R. H. Wijffels
(Ed.)

Immobilized Cells

Springer
Lab Manual

Description of the Immobilisation Procedures

DENIS PONCELET, CLAIRE DULIEU, and MURIEL JACQUOT

Introduction

Describing all the different methods of cell immobilisation would require a complete encyclopaedia. An exhaustive revue was however published four years ago [Willaert, 1996] and may be used as reference. In the present contribution, the most common techniques of microencapsulation will be described. The reader may easily extrapolate most other methods too.

Encapsulation of cells is based on two main steps: droplet formation followed by solidification (gelation, membrane formation). In a first part, methods required to form droplets will be described using simple drawings and equations. Most systems may be easily fabricated, using standard commercial items and small set-up. In the second part, bioencapsulation methods themselves will be described.

Droplet formation methods (Lab Scale)

The most simple method to form a droplet is to extrude a liquid through a nozzle using a syringe connected to a needle. However, it results in large beads (2 to 4 mm). The following advice may be applied to all devices:

- Continuous liquid flows may be obtained using a syringe pump (most lab suppliers) or by applying air pressure on the syringe or a vessel (EFD Inc., USA, see Figure 1a). The syringe pump allows to set up directly the

✉ Denis Poncelet, ENITIAA, Rue de la Géraudiere, BP 82 225, Nantes, 44 322, France (*phone* +33-251-785425; *fax* +33-251-785467; *e-mail* poncelet@enitiaa-nantes.fr; *homepage* BRG.enitiaa-nantes.fr)
Claire Dulieu
Muriel Jacquot

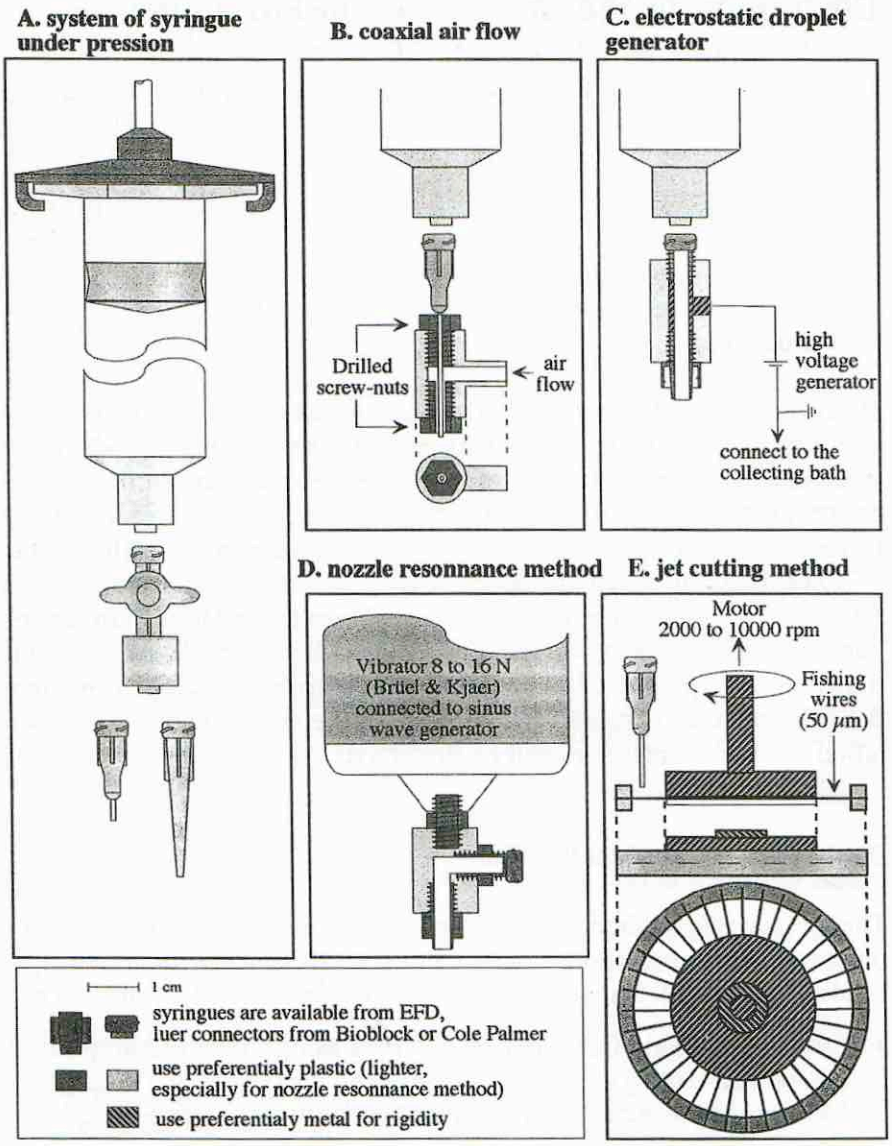

Fig. 1. Dropping devices

flow rate. An application of pressure on the syringe permits extrusion of fluids with higher viscosities and higher flow rates.

- Pressure required to obtain a desired flow rate is mainly proportional to the length of the needle and to the inverse of the internal nozzle diameter. It may be best to select the shortest needle cut at 90 °C (Hamilton, Switzerland or EFD Inc., USA) or, preferably, a tapered injector (EFD Inc., USA). Other injectors (such as those used for printer nozzles) may be more efficient (shorter length) but more difficult to set up.
- By using shorter and larger tubing connections and low resistance injectors, the necessary pressure may be decreased by a factor up to 5, allowing the handling of highly viscous liquid.

Different systems may be adapted to the nozzle to obtain smaller droplets, low size dispersions and larger flow rates (Figure 1b to d). Two working regimes exist to form droplets. At low flow rates ($\approx$ 30 ml/h), liquid exits the nozzle as droplets. For larger flow rates, the liquid forms a jet which is broken into smaller droplets.

Within the droplet formation dropping regime, smaller droplets may be obtained by applying a coaxial air flow around the needle (Figure 1b). While increasing the air flow rate, smaller droplets are obtained but it leads quickly to droplet size dispersion and spray dispersion [Poncelet, 1993]. This method is only recommended for low flow rate and droplet size larger than 800 µm.

An alternative method involves application of an electrostatic potential between the needle and the collecting solution (Figure 1c). The droplet diameter is mainly defined by the following equation [Poncelet, 1998]:

$$d^3 = d_0^3 \, (1 - U^2/U_c^2) \qquad (1)$$

where d is the bead diameter, do is the beads diameter without electrostatic potential (2 mm), U is the applied potential and Uc the critical potential ($\approx$ 4 kV). When U approaches Uc, the liquid forms a jet which breaks into droplet of 150 to 250 µm (independently of the potential electrostatic value). Narrow size distribution (less than 15 %) is obtained. Until now, experiments have only been conducted for low flow rate (30 ml/h). No commercial system is available.

In the jet regime, the nozzle resonance method (Figure 1d) involves application of a vibration to the liquid or nozzle. This method will be more fully described in Chapter 14. As a very simple approach, the size of the droplets is equal to 1.8 times the internal nozzle diameter and the size distribution limited to 5 %. The maximum flow rate is approxi-

mately equal to 3 l/h times the cube of the droplet diameter (in mm), allowing very high flow rate for large droplets. The jet may not break into small droplets (less than 800 μm) for high viscous liquid (more 0,5 Pa · sec) due to damping effects. Assembly set-up must avoid any external vibration. Several companies have proposed such systems (Inotech, Switzerland; Sodeva, France; Microdrop, Germany; Institute für Mikrotechnik Mainz, Germany).

Another simple method is to cut the jet using a vaned "wheel" (Figure 1e) [Pruesse, 1998].This device is simple to build and operate and its results in similar droplet size dispersion and flow rate as the resonance nozzle method. However, this technique may be less limited in regard to the solution viscosity than the resonance nozzle method. For droplet sizes lower than 500 μm, building of the device may be more difficult requiring a very high speed motor (10 000 to 40 000 rpm). As this method is useful for high flow, it will be more fully described in Chapter 13. geniaLab BioTechnologie (Germany) has proposed a commercial form of this device.

Emulsification methods (Lab Scale)

At the laboratory scale, emulsification is performed as a batch operation (Figure 2). A continuous system will be described in Chapter 13. It may be difficult to extrapolate directly from lab scale to pilot and industrial scales. It is necessary to respect some guidelines in building this device (see Figure 2 for an operational design):

- For expensive constituents, use not less than 100 ml beaker but larger volume (500 ml or even more) is preferable.
- In many cases, encapsulation involves polymers that will accumulate in every dead (unmixed) zones. Preference should be given to a round bottom reactor (Figure 2).
- Baffles (see Figure 2) enhance the effect of the mixing and allow the establishment of a relation between droplet size and impeller speed.
- Liquid height in the container must be approximately equal to reactor diameter.
- Turbine is the most efficient impeller. Marine would work at small scale but is more efficient at pumping liquid than creating shear breakage.

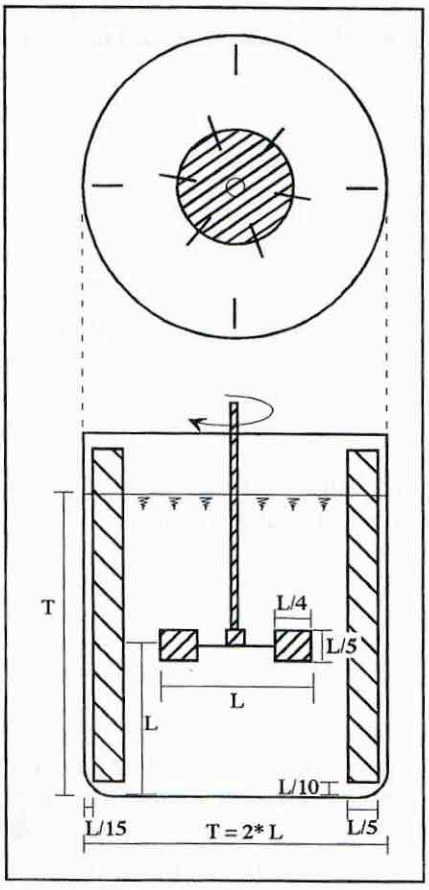

Fig. 2. Design for a turbine reactor

- Dispersed phase fraction may range from 15 to 25 % of the liquid volume. Higher values lead to larger size dispersion.
- As a first estimate, equations [Wang, 1986] may be used to compute the mean droplet diameter (50 to 500 μm).

$$\frac{D_{32}}{L} = 0.053 We^{3/5} \tag{2}$$

with $We = \rho_c N^2 L^3/\sigma$ and $D_{32} = \frac{\Re_i n d_i^3}{\Re_i n d_i^2}$ where D_{32} is the slauther or the mean surface diameter, L the turbine diameter, We, the Weber's number, ρ_C the density of the continuous phase, N the turbine rotational speed, σ the interfacial tension and d_i the individual drop diameter.

- Size distribution follows mainly a log normal law. But for simplicity, most authors have described it as a normal law with standard deviation generally ranging between 30 % and 50 %.
- To ensure equilibrium of the dispersion, mixing must be maintained for 5 min (low viscosity) to 15 min (high viscosity, polymer solutions).
- Addition of emulsifiers reduces diameters and size dispersion. For water in oil dispersion, Span 85 at 1 to 2 % may be used.
- Separation and washing of the microcapsules from oil may be a tedious problem, especially for small sizes. In most simple situations, after "solidification" of the droplets, microcapsules are filtered on buchner with a 50 μm nylon mesh and washed with a spray of water or adequate buffer.
- Rapid solidification process (at least at droplet surface) is necessary to get individual microcapsules and narrow size dispersion.

Subprotocol 1
Ionic Gelation (Alginate Beads)

Principle Alginate solution gels in presence of most di- and trivalent cations such as calcium, barium or aluminium. The simplest method to form alginate beads involves droplets extrusion of alginate solution into calcium solution (method 1: extrusion/external gelation) [Kierstan, 1977]. Another method involves dispersing alginate in oil phase and then releasing calcium from inside the droplets by gentle acidification (method 2: emulsification/internal gelation) [Poncelet, 1995].

Materials

- Droplet formation (method 1) or emulsification (method 2) device
- beakers, magnetic stirrer, 50 μm nylon mesh filtration device
- 100 mM calcium chloride solution
- 2 % alginate solution (Kelco, UK; SWK, France; Pronova, Norway):
  - In a beaker introduce 100 ml deionised water.
  - Mix gently with a magnetic stirrer.

  - Slowly disperse 2 g alginate powder on the liquid surface.
  - Leave to stand for two hours to allow alginate grain swelling.
  - Mix strongly for 15 min.
  - Adjust pH to 7.5 to 8 (method 2).
  - Leave to stand overnight to deaerate
- freshly prepared very fine 10 % calcium carbonate (2 μm grains, Setacarb from Omya, France) suspension in deionised water (method 2)
- canola oil mixed with 1.5 % span 80 (method 2)
- 0.5 ml acetic acid in 10 ml canola oil (method 2)

Procedure

Method 1: Droplet formation & external gelation

1. Introduce 20 ml alginate into the droplet formation device.
2. Introduce 100 ml calcium chloride into a beaker.
3. Mix with a magnetic stirrer at low speed.
4. Extrude the alginate solution dropwise into the calcium bath.
5. Leave to stand for 30 min to insure full gelation.
6. Filter the bead on a 50 μm nylon mesh and rinse with water.
7. Hold beads in calcium chloride solution.

Method 2: Emulsification & internal gelation

1. Introduce 100 ml oil phase into the emulsifying device.
2. Homogeneously disperse 2 ml of calcium carbonate suspension into 20 ml alginate solution.
3. Start the mixing and add the alginate mixture to the oil phase.
4. Let mix for 15 min, then add the acetic acid/oil solution.
5. Allow to gelify 10 min.
6. Filter on 50 μm nylon mesh and wash with calcium chloride solution.

Results

As alginate droplets shrink during gelation, approximately 10 g of spherical alginate beads may be obtained. Diameter and size distribution is a function of the dispersion device.

Comments

- Further information about this method may be found in Martisen 1989.
- Material to be encapsulated is introduced to the alginate solution. Procedure must then be adapted to keep around 2 % final alginate concentration.
- Bead strength is a function of the guluronic contents more than the viscosity or molecular weigh. Low viscous alginate solutions facilitate the handling and extrusion.
- Higher strength may be obtained by using barium, aluminium or higher concentration calcium solutions. Increasing concentration of the alginate solution may also enhance the bead strength but hinder the solution handling due to high viscosity.
- Internal gelation and for external gelation, with high calcium concentrations or addition of sodium ions in the gelation bath lead to homogeneous alginate concentration through the bead.

For method 2 (emulsification/internal gelation)

- Small calcium carbonate grain size ($\approx 2\ \mu m$) and good dispersion of the grains in the alginate solution are important to achieve good internal gelation.
- Initial alginate mixture pH must be higher than 7.5 to avoid internal pre-gelation.
- Complete gelation is reached at pH 6.5. Alginate mixture may be buffered to limit pH drop. But, quantity of added acetic acid must be kept constant (to maintain transfer rate).

Subprotocol 2
Thermal Gelation (κ-Carrageenan Beads)

Principle

Thermal gelation is based on the gelification of polymer solution by temperature decrease. κ-carrageenan beads may be obtained by dropping hot κ-carrageenan solution from a temperature controlled droplet extension device into a cold KCl solution (method 1: extrusion). An alternative method consists in dispersing the warm κ-carrageenan solution into vegetal oil and then dropping the temperature by adding cold oil (method 2: emulsification) [Audet, 1989].

Materials

- Droplet formation (method 1) or emulsifying (method 2) device with temperature control
- beakers, magnetic hot plate stirrer, 50 μm nylon mesh filtration device
- 200 ml vegetal oil (method 2)
- 200 ml KCl 0.3 M
- 3 % κ-carrageenan solution (Kelco, UK; SWK, France; Copenhagen Pectin, Denmark)
  - Heat 100 ml of deionised water at 80 °C in beaker.
  - Maintaining the temperature and mixing with a magnetic stirrer, add 3 g of κ-carrageenan.
  - Leave to stand until complete dissolution.
  - Reduce the temperature (to 45 °C for direct use).

Procedure

Method 1: Dropping method

1. Heat 20 ml of 2 % κ-carrageenan solution to 45 °C.
2. Introduce it into a temperature controlled dropping device.
3. Place 100 ml of cold (10 °C) KCl solution in a beaker.
4. Drop the κ-carrageenan solution in the KCl solution under gentle magnetic stirrer agitation.

5. Leave to stand for 15 min before collecting beads and keep in KCl solution.

Method 2: Emulsification method

1. Heat 100 ml of vegetal oil and 20 ml of 2 % κ-carrageenan solution to 45 °C.
2. Cool 100 ml of oil and 200 ml KCl solution to near 4 °C.
3. Place the warm oil in a temperature controlled emulsifying device.
4. Under agitation, add 20 ml of κ-carrageenan solution.
5. After 15 min, add 100 ml cold oil to the reactor.
6. After 5 min, pour the dispersion into 200 ml of cold KCl solution.
7. Let beads transfer in aqueous phase before removing oil.
8. Collect beads on a 50 μm nylon mesh and keep in KCl solution.

Results

Both procedures lead to around 20 g of κ-carrageenan beads.

Comments

- The main drawback of κ-carrageenan is the need to introduce the encapsulated material in a hot solution (45 °C). However, Copenhagen Pectin (Denmark) proposes κ-carrageenan with a low melting point (28 °C).
- Different additives have been proposed to enhance κ-carrageenan bead strength such as 10 % Locust bean gum [Arnaud, 1989] (SKW, France; Kelco, UK) or Konjac (FMC, USA).
- The method may be adapted for 2 % agarose or gellan gum in cold water (Kelco, UK) [Norton, 1990].

Subprotocol 3
Ionic Polymer Coating (Chitosane on Alginate Beads)

Principle

Polyanionic beads may be coated by polycationic membranes by simply suspending beads in the polycationic solution. This first coating may be followed by a second coating with a polyanionic polymer, and so on.

Materials

- beakers, magnetic hot plate stirrer, 50 µm nylon mesh filtration device
- 10 g of alginate beads
- 100 ml chitosane solution 0.2 % (Pronova, Norway; Primex, Norway)
  - Introduce 1 % acetic acid solution into a beaker.
  - Under agitation slowly, add 0.2 g of chitosane and let stand until dissolution.
  - Raise the pH to 6.
- 100 ml alginate solution 0.2 % (dilute from 2 % solution, see above for preparation)

Procedure

1. Introduce 100 ml of chitosane solution in a beaker.
2. Under gentle agitation, add 10 g of alginate beads.
3. After 30 min, filter and rinse with deionised water.
4. If required repeat 1 to 3 with alginate solution 0,1% then chitosane again, and so on.

Results

Coating leads often to reduction of the internal polymer bounds and then to swelling. 15 to 20 g of coated beads may be obtained from 10 g of alginate beads.

Comments

- Useful information about this method may be found in [Gaserod, 1998].
- This procedure has been mainly used with alginate beads coated but poly-L-lysine, polyethyleneimine and poly-ornithine have been tested as coating material.
- Multicoating allows a better control of the membrane molecular cut-off but may reduce mass transfer.
- Final polyanionic coating is preferred for biocompatibility in transplantation.
- Lower molecular weight of the coating material leads to heavier coating.
- In case of coated alginate beads, the alginate may be dissolved by suspending beads in 10 % sodium citrate.

Subprotocol 4
Coating by Transacylation Reaction

Principle By suspending hydrogel beads containing a polysaccharidic ester and a polyamine in an alkaline solution, transacylation between these components leads to a membrane formation at bead periphery [Lévy, 1996].

Materials

- beakers, magnetic plate stirrer, 50 μm nylon mesh filtration device
- 5 g of alginate beads prepared by droplet formation methods (see above) with a solution of 1 % alginate, 2 % propylene glycol alginate (PGA) and 5 % human serum albumin (HSA).
- NaOH 1 M and HCl 1 M

Procedure

1. Introduce 50 ml of deionised water and 5 g of alginate beads in a beaker.
2. Under gentle agitation, add 800 μl of NaOH 1 M.

3. After 15 min, neutralise with HCl 1M.
4. After 15 min, filter beads and rinse with deionised water.
5. Internal bead may be dissolved by suspension in 50 ml of 10 % sodium citrate.

Comments

- Coating thickness is controlled by the residence time in the alkaline buffer.
- pH remains neutral inside the beads. Its rises only in the microenvironement of the forming membrane.
- PGA may be replaced by pectin (less strong membrane) and HSA by several proteins such ovalbumin, haemoglobin (or most probably other polyamine materials).

Subprotocol 5
Polyelectrolyte Complex Membrane (Sulfoethylcellulose/Polydiallyldimethyl Ammonium Chloride)

Principle

By dropping a polyanionic solution in a polycationic solution, it forms a membrane around the droplets by polymer ionic interactions (and precipitation or coacervation) resulting in stable microcapsules [Dautzenberg 1996].

Materials

- droplet formation device
- beakers, magnetic stirrer, 50 μm nylon mesh filtration device
- 20 ml of 3 % sodium sulfoethyl cellulose (SSEC, Wolf Walsrode, Germany)
- 100 ml Polydiallyldimethyl ammonium chloride solution 0.5 % (PDADMAC, Wolf Walsrode, Germany; Clariant, Germany)
- 100 ml of 0,9 % NaCl solution

Procedure

1. Introduce 100 ml of PDADMAC solution in a beaker.
2. Introduce 20 ml of SSEC solution into the droplet formation.
3. Under gentle agitation, drop the SSEC solution into PDADMAC solution.
4. Leave to stand under agitation for 30 min.
5. Filter capsules and rinse with water.
6. Stock them in saline solution.

Comments

- Capsules have thin, strong and well defined porosity (molecular cut-off as low as 3000 Daltons).
- Viscosity of the droplet solution must be higher than collecting solution.
- Many material may be used to form such capsules [Hunkeler, 1997] but strong charges at least on one of the polymers are required to get good capsules. Excess charges may lead to capsule shrinking.
- Properties of the membrane are very dependent on polymer characteristics such as polymer molecular weight and its distribution.

References

Arnaud J. P., Choplin L. et al. (1989) Rheological Behaviour of Kappa-Carrageenan/ Locust Bean Gum Mixed Gels. J Texture Stud 19: 419-430

Audet P., Lacroix C. (1989) Two-Phase Dispersion Process for the Production of Biopolymer Gel Beads: Effect of Various Parameters on Bead Size and their Distribution. Proc Biochem 24: 217-225

Dautzenberg H., Arnold G., et al. (1996) Polyelectrolyte complex formation at interface of solutions. Progr. Colloid Polym. Sci. 101: 149-156

Gaserod O. (1998) Microcapsules of Alginate-chitosan: a study of capsules formation and functional properties. PhD Thesis, NTNU, Throndheim, Norway

Hunkeler D. (1997) Polymers for Bioartificial Organs. Trends in Polym. Sci. 5:286-292

Kierstan M., Bucke C. (1977) The Immobilization of Microbial Cells, Subcellular Organelles, and Enzymes in Calcium Alginate Gels. Biotechnol Bioeng 19: 387-397

Lévy M.-C., Edwards-Lévy F. (1996) Coating alginate beads with cross-linked biopolymers: a novel method based on a transcylation reaction. J. Microencapsul. 13: 169-184

Martisen A (1989) Alginates as immobilization materials, PhD Thesis, NTH, Trondheim, Norway

Norton, S., Lacroix C. (1990) Gellan Gum Gel as Entrapment Matrix for High Temperature Fermentation Processes: a Rhelogical Study. Biotechnol Technique 4(5): 351-356

Poncelet D., Poncelet De Smet B., Beaulieu C., Neufeld R.J. (1993) Scale-up of gel bead and microcapsule production in cell immobilization. in: Fundamentals of annimal cell encapsulation and immobilization. CRC Press, Boca Raton, USA, pp 113-142

Poncelet D., Poncelet De Smet B., et al. (1995). Production of alginate Beads by Emulsification/Internal Gelation II: physicochemistry.Appl Microbiol Biotechnol 42: 644-650

Poncelet D., Neufeld R.J., et al. (1999) Formation of Microgel beads by electric dispersion of polymer solution. AIChE J. 45(9) 2018-2023

Prusse U., Fox B., et al. (1998) The jet cutting Method as a new immobilization technique. Biotechnol. Techn. 12, 105-108

Wang C.Y., Calabresse R.V. (1986) Drop Breakup in turbulent stirred-tank contactors. AIChE J. 32: 657-676

Willaert R.G, Baron G.V. (1996) Gel entrapment and microencapsulation: methods, applications and engineering principles. Reviews Chem. Eng.12: 1-205

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