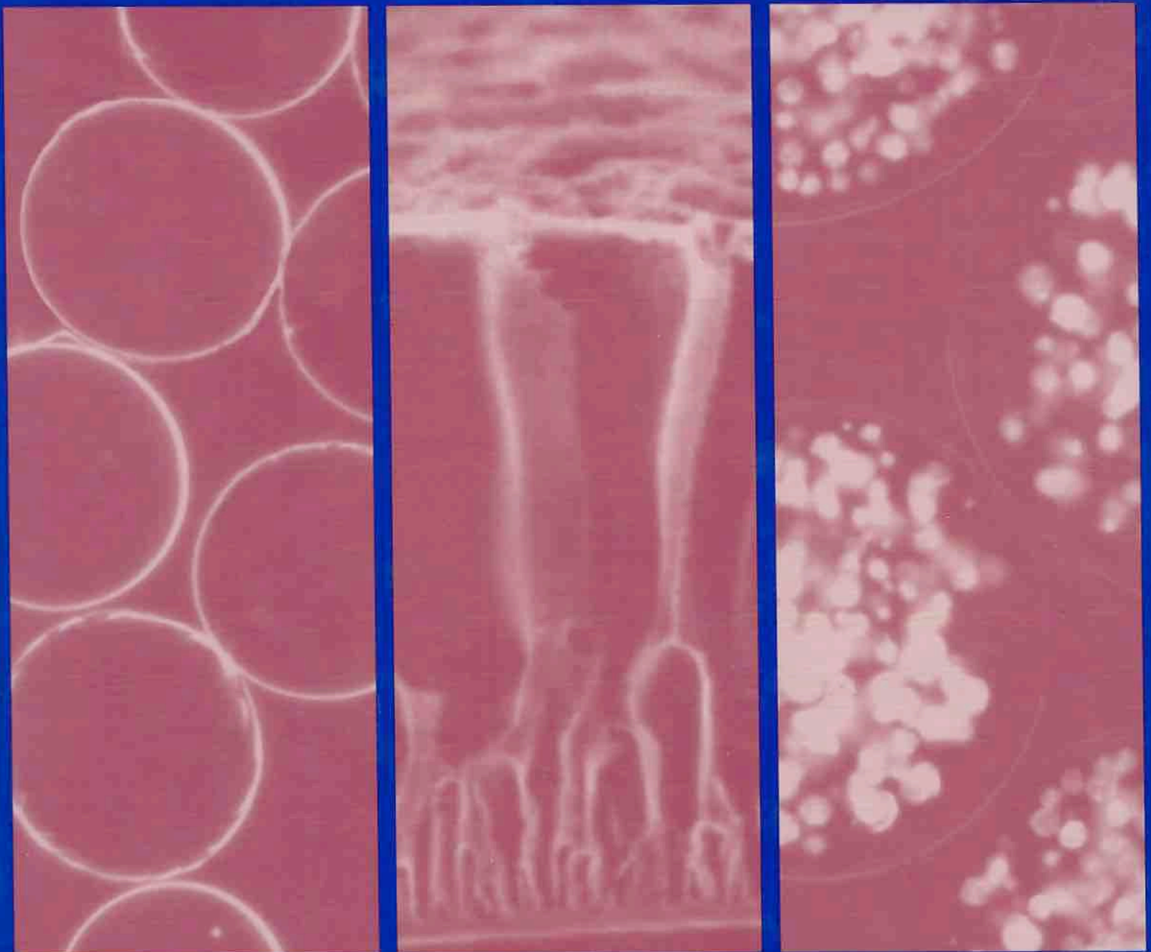
Tissue Engineering

Cell Encapsulation Technology and Therapeutics



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Encapsulation and Immobilization Techniques

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Introduction

Cells are generally found in an immobilized state in natural environments. In biotechnology, immobilization provides protection to the cell from unfavorable conditions, washout, shear, and immunological rejection. Moreover, immobilization provides for an organization of the cells, enabling synergistic interactions between adjacent cells. Multicellular organisms result from higher levels of cell organization and structure that result from a form of cell immobilization. It is therefore not surprising that scientists and engineers seriously consider cell immobilization as a means of solving technological problems involving the handling and processing of cellular materials.

Cell immobilization methods may be classified into three categories, as illustrated in Figure 1.1. This classification is mainly based on the methods used for immobilization. In aggregation, adhesion, and porous carrier adsorption, immobilization results from attachment between cells and/or between cells and the support. In the case of porous carriers, cells first migrate into the porous matrix before being fixed to the carrier. Attachment may be due either to the production of adhesive polymers by the cells or to external ionic or covalent cross-linkers. In some cases, cell selection or genetic modifications are performed to promote cell aggregation.

Cells may also be maintained in a defined volume by use of preformed devices. The barrier may be formed by a membrane dividing the reactor into two parts (membrane reactor) or through the use of hollow fibers. The volume restriction may also be obtained by recycling cells in the reactors after centrifugation, settling, or ultrafiltration. The immobilization device is built in the absence of cells, and immobilization is initiated by addition of the cell suspension.

Bioencapsulation, as the third alternative, involves immobilization of the biologically active component in hydrogel beads or microcapsules. Cells are mixed into the hydrogel or a membrane-forming material, then dispersed dropwise. Gelation or membrane formation is obtained through physical (temperature) or chemical (cross-linker) modifications.

Each method of immobilization has advantages and drawbacks. Selection is a function of the application, the cell line, and other scientific, technical, and economic criteria. For example, the cost of immobilization is more important in food production, compared to some medical or pharmaceutical applications. The food industry may be motivated to reduce investment cost while maintaining strong attachments to traditional technologies. Moreover, food and drug regulations, and the effects of toxic reagents on the cells, may limit the methods and the materials to be applied for immobilization. It is therefore difficult to outline clear guidelines for selecting an immobilization method. In many cases, more than one method, or a combination of methods, may serve the specific requirements.

Table 1.1 summarizes the major reviews on cell immobilization. The review by Willaert and Baron (1996) is particularly exhaustive, with over 1000 references. This chapter will deal more specifically with bioencapsulation. Additional information may also be found through the Bioencapsulation

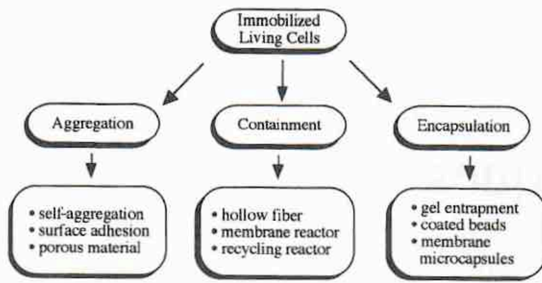

FIGURE 1.1. Classification of the cell immobilization systems.

Research Group Web site located at *http://ensaia.u-nancy.fr/BRG/BRG.html.*

Bioencapsulation Methods

Bioencapsulation methods involve two main steps. First, the internal phase containing cells is dispersed into small droplets, which are then solidified by gelation of the droplets or membrane formation at the droplet surface.

Droplet dispersions are formed by dropwise extrusion, or emulsification of the internal phase into an immiscible external phase. Many methods may be used to solidify the capsules, including gelation by ionic binding, temperature change, or polymerization, or by cross-linking of prepolymers. A membrane coat may then be applied to the gel bead by ionic polymer coating, polymer transacylation, or spray coating, or via direct membrane formation by coextrusion and external layer precipitation or interfacial polymerization or coacervation. These methods will be described more fully in subsequent chapters of this review.

Diameter and Size Distribution of Microcapsules

Diameter is one of the more important properties of gel beads and capsules. Most formulation processes include the diameter as an input parameter. It is therefore necessary to clearly define the optimum size as a function of the application and to choose a droplet formation process that ensures both correct diameter and minimum size distribution.

The capsule itself must be sufficiently large to contain the cell, cell aggregate, or product in the case of growing cells. There are also practical advantages to larger capsules or beads, as they are easier to handle during washing and settling operations and in reactor operations such as fluidization. In many cases, the cells must be homogeneously distributed within the internal capsular matrix. The probability of finding cells in the microcapsule is therefore related to Poisson's law (Nir et al 1990). To ensure at least one cell per capsule, the mean cell number per capsule must be higher than four.

On the other extreme, excessively large capsules create an internal dead volume. Implantable microcapsules must be smaller than half the internal diameter of the injection needle. In the case of a bioreactor, the shear or abrasion effects on capsules increase dramatically with diameter (Dos Santos et al 1997, Poncelet and Neufeld 1989), and large capsules may lead to mass transfer limitations.

TABLE 1.1. Reference books and reviews on cell immobilization.

Title	Reference
Immobilized cells and organelles	Mattiasson, 1983
Immobilized cells and enzymes	Woodward, 1985
Immobilizierte biokatalysatoren	Hartmeier, 1986
Process engineering aspects of immobilized cell systems	Webb et al, 1986
Immobilized cells: principles and applications	Tampion and Tampion, 1987
Bioreactor immobilized enzymes and cells	Moo-Young, 1988
Fundamentals of animal cell encapsulation and immobilization	Goosen, 1993
Wastewater treatment with microbial films	Iwai and Kitao, 1994
Immobilized biosystems: theory and practical applications	Veliky and McLean, 1994
Special issue on immobilized cell technology in food processing	Champagne, 1994
Immobilized living cell systems: modeling and experimental methods	Willaert et al, 1995
Gel entrapment and micro-encapsulation: methods, applications, and engineering principles	Willaert and Baron, 1996

The optimum size is often a compromise. In fermentation, the proposed size is generally around 2 mm to facilitate handling, or 800 μm to reduce the mass transfer limitation. In transplantation, the microcapsule diameter is generally in the range of 300 to 800 μm, and many authors limit the size to less than 500 μm.

Size dispersion may also play an important role in microcapsule behavior. Although uniform diameter, or monodispersed, capsules provide zero-order kinetics for product release, polydispersed capsule preparations result in first or apparent second-order kinetics (Poncelet et al 1988). Also, in a bioreactor, microcapsule mechanical resistance decreases with increasing diameter, and level of mixing or shear, resulting in the mass transfer efficiency. If the capsules are size-dispersed, the level of shear must be limited by the largest-diameter capsules.

It is therefore important to limit the size dispersion as much as possible. Although production of large monodispersed capsules is now feasible, it is still a tedious problem to produce diameters less than 800 μm, especially on a large scale. The size dispersion, expressed as standard deviation may range from 5% of the mean for 3 mm capsules to more than 50% for 300 μm capsules (Poncelet et al 1993).

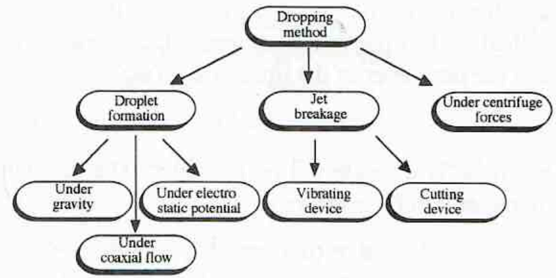

FIGURE 1.2. Dropping methods.

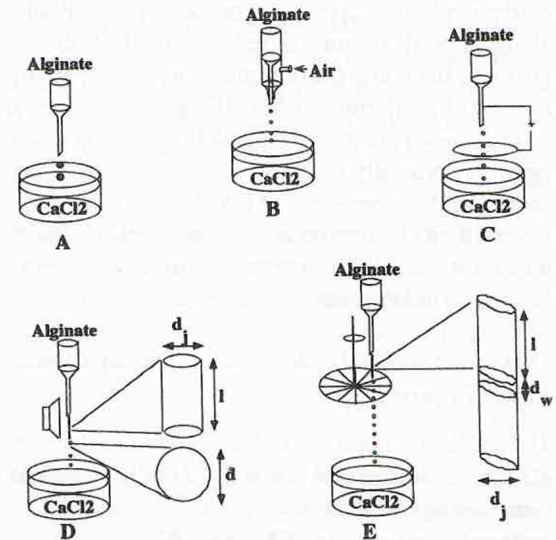

FIGURE 1.3. Dropping devices.

Droplet Formation and Bioencapsulation

Capsules are formulated by droplet extrusion or emulsification. In selecting an appropriate method, the following parameters must be taken into account: desired mean size, acceptable size dispersion, scale of the production, and the maximum level of shear that the cells may tolerate. Although important, tolerable levels of shear for cells is difficult to define, particularly when they are suspended within rheologically complex internal phase media. The following sections outline the various techniques used to form droplets.

Droplet Extrusion

When a liquid is forced through a nozzle or needle, it is extruded initially as individual droplets. With increasing flow rate, the pendent droplet tends to stretch before detachment, becoming more pronounced until the extruded liquid forms a jet or continuous stream from the needle tip. The jet stream will then break naturally into small droplets. Extrusion methods may be divided in two classes: drop formation/extrusion and jet breakage. The limit between the two techniques or regimes is determined by the minimum jet velocity, $u_{j,min}$:

$$u_{j,min} = 2(\sigma / \rho dj)^{0.5} \tag{1}$$

where σ is the surface tension, ρ the density of the flowing liquid, and d_j the jet diameter. Figure 1.2 summarizes the different extrusion methods proposed in the literature, and Figure 1.3 illustrates the devices used for producing droplets.

Drop Formation Under Simple Gravity (Figure 1.3A)

The simplest method to form individual droplets is to let a liquid droplet fall from the tip of a needle. The mass of the droplet, *m*, will then be determined by the equilibrium between the gravity force m g

and the forces acting to maintain the droplet attached to the tip (product of the surface tension, γ, and the perimeter of the tip, $2 \pi d_e$, Tate's law):

$$mg = 2\pi d_e \gamma \qquad (2)$$

where d_e is the external tip diameter. The droplet diameter will be given by

$$d = (6m / \pi\rho)^{1/3}. \qquad (3)$$

The real diameter of the capsule needs to be corrected by a swelling or shrinkage factor due to the entrapment or encapsulation process. For example, alginate bead volume is reduced by half during gelation. In contrast, nylon microcapsules swell by a factor of 1.3 during washing (Poncelet et al 1988).

The droplet diameter obtained by extrusion under gravity is typically larger than 2 mm, even for very small needle diameters. Also, the flow remains limited by jet formation at an order of magnitude of mL/h. Interest in this simple system may therefore be limited to laboratory-scale research.

Drop Formation Under Coaxial Air or Liquid Flow (Figure 1.3B)

The application of a coaxial air jet around the needle has been proposed (Lane 1947) to increase the force acting on nascent drops. The air jet may be replaced by a liquid jet (Charwat 1977), permitting a better control of the viscosity, surface tension, and density of the entraining phase, through selection of an appropriate liquid.

Many laboratory studies on cell encapsulation are largely based on air-jet systems, while liquid jets have received limited interest (Dupuy et al 1988). Both methods produce beads or microcapsules ranging from a few micrometers to one millimeter. However, the flow rate remains very limited, to less than 30 mL/h, to avoid formation of a liquid jet. The size dispersion increases drastically when the droplet diameter is decreased (Poncelet et al 1993). For these reasons, the coaxial fluid jet systems have not been considered for scale-up. Even on the laboratory scale, this method is being replaced by the technologies described below.

Drop Formation Under Electrostatic Potential (Figure 1.3C)

Drop formation is greatly improved by replacing the drag force with a high electrostatic potential between the capillary and the collecting solution (Burgarski et al 1994a, 1994b; Poncelet et al 1994). Alternately, electric potential may be applied between the capillary and a stainless steel ring placed below the capillary. Increasing the electrostatic potential, U, to a critical value, U_{cr}, leads to a decrease in the droplet size, d. For higher values, the liquid exits the tip as a jet that breaks itself into small droplets.

The mass of the droplet detaching from the tip is given by equating the sum of gravity forces, $m\,g$, plus electric forces, Fe, to the surface tension forces, $2\,p\,d_s\,\gamma$ (Poncelet et al 1998):

$$mg + Fe = 2\pi d_s \gamma \qquad (4)$$

with

$$Fe = \pi\epsilon_0 (d / 2h)^2 U^2 \qquad (5)$$

where ϵ_0 is the electric permittivity of air, h the distance between the pendent droplet and the collecting solution, and d_s the diameter of the droplet detachment section.

In fact, the electric force is relatively small, and thus plays a secondary role in reducing droplet diameter. The primary reason is that charged molecules moving to the droplet surface create a repulsion between molecules at the air-liquid interface, counteracting the surface tension. The resulting decrease of surface tension force is then

$$\gamma = \gamma_0(1 - \epsilon_0 U^2 / d_c) \qquad (6)$$

(where γ_0 is the surface tension at $U = 0$), resulting in droplet size reduction with increasing electrostatic potential (Poncelet et al 1998).

When the surface tension approaches zero, the liquid tends to form a jet rather than drops. Equation 6 represents an equilibrium state. In some cases, migration of the molecules to the surface is slower than the rate of drop surface formation. The real surface tension, γ, is then an intermediate value between the value given by Equation 6 and γ_0. Larger drops are therefore obtained when the flow rate is increased. Most encapsulation processes involve an interaction between a polymer and a counter-ion. The counter-ion migrates faster than the higher molecular weight polymer. Smaller drops are indeed obtained if the charge of the droplet has same sign as the counter-ion.

Bead size distribution obtained with an electrostatic generator is generally better than that obtained with coaxial air flow (standard deviation = 15%). However, satellite peaks may be observed in

the size distribution profile. Satellites are formed by breakage of the fine filament between the droplet and the needle tip just before separation, resulting in secondary peaks. The flow rate is still limited by the formation of the jet. The electrostatic potential droplet generator is a promising technique to obtain small microdroplets (down to 200 μm), at least at laboratory scale.

Vibrating Capillary Jet Breakage (Figure 1.3D)

If liquid in the capillary exceeds a certain velocity (Equation 1), it exits from the tube as a jet. Capillary jets are unstable and fracture easily, forming small droplets. Experiments have shown that the jet breaks with a specific natural frequency (Savart 1833) equal to

$$f = u_j / \alpha d_j \quad (7)$$

where u_j and d_j are the linear velocity and the diameter of the jet, and α is a factor equal to

$$\alpha = 4.44\,(1 + 3\mu / \rho\sigma dj)^{0.5}. \quad (8)$$

Rayleigh (1878) showed that if an external wave of the natural frequency, f, is applied to the jet, the jet breaks into monodispersed droplets (standard deviation equal to 5%). The jet breaks into cylinders with radius d_j and length, $\lambda = u_j/f$. From geometric considerations, the final spherical droplet diameter is given by

$$d = 1.15\alpha d_j. \quad (9)$$

The system is, therefore, simply driven by Equations 7 and 9. One could expect to produce droplets from a few microns to 3 mm at relatively high flow rate (24 L/h for 3 mm diameter carrageenan beads (Hunik et al 1993). However, with increasing viscosity, the pressure required to ensure a jet increases proportionally to the viscosity and to the fourth power of the internal nozzle diameter. The flow rate decreases drastically while producing small droplets with the highly viscous fluids generally encountered in encapsulation processes. The jet diameter, d_j, is 0.8 times the internal nozzle diameter (d_i) for water, but 1.3 times d_i for alginate solution.

Vibrating jet breakage is one of the most efficient techniques to produce large capsules (1 to 3 mm) with a narrow size distribution. A multinozzle system would enable production in the order of hundreds of liters per hour. However, it appears more difficult to use this process for microcapsules less than 800 μm in diameter.

Rotating Systems for Capillary Jet Breakage (Figure 1.3E)

To overcome the limitations of the vibrating system, Prusse et al (1996) proposed a rotating device to cut the jet into small droplets. The diameter of the cutting wires, d_w, is the main parameter determining the effectiveness of this method. To reduce loss due to the cutter, the linear velocities of the jet, u_j, and of the cutting wire, u_w, must be equal. Then, the lost fraction is approximately equal to

$$\text{Lost fraction} \approx 2\,d_w / \lambda \quad (10a)$$

where λ is the length of cut jet section. To limit the loss to 5%, λ must be higher than 40 times the cutting wire diameter, d_w. Through geometric considerations, the droplet size, assuming a negligible loss, is equal to

$$d \approx (3d_j^2\,\lambda / 2)^{1/3}. \quad (10b)$$

With a capillary of 400 μm internal diameter and 25 μm wires, the droplet diameter would be equal to 600 μm. The flow rate will only be limited by the pressure applied. Rotating jet breakage appears to be an easy, efficient, and scalable device for producing large quantities of relatively small microcapsules with narrow size distribution.

Rotating Capillary Jet Breakage

Replacing gravity by centrifugal force has been applied in the microencapsulation of food, chemical, and pharmaceutical ingredients. However, very few studies have concerned cell encapsulation. In general, the size of the collecting reservoir is very large with diameters up to 10 meters, complicating recovery of the formed microcapsules and maintaining sterility. Moreover, such systems involve high levels of shear, potentially damaging fragile encapsulants. It would be necessary to address these points before applying the technology to bioencapsulation.

Rotating extrusion devices operate by extruding liquids through nozzles at the periphery of a cylinder, or by dispersing liquids onto a spinning disk. In the first system, the liquid flows through nozzles

mainly as jets. Schlameus (1995) reports productivity levels up to 60 kg/h. The droplet diameter ranges from 500 μm to 2 mm with a standard deviation of around 15%. The limitations of jet formation involving pressure and jet diameter have to be considered.

With the spinning disk device, liquid flows on a rotating disk. Liquid exits the disk as droplets, filaments, or films, as a function of the working conditions (Chicheportiche 1993). The filament regime is of particular interest. Filaments break into small droplets in a manner similar to that of jets. Droplets as small as 50 μm may be obtained (unpublished data). No pressure is required to ensure flow, and very limited shear is applied to the cells.

In both devices described, vibration may be applied to facilitate droplet formation. In the case of spinning disks, Chicheportiche (1993) observed a very narrow size distribution (standard deviation of 5% without satellite peak formation).

Emulsification Methods

Although they are quite promising for large-scale production of small capsules, little data exist regarding emulsification methods for cell encapsulation (Audet et al 1989, Poncelet et al 1993). It is necessary to refer to the general theory of emulsification and to a limited number of papers on the encapsulation of chemicals or biochemicals by emulsification methods (Poncelet et al 1989, Ogawa et al 1972).

Emulsification methods provide capsules from a few micrometres to a millimeter in diameter. In all cases, the size dispersion is higher than with extrusion devices, ranging from 30 to 50% of the mean diameter. However, the potential for scale-up is the main advantage. Emulsification is generally performed in a reactor by means of a turbine. However, a more promising technology involves passing the two immiscible phases through a tube containing deflectors or stationary baffles, known as static mixers (Poncelet et al 1993), as illustrated in Figure 1.4. Such a system improves the size distribution and reduces shear. As concerns industrial-scale applications, it permits continuous processing, and the enclosed plumbing enables the maintenance of aseptic conditions, while not being limited by scale.

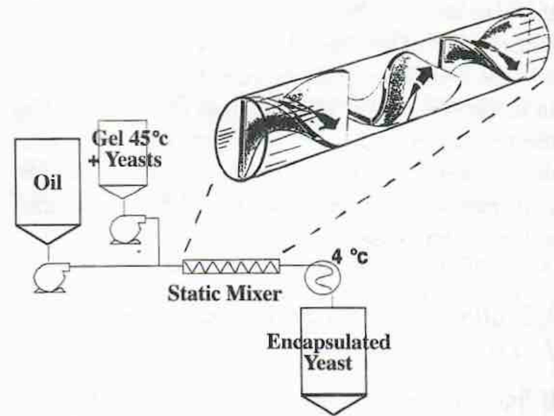

FIGURE 1.4. Static mixer.

Gelation and Membrane-Formation Methods

The most common method for producing capsules is to form gels from liquid droplets. The resulting hydrogel beads are very porous. Polymer coats are often applied to ensure better isolation and retention of the encapsulated material. The gel core of coated beads may also be liquefied, resulting in a liquid droplet retained within a membrane coat. To simplify the encapsulation process, direct membrane formation around liquid droplets has been proposed. Figure 1.5 summarizes the various bioencapsulation procedures as developed in the following paragraphs.

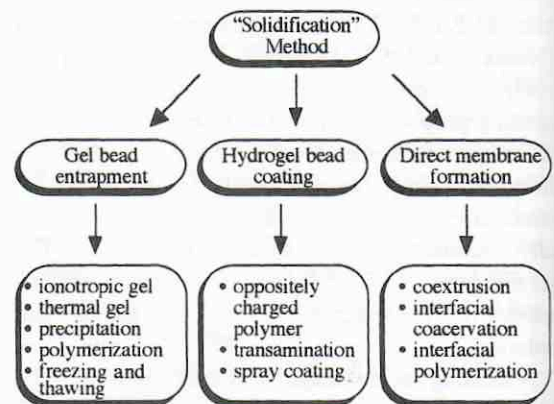

FIGURE 1.5. Bioencapsulation "solidification" methods.

A unique feature of encapsulation, in regards to other cell immobilization technologies, is that a matrix is built around the cells. Thus, the concept of biocompatibility concerns not only the encapsulating material but also the encapsulation process. Materials that are considered to be biocompatible as implantable devices, for example, are not suitable for cell encapsulation, because they may require formulation conditions that are unfavorable for cell survival, as is the case with the polymers used for hollow fiber production. Many chemical encapsulation methods would also be rejected because of the use of organic solvents or cross-linkers. The range of bioencapsulation methods is reduced for these reasons. Moreover, any process has to be carefully optimized to reduce toxic effects and to carefully and reproducibly regulate capsule structures. Cells are very sensitive to their environment; thus, the presence of chemical traces and microstructures within the gel may affect their behavior (Barbotin et al 1990).

Cell growth is possible and common in capsules. Cultivating encapsulated cells in appropriate media may make up any loss resulting from the encapsulation process, or from undesirable release through the porous matrix structure (Groboillot et al 1993). The challenge is to ensure and maintain appropriate cell behavior after encapsulation. Optimizing a bioencapsulation method is in fact a delicate process even if the technology appears simple. As an example, the development of the Moet & Chandon process to produce champagne with encapsulated yeast is the result of a 14–year research program. Also, the artificial pancreas based on islet encapsulation was initially proposed in 1979, but is still in developmental stages.

Hydrogel Bead Entrapment

Entrapment in hydrogel beads is the most commonly used cell immobilization technology, because of its simplicity and gentle formulation conditions. Numerous studies have been conducted to select encapsulation materials, to optimize processing conditions, and to characterize hydrogel beads. A large number of studies have also been devoted to the physiological behavior of hydrogel-entrapped cells.

Gel-forming materials may be classified as natural or synthetic polymers. Natural polymers are composed of polysaccharides or proteins, and synthetic polymers may be preformed, polymerized *in situ* from monomers, or cross-linked from prepolymers.

Ionotropic Gelation

Several charged polymers form gels when introduced dropwise into an oppositely charged multivalent counter-ion solution. The success of ionotropic gelation is mainly due to the mild formulation conditions, involving no pH or temperature changes, reagent toxicity, or residues. It is a simple, fast, and low-cost technique.

Alginate is the most widely used and investigated polymer for cell entrapment, by a large measure. Alginate constitutes a family of unbranched polysaccharides, mainly extracted from algae. It is composed of 1,4–linked β-D-mannuronic (M) and α-D-guluronic acid (G) residues. The monomers are sequenced in homopolymeric blocks (M-M or G-G blocks) with alternating structures (M-G blocks) (Smidsrod et al 1972, 1974). Divalent and trivalent cations, generally calcium, induce gelation by binding mainly to the guluronic blocks. Divalent cations bind within two guluronic blocks, forming a series of electronegative cavities. As it is a cooperative process, stronger gels and improved gelation are obtained with high guluronic alginate (up to 70%) containing long guluronic blocks (up to 15 residues; Martinsen et al 1989). The molecular weight of the chains has a limited impact on the gel properties.

Alginate beads are produced by dropping cell-loaded alginate into calcium chloride solution, in a procedure known as external gelation. Calcium ions diffuse into the alginate drops, forming a three-dimensional lattice of ionically cross-linked alginate. Most studies have been conducted with large beads (2 to 3 mm), produced by droplet extrusion from a syringe. The resulting beads are not homogeneous, because the alginate concentration increases from the center of the beads (2%) to the surface (up to 10%; Skjak-Braek et al 1989). Moreover, Nava Saucedo et al (1996) showed that microchannels are formed between the surface and the bead core.

To obtain higher gel homogeneity with smaller beads on a large scale, Poncelet et al (1992) formed alginate microspheres by internal gelation. Alginate solution containing dispersed insoluble calcium carbonate microcrystals was emulsified in

oil, then acidified gently by addition of oil-soluble acetic acid to liberate calcium. Internal gelation may also be obtained by dropping the alginate/calcium carbonate mixture into an acidic solution. The resulting beads are more homogeneous in composition, and the slight pH reduction during formulation (7.5 to 6.5) is unlikely to damage cells.

The major disadvantage of alginate beads is sensitivity to calcium chelators such as phosphate, lactate, or EDTA, and to cations such as sodium or magnesium, which are able to displace calcium. Because these compounds are often required in fermentation media, use of stronger gelling agents such as barium or aluminum ions and higher guluronic content alginates will improve stability of the beads (Martinsen et al 1989). Alternately, chelators are often used to liquefy the alginate when forming liquid-core microcapsules, or when recovery of the encapsulant is desired.

Chitosan is a polyglucosamine polysaccharide, obtained by deacetylation of chitin (Muzzarelli 1977). It forms gels in the presence of polycations such as phosphate (Moore and Roberts 1980). Beads are produced by dropping chitosan solution into phosphate solution (Vorlop and Klein 1981, 1987). Hydrophobic gels may also be obtained by gelation in more hydrophobic anions such as octyl or lauryl sulphate (Vorlop and Klein 1987). Mechanical stability of chitosan beads is comparable to that of alginates (Klein and Kressdorf 1989), and chitosan beads are stable in phosphate buffer. However, chitosan is water soluble only for pH levels lower than 6.5, and chitosan may interact with cell membranes, leading to loss of cell viability or activity. Use in cell encapsulation has been limited thus far.

Pectins are acidic polysaccharides extracted from plant cell walls. The extracted forms are predominantly linear polymers of 1,4 α-D-galacturonate backbone. Acidic groups are partially methoxylated. Pectic acids have less than 5% methoxyl groups. Other pectinic acids are also divided into high-methoxyl (substitution higher than 50%) and low-methoxyl pectins (LM pectinate).

Pectins (pectate and LM pectinate) gel by strong binding of calcium or aluminum ions, as is the case with alginate (Thibault and Rinaudo 1985). Pectate and pectinate gel beads are produced by dropping pectin into calcium (Gemeiner et al 1989, Toth et al 1989) or aluminum ionic solutions (Berger and Ruhlemann 1988, Navarro et al 1983). Beads are inhomogeneous, like alginates, with decreasing concentration from the surface to the center of the beads (Skjak-Braek et al 1989). Calcium pectate beads are much less sensitive to calcium chelators or competitors than alginate beads (Berger and Ruhlemann 1988). Pectate-alginate mixtures have been proposed to provide highly stable beads (Toth et al 1989).

Polyphosphazene has been proposed as a synthetic polymer to replace alginate (Bano et al 1991). Synthetic materials will reduce problems associated with the purification of natural polymers such as alginate. However, development of this system has been limited thus far.

Thermal Gelation

Thermal gelation is achieved by cooling a warm aqueous polymer solution. Beads are obtained by dropping the polymer solution into cold water, but most often the polymer solutions are emulsified in warm oil and cooled by addition of cold oil or water (Audet et al 1989) or by using heat exchangers (Neufeld et al 1991). Thermally gelled beads are generally less sensitive to destabilizing ions than ionotropic gels. However, the need to heat the polymer solution and encapsulant before gelation can limit its use for fragile cell encapsulation. Some gel manufacturers are responding by developing low-temperature gelling materials. Also, thermally gelled beads may not be suitable for mesotrophic fermentation or implantation, because they would dissolve.

Agar is obtained from red algae and consists of alternating 1,3–linked β-D-galactopyranose and 1,4–linked 3,6–anhydro-α-D-galactopyranose monomers. Substitutions (sulphate, methylether) on the β-D-galactopyranose give different gelation behavior to agar (Clark and Ross-Murphy 1987). Methylether content leads to higher gelling temperature (from 30 to 40°C). Neutral agar is the main gelling fraction, and purification of this fraction provides agarose. The gel structure is maintained mainly by hydrogen bonding.

Carrageenan has often been proposed as an alternative to alginate in fermentation processes. Carrageenans have a structure similar to agar. The 1,4–linked β-D-galactose is only partially in anhydrous form, and the percentage of sulphate substi-

tation is higher. Carrageenans are extracted from red seaweed and are divided in three types (λ, κ, and τ) (Landau 1992, Thomas 1992). κ-carrageenan forms strong gels upon cooling in the presence of potassium ions (Chibata et al 1987). Gelation may also be induced by contact with different ions such as calcium or copper, but thermal gelation is the most common method.

κ-carrageenan beads may be produced by the droplet formation technique (Buitelaar et al 1990, Woodward et al 1988), or, for large-scale production, by emulsification (Audet et al 1990). Higher gel mechanical resistance may be obtained by mixing κ-carrageenan with other gel-forming polymers such as galactomannans (Guiseley et al 1989), locust bean gum (Audet 1989), or taragum (Cairns et al 1986).

Gellan gum is a gel-forming polysaccharide produced by bacterial fermentation (Gibson 1994). It is composed of linear tetrasaccharide units that include two glucoses, one rhamnose, and one guluronic acid (O'Neil et al 1983). Guluronic residues confer anionic charges to the gellan gum. Different substituants may be attached to the chains (Kuo et al 1986) resulting in soft, elastic, and cohesive gels, while de-esterified polymers form strong, hard, and brittle gels (Gibson 1994, Rinaudo 1988). Unsubstituted gels have been reported as stronger than most other gels (alginate, agar, κ-carrageenan; Sanderson et al 1989).

Gellan gelation is achieved by cooling in the presence of stabilizing divalent cations (Norton and Lacroix 1990). Gelation temperature is a function of the gum concentration, ionic strength, and type of counter-ions, and may vary from 35 to 55°C. Stronger gels are obtained with gum mixtures of high gelation temperature. Gellan gum was first proposed for encapsulating thermophilic bacteria (Grasdalen and Smidsrod 1987, Norton and Lacroix 1990); however, by using citrate, phosphate, or EDTA sequesterants, the gelation temperature may be decreased and mesophilic bacteria may be entrapped (Camelin et al 1993) Gellan gum beads may be obtained by droplet extrusion or emulsification methods (Grasdalen and Smidsrod 1987, Norton and Lacroix 1990).

Some polymers can also gel upon slight heating (Perols et al 1997; Markvicheva et al 1991). As an example, polyvinyl capralactam is used to encapsulate enzymes and hybridoma cells (Donova et al 1993, Markvicheva et al 1991). This synthetic polymer is water-soluble, nontoxic, and inexpensive. An increase in temperature from 10 to 40°C permits gelation. Gel characteristics and gel reversibility may be modulated using various stabilizers. For example, monoclonal antibodies produced by hybridoma cells may be concentrated and recovered through bead, liquefaction initiated by a temperature drop (Markvicheva et al 1991).

Reticulated Protein Gels

Applications of proteins for cell encapsulation are relatively limited. Collagens, a family of animal fibrous proteins, are rich in glycine and proline, favoring stable triple helix formation (Bornstein and Sage 1980). In the presence of water, collagens swell and gel through ionic, hydrogen-bonding, and other interactions. Collagens dissolve at low pH; thus, encapsulation requires mixing cells with collagen at low temperature, ionic strength, and pH. Gelation is initiated by raising these three parameters. In most cases, reticulation with glutaraldehyde is necessary to obtain strong gels.

Gelatin is a hydrolyzed derivative of collagen. Reversible gelation is obtained by cooling the solution below 30–35°C (De Alteriis et al 1988). Beads are formed by dropping gelatin solution into a cool hydrophobic fluid. Addition of a cross-linker such as glutaraldehyde, formaldehyde, or chromium salt is necessary to stabilize the gel (Sungur and Akbulut 1994).

Synthetic Polymers Formed In Situ

Synthetic polymers are more flexible and have more reproducible characteristics than natural polymers, for the purpose of developing specific properties. Porosity, hydrophobicity, mechanical strength, and stability of the gel may be more precisely modified. In most cases, *in situ* polymerization leads to loss of cell viability as a result of monomer toxicity and the reactive environment. Careful control of the gel formation process is therefore important to maintain high cell viability.

Polyacrylamide gels obtained by free radical linear polymerization of acrylamide (single unsaturation) in the presence of bisacrylamide (double unsaturation) form a three-dimensional network. The

ratio between monomer and bisacrylamide determines gel porosity and strength. Free radical reaction is initiated chemically (i.e., by using persulphate) or photochemically (i.e., with riboflavin as photoinitiator). Acrylamide monomers are toxic (Lusta et al 1990), but well-controlled conditions during polymerization (low temperature, minimum polymerization time) enable high cell viability (Skryabin and Koshcheenko 1987). Spherical beads are obtained by dispersing polymerization medium that contains cells into a hydrophobic oil before polymerization (Mosbach 1984, Nilsson et al 1987).

As an alternative to acrylamide, methacrylate monomers can be used and polymerized using a cross-linking agent (Cantarella et al 1983). Again, cell toxicity results in low cell viability. Polymerization using γ-radiation at freezing temperature has been described. This process, while successful in a few cases (Carenza and Veronese 1994) has limited application for cell encapsulation.

Prepolymer resins as gel-forming materials appear to have important advantages (Fukui and Tanaka 1984), because the entrapment procedure is simple and performed under mild conditions, since the use of monomers is avoided. The gel structure may be controlled by varying polymer chain length or hydrophobic/hydrophilic balance. As an example, prepolymerized acrylamide chains, partially substituted with acylhydrazide groups (Freeman 1987), are cross-linked by dialdehydes such as glyoxal and glutaraldehyde. Prepolymer and cross-linker concentrations determine the porosity and strength of the gel. Glyoxal appears to be the best cross-linker both for gel structure and cell viability (Freeman 1987), and several other prepolymers have been proposed. An interesting development has been to clone *E. coli* to increase resistance to acrylamide monomers (Lusta et al 1990).

Freeze-Thawing Polyvinyl Alcohol Gels

Polyvinyl alcohol (PVA) is a low-cost, nontoxic polymer that becomes gelatinous upon freezing. In fact, repeated freeze/thaw cycles strengthen the gel (Nambu 1983). The gel forms by PVA exclusion from water crystals during freezing, resulting in a concentration of the polymer. The high-PVA phase forms a continuous three-dimensional network. Cells are protected during freezing with cryoprotectants such as glycerol. Temperature profiles during both freezing and thawing determine both gel structure and cell viability. PVA gels are stable to 65°C and can be used for thermophilic microorganisms (Varfolomeyev et al 1990).

PVA gels are also formed by cross-linking with boric acid (Ochiai et al 1981). The highly acidic conditions (pH 4) limit the scope of this method. Mixing PVA and alginate has been proposed, mainly to reduce the sticking tendency of PVA gel beads (Wu and Wisecarver, 1992). PVA beads can also be treated with phosphate solution to esterify PVA, strengthening the beads.

Hydrogel Bead Coating

The gel bead structure is porous, allowing for the diffusion of lower molecular weight molecules in and out of the beads. Cell immobilization often requires a reduced permeability, to further isolate cells from the external medium, as in cell transplantation. Beads can also be sensitive to their environment, as are alginate beads, which dissolve readily in citrate or phosphate. The external surface of the beads may also be modified to improve biocompatibility or bead strength. These factors have led researchers to consider applying membrane coatings to beads.

Most types of beads described above may be treated with cross-linkers such as glutaraldehyde to strengthen the external layer. Process conditions are very important to enhancing bead mechanical properties without reducing cell viability. Moreover, the use of dialdehydes can be a problem for food and medical applications, although glutaraldehyde has FDA approval. Diamines or low molecular weight polyamines could also be used to cross-link the external layer of the beads.

High molecular weight polyamine coatings are applied under gentle conditions. Alginate beads are first coated by suspension in poly-L-lysine (PLL) solution (Lim and Sun 1980), and a second alginate coat is then applied by suspending the coated beads in alginate. The PLL/alginate double coat provides a stronger membrane and enhances bead biocompatibility for transplantation (O'Shea et al 1984). The alginate core can be liquefied in citrate if a liquid core is desired. All of these steps are performed at neutral pH and room temperature, and in physiological solution, ensuring mild encapsulation conditions.

Limited alternatives have been proposed to PLL coating. Polyethyleneimine (PEI) has been tested but rejected because of biocompatibility problems following transplantation (Sun and O'Shea 1985). Poly-L-ornithine, poly-L-glutamate (Burgarski et al 1993, Young et al 1993), chitosan (McKnight et al 1988), and modified chitosan (Pandya and Knorr 1991) have all been evaluated as alternatives.

Most studies related to the control of the alginate bead coating involve PLL membranes and were performed by Goosen and coworkers in Canada (Goosen et al 1985, King et al 1987, Okhamafe and Goosen 1993). The main concerns were the molecular cut-off and mechanical strength controlled by selecting PLL molecular weight, concentration, and coating time. Similar studies have also been conducted by Vandenbossche et al (1993).

An innovative process has been proposed by Lévy et al (1996), in which alginate beads containing a protein and PEG-esterified alginate were suspended in an alkaline solution where transamination takes place between the protein and the esterified alginate. Strong covalently bonded membranes result without loss of cell viability, even for fragile cells.

Hydrophobic polymers can also be used to coat beads. The simplest method is to coextrude a hydrogel with a polymer solution (Dupuy et al 1988). Alternately, the beads may be coated by spray application of a polymer solution or a latex (fine polymer dispersion in water) in a fluidized bed reactor (Sun et al 1997).

Direct Encapsulation

A popular encapsulation procedure involves forming alginate beads, coating them with PLL and alginate, and then performing core liquefaction. This procedure is simple and involves mild process conditions, yet requires several steps, which increases cost from a process point of view and increases risk of contamination because of the increased number of operations. It would be desirable to formulate membrane-bound microcapsules in a single step. Most methods require the use of solvents or other toxic reagents or conditions. The following review is limited to techniques that have been successfully applied for cell encapsulation.

The simplest method, at least at laboratory scale, is to coextrude droplets of an internal phase that contains cells in an external polymer solution into a gelification bath (Udulag 1994). The receiving bath may be a gel initiator, or a solvent extraction medium in the case of a hydrophobic external phase. This technology has been developed by Sefton et al, using polyacrylates (Babensee et al 1992, Douglas and Sefton 1990, Sefton et al 1987), for the encapsulation of fragile cells. The main drawback of this method is the control of the process from the hydraulic point of view. Stevenson and Sefton 1993 described the different technological difficulties that must be overcome to obtain spherical microcapsules with a uniform membrane and appropriate diameter. Although scale-up may be difficult, the process is usable with a large variety of membrane materials.

Interfacial polymerization was proposed in 1964 as a process for producing artificial cells (Chang 1964). A diamine solution is emulsified within an organic phase, and an acid dichloride is added to the emulsion to initiate membrane formation. Diamine and dichloride polymerize at the droplet interface, providing microcapsules with thin but strong membrane coats. Initially, the process involved high pH levels (>10), high concentrations of diamine (0.4M), and the use of polar solvents (chloroform). Use of an internal phase containing polyamines such as gelatin, polyethyleneimine, or chitosan improves the biocompatibility of the process (pH 8 and vegetable oil as dispersing phase; Groboillot et al 1993, Larish et al 1994, Poncelet et al 1990). Higher viability was obtained with gelatin (Hyndman et al 1993). Improvements are still needed to ensure high levels of cell viability.

Microcapsules may also be obtained by dropping a charged polymer in a solution of an oppositely charged polymer, in a process known as interfacial coacervation. Polymer coacervate forms at the droplet interface, forming a continuous membrane. Many polymer combinations may be used to create such capsules. Alginate with chitosan was first proposed by Rha et al (1984), and alginate may be replaced by κ-carrageenan (Pandya and Knorr 1991). Dautzenberg (1985) used cellulose sulphate and poly(dimethyldiallylammonium chlorides) to study parameters such as the impact of the polymer molecular weight, degree of substitution, and polymer concentration on the membrane mechanical resistance or molecular cut-off (Dautzenberg 1996). Interfacial coacervation provides molecular weight cut-off values to as low as 3000

daltons. Hunkeler et al (1996) tested 1300 combinations of polymers and defined guidelines for selecting the best combinations. However, multicomponent systems, including for example capsule fillers, improve the microcapsule properties (Hunkeler 1997). Interfacial coacervation is one of the more biocompatible processes for cell encapsulation and may be the most promising alternative to the commonly used alginate-PLL system.

Applications

When considering the possibility of immobilizing cells for a particular application, the cost and technological complexity must be carefully taken into account. Mass transfer limitations, changes to cell behavior, and effects on cell viability may all affect and generally reduce the performance of the immobilized cell system. However, in many cases, immobilization is required to reach specific objectives.

In fermentation, cell immobilization (1) enables continuous operation without relying on cell growth to maintain cell density, (2) simplifies the downstream processing as it facilitates cell separation, and (3) enables the use of mixed and spatially localized microbial cultures to obtain higher yields, especially for secondary metabolites. Immobilization also protects cells from the surrounding environment, stabilizes the cells, reduces inhibition from substrates, and protects implanted cells from immunorejection.

In the next section, the main types of immobilized cell applications are summarized in relation to therapeutics. The review by Willaert and Baron (1996) may be consulted for an in-depth review.

Many antibiotics such as candicidin (Constandinides and Mehta 1991), cyclosporin C (Foster et al 1983) and oxytetracycline (Farid et al 1994) are produced by microbial processes. Immobilization of cells is mainly considered for continuous production of the antibiotics. The main objective is to obtain stable antibiotic production with limited cell growth (Furusaki and Seki 1992). Polyacrylamide, alginate, and carrageenan beads are the most usual immobilization matrices.

Steroids serve as the basis for producing many hormones (Larsson et al 1976). The biotransformation is complex, involving oxygen activation and continuous supply of reductive power. The enzymes involved in these transformations, which include hydroxylases and deshydrogenases, are often unstable. As these biotransformations are generally based on the activity of one or two enzymes, cell viability may be less important. However, maintaining cell structure offers protection for the enzyme. Many transformations of steroids use photo-cross-linked resin beads (Sonomoto et al 1981, Tanaka et al 1984), polyacrylamide (Vlahov et al 1990), and alginate or carrgaeenan beads (Hocknull and Lilly 1990).

Animal and plant cells may be immobilized to produce therapeutics, vaccines, and monoclonal antibodies. Immobilization is required not only to provide protection of cells but also to mimic the natural cell environment. For both types of cells, alginate and agarose beads are the most usual systems of immobilization. However, microcapsules produced by alginate bead coating (Koo and Chang 1993), coextrusion (Uludag et al 1994), or interfacial coacervation (Mansfeld et al 1995) are also commonly used.

Animal cells may also be immobilized as artificial tissues and used for testing different drugs, which makes systematic studies of drugs easier to perform. However, the major application of animal cell encapsulation is the development of artificial organs. Treatment of diabetes with encapsulated pancreatic islets is a major subject, but if successful, it will have important applications in the treatment of many other diseases (Alzheimer's, Parkinson's, hemophilia). PLL-coated alginate beads are used in over 90% of the studies on the subject. However, coextrusion (Sefton et al 1992), interfacial coacervation (Hunkeler et al 1996), and transacylation (Lévy and Edward-Lévy 1996) are very promising technologies. Although their basic principle is relatively simple, development is required to ensure biocompatibility and scalable formulation. This subject will be developed in Part Three.

Conclusions

Cell immobilization and encapsulation has a broad range of applications. Although simple and biocompatible conditions are required for cell encapsulation, technological development thus far is

time-consuming, requiring collaboration between scientists and engineers from many disciplines.

A discussion about the application of encapsulated cells is a discussion about the future. It represents an important objective for scientists and industry for the benefit of all.

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