratio of monovalent ions (Na^+, K^+) to bivalent ions (Ca^{2+}, Mg^{2+}) between 20:1 and 50:1.

Figure 5: Re-suspension of cells in buffer.

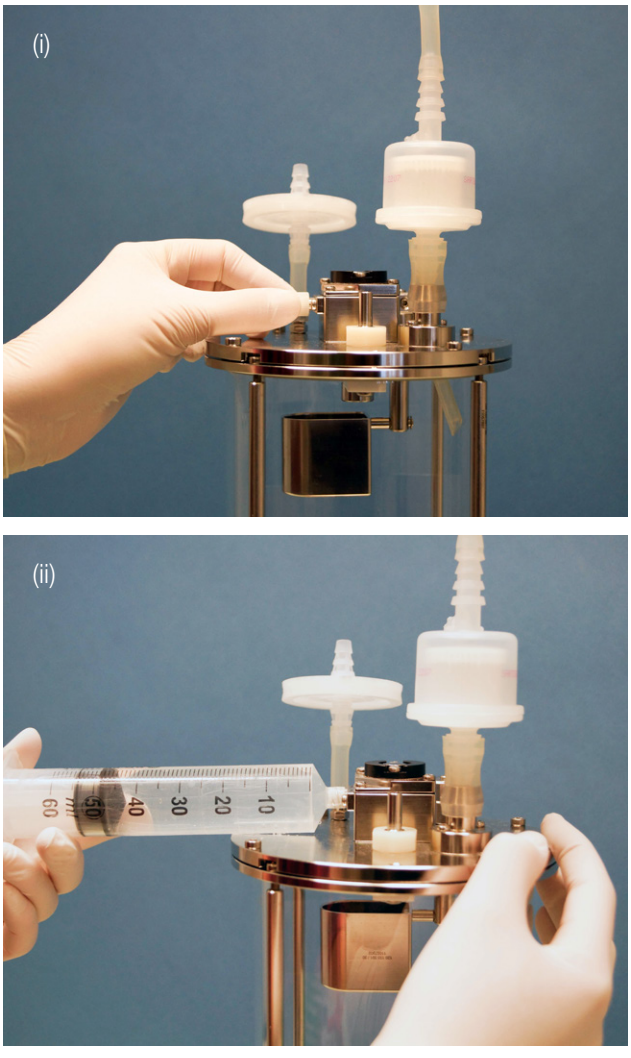


Figure 6: (i) Removal of the luer lock blind plug and (ii) attachment of the syringe to bead producing unit.

Special Recommendations for Cells

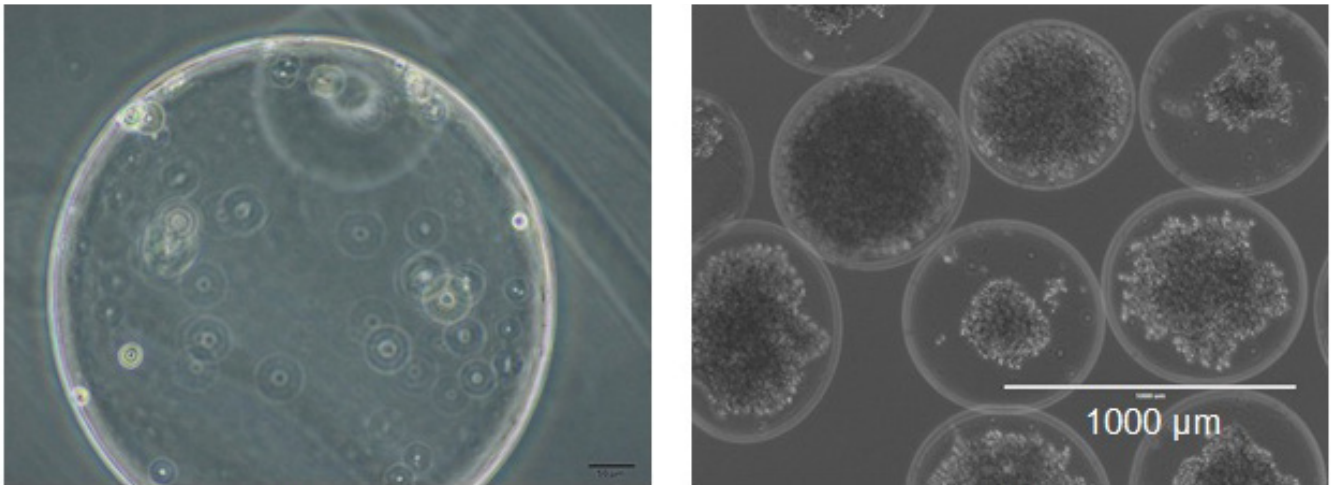
For dividing cells - dissolve the alginate core, then maintain Na/Ca-ratio > 20:1 in the culture medium so that the core will not re-solidify by combining with the free divalent ions.

For resting cells –the gelated alginate core structure can be maintained. In addition Ba^{2+} can also be used, which is a stronger gelating ion compared to Ca^{2+}. Ba-alginate is extremely stable and can even withstand dissolution by 50 mM citrate-solution for many days.

Quick-start Guide to Encapsulate Cells in an Alginate-Poly-L-Lysine-Alginate Membrane

Procedure	Time (min)
Re-suspending cells in 2 mL of MOPS buffer and mix with 10 mL of BUCHI alginate	-
Produce alginate droplets on BUCHI Encapsulator and hardening in 200 mL of gelling solution	10
Washing beads with 200 mL of MOPS buffer	5
Incubate beads in 50 mL of poly-L-lysine solution	15
Washing beads with 200 mL MOPS buffer. Repeat X2	5 (15 in total)
Incubate capsules in 100 mL of 0.03 % alginate solution	5
Washing capsules with 200 mL MOPS buffer. Repeat X1	1 (2 in total)
Incubate capsules in 200 mL of solubilization solution	15
Washing capsules with 200 mL MOPS buffer. Repeat X2	5 (15 in total)
Addition of media and recovering capsules in collection flask	-

Results



(i) (ii)

Figure 7: Light microscope image of encapsulated CHO cells in alginate-poly-L-lysine-alginate microcapsules using the outlined procedure on the BUCHI Encapsulator B-395 Pro. (i) Capsules immediately after performing the experiment. (ii) Cell (CHO) containing capsules after 192 hr in culture.

References

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[7] http://www.stockmarketmediagroup.com/nuvilex-inc-will-enter-phase-iii-trials-with-advantages-using-cell-encapsulation/

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