ENCYCLOPEDIA OF BIOPROCESS TECHNOLOGY: FERMENTATION, BIOCATALYSIS, AND BIOSEPARATION

VOLUME 4

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VOLUME 4

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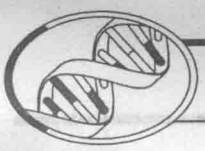

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BIOPROCESS TECHNOLOGY: FERMENTATION, BIOCATALYSIS, AND BIOSEPARATION

VOLUME 4

MICROCARRIER CULTURE

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KEY WORDS

Anchorage-dependent cells
Bioreactor
Cell aggregate
Cell attachment
Cell immobilization
Mammalian cells
Microcarrier
rDNA protein
Shear damage
Vaccine

OUTLINE

INTRODUCTION

Most recombinant DNA–derived mammalian cell culture products are manufactured using cells grown in suspension. Commonly used cells include Chinese hamster ovary (CHO), baby hamster kidney (BHK), hybridoma, and myeloma cells. Those cells can be cultivated in fermentors similarly to microorganisms. However, the vast majority of cells grown in research laboratories, and many of those used in the manufacturing of viral vaccines for human and veterinary use, are anchorage-dependent or anchorage-preferred cells. This means that they need to attach to a compatible surface to survive and multiply, or, if given a compatible surface, they will readily attach to it and multiply rather than stay in suspension. Frequently used anchorage-dependent cells include MDCK, vero, mouse L, fibroblasts such as MRC-5 and WI-38, and almost all differentiated primary cells from nonblood tissues, such as hepatocytes, neuronal cells, and chondrocytes. These cells are grown on petri dishes, flasks (T-flasks), and roller bottles in research laboratories. Traditionally roller bottles are used in the production of biologics, such as cytokines and viruses, derived from those cells. In a typical production plant of those biologics, it is common to see tens of thousands of roller bottles being used for cell cultivation. The amount of labor requirement for roller bottle handling is daunting. Many of those processes manufacture products that were approved by regulatory agencies long ago. If the products were developed more recently, many, if not most, would have been manufactured with an alternative and improved method.

The need to establish a better method of culturing anchorage-dependent cells in large scale prompted the development of microcarriers. The first microcarrier culture was demonstrated by van Wezel in 1967 with diethylaminoethyl (DEAE) Sephadex A50 particles originally designed as column packing for ion exchange chromatography (1). The first industrial-scale product (inactivated polio vaccine) produced in microcarrier cultures was subsequently introduced by van Wezel in 1972 (2). This new way of culturing anchorage-dependent cells spurred other investigators to discover novel techniques to improve cell attachment to the beads, to test new types of microcarriers, and to increase the cell density. Serum coating of DEAE Sephadex beads (3), electric corona discharge for treatment of plastic (polystyrene) beads, and use of dextran microcarriers with a lower charge density (4) led to improvements in microcarrier technology. Since then, a variety of microcarriers have been developed and much research has provided insights into optimization of culture conditions. Table 1 presents a list of the most commonly used commercial microcarriers. The advantages of microcarrier culture over conventional monolayer culture methods (e.g., roller bottles, stacking flat plates) are numerous. Using microcarriers to provide a surface for cell attachment, a large surface area can be contained in a given reactor volume with a relatively uniform environment. Using a reactor similar to conventional fermentors, environmental monitoring and control become possible, thus opening up the possibility of further process optimization.

Most conventional microcarriers have a diameter of approximately 200–250 μm when suspended in medium and

Table 1. Commercially Available Conventional and Macroporous Microcarriers

Microcarrier type	Supplier
Conventional microcarriers	
Biosilon (polystyrene based)	Nunclon
Cytodex 1 (DEAE dextran)	Pharmacia
Cytodex 2 (DEAE dextran)	Pharmacia
Cytodex 3 (gelatin)	Pharmacia
Glass-, collagen-, and hydroxyapatite-coated plastics	Solohil Engineering
Macroporous microcarriers	
Cultisphere (collagen or gelatin)	Hyclone
Cytoline (polyethylene)	Pharmacia
Cytopore (cellulose)	Pharmacia

a specific density of 1.02–1.03 g/cm^3, slightly higher than that of the medium. This higher density allows a minimum agitation to be used to keep the microcarriers in suspension. It also allows for ready settlement after agitation is turned off, which is ideal for culture harvest or exchange of medium for supporting higher cell growth. A typical culture uses a microcarrier concentration equivalent to a settled bead volume 5–15% of total culture volume. This gives a surface area of about 0.5–1.5 m^2/L culture volume. Each liter of culture is equivalent to 5–150 roller bottles. The potential of increasing cell growth surface area was further advanced by the development of macroporous microcarriers (5). These are highly porous convoluted spheres with internal cavities for cell attachment and growth. Macroporous microcarriers can be used for culturing both anchorage-dependent and suspension cells.

Over the past 30 years, microcarrier culture technology has developed and matured to its present-day status. It allows for an easy way of producing a large quantity of cells both in laboratories and for industrial manufacturing. The ease of cell retention in microcarrier cultures also facilitates the employment of a perfusion system, which increases the maximum achievable cell density significantly. For large-scale cultivation of anchorage-dependent cells, microcarrier culture is still the best method.

CONVENTIONAL MICROCARRIERS

The microcarriers developed in the 1970s were initially based on cross-linked dextran. Soon after their introduction they were used successfully for the production of various vaccines and biologicals (6). Further development led to the use of other materials as microcarrier supports. These materials include polystyrene (7), glass (8), cellulose (9), and gelatin (10). The original dextran-based microcarriers require tedious procedures for detachment of cells (11) for subsequent inoculation, which is not the case for some of the other materials developed (8). Several studies have been directed at the effect of surface charge on cell attachment (12,13) and the kinetics of attachment (12).

Cell Surface Interactions and Attachment Kinetics

A microcarrier culture is typically initiated by trypsinizing cells that are adherent on a surface, either on tissue culture flasks or on microcarriers. Sometimes cells for inoculation are cultivated in suspension, and the production scale is conducted with microcarriers. Inoculation from a suspension culture eliminates the need for trypsinization. Once inoculated into a microcarrier suspension, the cells can either attach to the microcarriers or agglomerate to each other to form aggregates. It is essential that the rate of cell attachment to the microcarriers follows rapid kinetics to prevent them from agglomerating. The need for rapid attachment is even more pronounced in cases of primary cells such as hepatocytes or in case of other adherent cell lines that lose their viability if maintained in suspension over extended periods of time. For single cell populations, several studies have demonstrated that the initial rate of attachment of cells to microcarriers is essentially a first-order process, meaning the rate of increase in attached cells is proportional to the concentration of unattached cells, provided that the surface area available for attachment is not limiting (12). The process of cell attachment to any foreign surface can be viewed as a combination of two separate processes: initial adsorption of cells to the microcarrier surface followed by adhesion molecule–mediated attachment and cell spreading. The affinity of the binding of the cells to the microcarriers depends on the cell line, the microcarrier characteristics, the growth phase of the culture, the medium composition, and the cell loading per microcarrier. Cells initially adsorbed can possibly desorb from the microcarrier surface without really developing cell adhesion; the surface should therefore have sufficient affinity so as to allow the cells to remain attached to develop a firm grip by cell adhesion molecule–mediated interactions.

The initial adsorption of cells onto the microcarrier surface is a physical process and is believed to be facilitated by various attractive or affinitive interactions between the cell surface and the microcarriers. Cells in general fail to attach to unmodified crossed-dextran beads such as Sephadex but attach readily to DEAE Sephadex, although the optimal charge density for cell spreading and growth is less than that in DEAE Sephadex (4). Plastics such as polypropylene and polystyrene, in their native state, have a lower surface energy and are therefore incompatible for cell attachment. When treated with an electric corona discharge, their surface energy rises to that of tissue culture dishes, which enhances their ability for cell attachment. It has been demonstrated that corona discharge leads to the unmasking of carboxylic groups on the polystyrene surface, leading to this increase in surface charge density (14). Also, the attachment of cells has been shown to be affected by the length of the carbon chain on the unmasked surface groups (15). The cell surface is negatively charged due to the presence of negatively charged residues on the side chains of membrane-associated cell surface proteins. The interaction of the cell surface with positively charged surfaces can be attributed to the electrostatic interactions. The attachment of cells to negatively charged surfaces is likely due to Lewis acid type reactions in which short-range electron donor–acceptor interactions lead to coupling. In some cases microcarriers coated with cell adhesion factors, such as fibronectin, collagen, and laminin (16), have also been used to facilitate cell adhesion. It is interesting to note that for many cell types the initial attachment rate to charged microcarriers such as DEAE-modified cross-linked dextran beads (Cytodex-1) is faster than to those coated with collagen, which is an excellent surface for cell adhesion on petri dishes.

Furthermore, although serum provides many factors that promote cell adhesion, the initial attachment to charged microcarriers is often faster in the absence of serum (12). These observations suggest that electrostatic interactions between the surfaces of cells and microcarriers is a dominant factor for initial cell attachment. The provision of adhesion molecules, either covalently bound to microcarriers (16) or in soluble form in medium (17), promotes subsequent development of firm grips of cells on microcarriers and facilitates cell spreading. In many cases, no exogenous adhesion molecules are necessary because

cells synthesize their own. For some cell types the omission of these factors impedes cell spreading despite successful cell attachment to microcarriers (16). It is possible then to coimmobilize charged groups and cell adhesion molecules on the microcarriers to promote both initial physical attachment and subsequent biological events of development of adhesion plaques and cell spreading. Irrespective of the surface charge and the hydrophobicity or hydrophilicity of the microcarriers, it is essential that the surface have good wettability characteristics (18).

It has also been shown that a correlation exists between the exchange capacity of the microcarriers and the rate of cell attachment. A higher exchange capacity of the microcarriers leads to a higher first-order rate constant for cell attachment. However, at physiological pH, a higher exchange capacity is not correlated to a higher charge density. Moreover, a difference in cell spreading is observed at different exchange capacities (12). It appears that higher exchange capacities lead to increased cell attachment, but one must be cautioned that a higher exchange capacity may not lead to optimal growth of cells. The optimum exchange capacities for cell attachment and for cell growth are thus very different, and it is advisable to perform preliminary experiments to determine what exchange capacity is best suited for a particular cell type and a particular microcarrier type.

Inoculum Cell Density

After inoculation, cells attach to microcarriers randomly and the number of cells per microcarrier is distributed over a range. Once the adhesion plaques are developed and spreading occurs, most cells do not detach readily and reattach to other microcarriers. They grow until they cover the surface of the bead. For cells subject to contact inhibition, growth ceases then. Many cell types can form multiple layers, but growth rate often slows down after reaching confluence. Therefore, cells on beads with more cells initially reach confluence and stop growing or slow their growth rate faster while others are still growing. Once this happens the overall growth rate in the culture slows. The initial cell distribution on microcarriers thus affects the overall growth kinetics of microcarrier culture. The narrower the initial cell distribution is, the better the growth kinetics will be. Furthermore, microcarriers that do not receive any cells initially will remain barren. Thus, the initial cell distribution after inoculation also affects the maximum cell concentration attainable in the culture.

The initial random distribution of cells on the microcarriers can be simulated by a Poisson distribution, which predicts the probability of a microcarrier having a certain number of cells given the average number of cells per microcarrier (19). Ideally the average number of cells that attach per bead should be such that the fraction of beads without any attached cells is kept at a minimum. It has been shown that a minimal average of about five to six cells per microcarrier is a necessary inoculum size (19,20). With this inoculum ratio, the percentage of microcarriers without any cells attached to them is less than 1%, as estimated from the Poisson distribution.

Cell Shear

Adequate agitation in microcarrier cultures is critical for providing a homogeneous environment and thus facilitates oxygen transfer. The hydrodynamic forces that result from agitation can be detrimental to cells grown on microcarriers. Cells grown microcarrier culture are more shear sensitive than those grown in suspension. The dominant cell damage mechanism in microcarrier bioreactors is believed to be due to forces generated by the collision of microcarrier beads with each other and by small turbulent eddies, which are motions generated by the random variations of fluid direction and velocity in a turbulent flow field (21). In general, except in the spinner flasks used in laboratories, a pitch-blade or marine-type impeller is used for agitation. The ratio of impeller diameter to tank diameter is typically larger than that used in microbial fermentation. The larger impeller provides a larger capacity in liquid pumping for suspending the microcarriers. Because of the low solubility and mass transfer rate of oxygen in culture medium, external aeration is needed at larger scale. In many cases oxygen is supplied indirectly through membrane or silicone rubber tubing, which has a high permeability for oxygen. Use of such tubing is practical even up to a reactor of hundreds of liters. Direct sparging with air or oxygen is used in large stirred tank bioreactors. It is well known in suspension cultures that shear force from rupturing bubbles in a sparged system is the most dominant cell damaging mechanism. This is also likely the case in microcarrier cultures (22). With direct sparging surface active agents, such as Pluronic F-68, are used to reduce the attachment of cell-laden microcarriers to rising bubbles. Attaching to these bubbles and rising with them at a high velocity is often detrimental to cells attached to microcarriers. Furthermore, it is necessary to employ antifoaming agents. Otherwise, cell-laden microcarriers may rise to the top of the foam layer and accumulate there, eventually losing viability.

MACROPOROUS MICROCARRIERS

The cell concentration achievable in a conventional microcarrier system is limited by the available surface for growth. Increasing the microcarrier concentration beyond a certain limit does not lead to an increase in cell concentration because the increased agitation needed to suspend the microcarrier slurry becomes damaging to cells. Macroporous microcarriers were developed with an aim of circumventing some of these limitations of conventional microcarriers.

Macroporous beads are essentially convoluted spheres with relatively large pores for cell attachment and growth. The commonly used materials are gelatin (5), collagen (23), cellulose, glass, polyethylene (24), and polystyrene. The desirable properties of the materials of construction for macroporous microcarriers were discussed earlier. After inoculation of cultures, the cells attach to the external surface of the microcarriers and subsequently migrate inward to populate the interior of the beads. If large open channels leading to internal pores exist, cells may also populate the interior soon after inoculation. Thus, these macroporous

microcarriers provide extended surface area in the interior and potentially support a higher cell concentration with an equivalent number of beads. Also, the cells in the interior of the macroporous microcarriers are shielded from fluid flow and are hence not susceptible to detrimental shear effects (22). However, there are two areas for potential concern. First, in contrast to conventional microcarriers the surface of the macroporous microcarriers is full of cavities and is highly irregular. This irregularity interrupts the flow pattern in the bulk fluid in the immediate vicinity of the beads and gives rise to local microeddies that may damage the cells attached on the surface. Therefore, to avoid shear damage, once attached cells need to migrate inward at a relatively fast rate or the internal pore must be relatively open to allow for cells be carried into the interior by fluid convection during the cell attachment period after inoculation. The second cause for concern is the potential gradients of nutrients, oxygen, and metabolites along the radius of the microcarrier which may cause growth limitation. As a result, necrotic core could be induced within the beads (24,25). Use of intraparticle convection has been postulated as a method to overcome the formation of heterogeneous environment. Numerous cell types have been successfully cultivated on macroporous microcarriers for purposes of product production and virus production (26). Apart from stirred tank reactors, fluidized-bed reactors (27) are also used for cultivating cells in macroporous microcarriers.

Spatial Distribution of Cells and Cell Viability

To ensure efficient use of macroporous microcarriers the internal pores should be open to the exterior through open channels that allow for cell movement into the interior. Some collagen-based microcarriers have leaflike interiors with open and flat surfaces, and others have open pores interconnected by tortuous channels. The materials that the microcarriers are made of also affect the rate of cell movement. Thus, both the structure and the material may affect the performance of the culture. Ideally, macroporous microcarriers should have very open structures to allow cell penetration into the interior immediately after inoculation. Architectural staining of paraffin-embedded thin sections of cell-laden macroporous microcarriers with hemotoxylin and eosin (H&E) indicate that the distribution of cells in the interior is usually not uniform. Some of the internal cavities are packed to confluency, whereas others are devoid of cells.

Traditional methods of examining the pore structure and cell behavior in the interior require fixation and sectioning, which cause aberation. Furthermore, reconstruction of the three-dimensional image is difficult. By digitizing sectioned macroporous culture in three colors, it is possible to classify pixels as conforming to either background, cytoplasmic, microcarrier matrix, or nuclear parameters, based on a set of classification rules determined by statistical analysis (28). When relating the number of pixels occupied by cellular material to the total number of pixels in the sectioned microcarrier, quantification of cell growth can be generated. To examine the accessibility and geometrical features of the pores, confocal laser scanning microscopy (CLSM) can be used to optically section the beads (29). CLSM requires fluorescent samples. Most microcarriers can be rendered fluorescent by appropriate staining techniques. It is also possible to stain cells with a vital dye that has different excitation wavelengths than the dye used for microcarrier staining. This dual stain allows simultaneous observation of cells and microcarriers. Individual beads can be optically sectioned serially through their depths. A three-dimensional image can be reconstructed by combining the sections for examination of the openness and connectivity of the pores. To further evaluate the geometry of the pores, the solid structure of the beads can be removed from the reconstructed three-dimensional images to allow for direct visualization of the pores. Cell distribution, divisional, and other morphological characteristics can be observed directly. This confocal microscopic technique for direct evaluation of pore structure of macroporous beads is noninvasive and requires minimal sample preparation. It can easily be adapted for in situ observation of cell behaviors and to provide information to optimize the structure of those microcarriers.

MICROSPHERE-INDUCED AGGREGATES

A possible way to increase cell concentration in conventional microcarrier systems is by increasing the microcarrier concentration. However, a potential drawback of this scheme is the large ratio of settled bead volume to the culture volume. This large volume of settled beads calls for sophisticated methods of agitation to keep the microcarriers in suspension. Compared with these systems, the settled volume of cell aggregate with an equivalent cell concentration is relatively small, and the agitation requirements parallel those of simple suspension cultures. Also, some adherent cell lines have been engineered to grow in suspension or as aggregates by processes of adaptation or media adaptation. It has been demonstrated that agglomeration can be induced by lectin, concanavalin A, and wheat germ agglutanin (30). However, these processes are relatively slow, and a significant number of the cells initially inoculated lose their viability because of the duration required for aggregate induction.

Microspheres made of DEAE-derivatized Sephadex beads with diameters of 20–50 μm have been demonstrated to increase the rate of aggregate formation (31). Hence, this process can be thought of as a process similar to nucleation and growth in crystallization. After the initial attachment phase, these cell-laden microspheres come into contact with each other to form larger clumps, which serve as nucleation sites for aggregate formation via growth of these attached cells. Over time, aggregates emerge, each consisting of a few microspheres surrounded by multiple layers of cells.

A variety of cells, including CHO, vero, human embryonic kidney cells (293 cells), and swine testicular cells have been successfully grown on these microspheres (31) for virus and protein C productions (31). The drawback of employing aggregate cultures is that little is known about controlling the size of aggregates. As the aggregate size increases, nutrient limitation and a decreased viability can be expected.

CONCLUSION

Microcarrier processes, because of their ease of operation and control, are ideal for cultivating anchorage-dependent cells in both the laboratory and in manufacturing. The bioreactor operation requirements are virtually identical to those for a typical suspension culture in fermentors except that the upper limit for agitation is lower. Operating modes such as batch, fed batch (32), and perfusion are routinely being used in microcarrier cultures. The prevailing use of microcarriers in industrial scale is for the manufacture of viral vaccines and reagents. Depending on the cell type, serum-free cultivation is also possible. Because most newly developed cell culture processes for rDNA products employ suspension cells, microcarrier culture processes will see only moderate growth in the future. However, with increased efforts in research and product development employing differentiated cells, we may see microcarriers play an increasingly important role in small to moderate scales. This technology—developed three decades ago—is still intriguing to cell culture technologists. Its versatility in supporting the growth of a variety of anchorage-dependent cells ensures it a unique place in bioprocessing.

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See also MAMMALIAN CELL BIOREACTORS; MAMMALIAN CELL CULTURE REACTORS, SCALE-UP.

MICROENCAPSULATION

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KEY WORDS

Alginate
Beads
Cellulose sulfate
Coated beads
Encapsulation techniques
Hollow microspheres
Immobilization
Microencapsulation
Polyvinyl alcohol

OUTLINE

INTRODUCTION

The immobilization of active biological substances or living cells has become a universal tool in biotechnology over the past decades. Immobilization can be defined as any procedure that confines substances or cells inside a given system and limits its free diffusion or migration. In biotechnology, the concept of microencapsulation is related to a special immobilization system, where the biological material is confined inside particles, beads, or hollow spheres.

Spherical particles can be considered to be the best-suited immobilization system for use in bioreactors, due to their optimal hydrodynamic properties and abrasion resistance. Because microencapsulation is a very powerful and universal tool, the applications for microencapsulated systems cover a very wide field. To date, it has been used to improve the relatively outdated biotechnological processes in the food industry (1,2), wastewater treatment (3), leaching (4), and environmental detoxification (5). In modern biotechnology it has been applied to cell culture processes for the production of high-value substances such as antibodies (6,7), erythropoetin (8), and the anticancer drug taxol (9), as well as for the production of artificial seeds (10,11) and cryopreservation (12). In medicine, microencapsulated cells have been used as artificial organs to treat diabetes (13,14), as a delivery system for gene therapy (15,16), as an intermediate step in cancer therapy (17), and for the treatment of Parkinson's disease (18,19), to name a few. Furthermore, microencapsulation can serve as a controlled release system for drugs in medicine (20–22) and pesticides in agriculture (23,24).

MICROCAPSULES

Although there are a variety of encapsulation techniques and suitable materials, the resulting microcapsules can be divided into only three main groups: beads, coated beads, and hollow spheres.

Beads

Beads can be produced by cooling liquid drops of gelling agents (gelatine, agarose) under their melting point, thus transforming the polymer solution into a stable cryogel by internal hydrogen bonding, or by chemically or ionically cross-linking polymers to produce a hydrogel. Because bead formation is a simple one-step process, it is relatively easy to develop a large-scale production process (25). The most common bead formation system is alginate cross-linked with calcium ions or any other divalent metal ions.

The fully developed three-dimensional internal network structure enables the beads to withstand extreme mechanical stress. Permeability of hydrogel beads is excellent, and they are often used for culturing cells and microorganisms. The disadvantage is their lack of a real barrier on the surface, so cells can expand during cultivation.

Coated Beads

Providing beads with one or several additional walls leads to the formation of coated beads, which have been developed to overcome the problems associated with the open porous structure of bead surfaces. To produce a membrane, the beads from charged polymers (e.g., alginate, agarose) are treated with a diluted solution of an oppositely charged polyelectrolyte (e.g., poly-L-lysine, poly(ethylene imine), poly(*N*-vinylamine), chitosan) thus forming a simplex membrane on the bead surface. The use of several alternatively charged electrolytes leads to multilayer membranes (26–28). Alternatively, beads can be soaked with a photosensitive substance that cross-links when irradiated (29–31).

A proper coating process produces an additional membrane without incorporation of the material to be immobilized and has the advantage that the membrane properties can be engineered independently with respect to diffusion, molecular weight cut-off, cell retention, or immunoprotection from the internal bead structure and material. Possibly the most well-investigated clinical application of microencapsulation is based on poly-L-lysine-coated calcium alginate beads in the treatment of diabetes (32,33), which has recently been applied to humans (34,35).

Hollow Spheres

Hollow spheres can be produced in a one-step process using two membrane-forming materials that cannot penetrate each other due to diffusional limitations. The cross-linking reaction is then limited to the interface area of the hollow sphere, forming drops and producing a stable membrane around a liquid core. Typical hollow-sphere-forming materials are all polyelectrolyte combinations, for example, cellulose sulfate (CS) and poly(diallyl dimethyl ammonium chloride) (PDADMAC), and CS and poly(ethylene imine) (PEI), able to produce a simplex membrane (36). Another method for the production of hollow spheres is the use of two materials such that the cross-linking material of the drop can diffuse into the surrounding solution. The membrane starts forming at the surface of the droplets and then proceeds outward, creating cell-free membranes. The membrane thickness can be controlled by the electrolyte concentration of the droplet. In principle it is a reversal of the bead-forming process (37). Of course, hollow spheres can also be obtained from a multistep process in which the

core of the coated beads is solubilized. This method has been used for controlled release systems in agriculture and the pharmaceutical industry. It is applied on shear-sensitive compounds such as animal cells in particular (38,39).

Recently simplex membrane systems have gained importance for medical applications such as artificial liver support (40) and gene therapy (41). Considerable progress has been made through a U.S. National Aeronautics and Space Administration study in which over a thousand combinations of polyanions and polycations were tested to identify new polymer candidates that could be suitable for the encapsulation of living cells, especially pancreatic islet cells for treatment of diabetes in humans (42,43).

FUNDAMENTAL METHODS IN MICROENCAPSULATION

The basis of all microencapsulation processes is the formation of a liquid drop followed by gelation (44,45), a cross-linking reaction, or membrane formation. The liquid drops can be obtained from extrusion of a liquid through a small needle or orifice, or from emmulsification of the drop-forming solution in a second immiscible solution by use of dynamic or static mixers. Scale-up ability of the process and a uniform microcapsule size and shape are the main parameters in encapsulation technology (46).

Dropping Methods

A liquid ejected with low velocity from a needle will break into individual drops. If the velocity is increased, drop formation increases until the maximum velocity is reached and the liquid begins to form a jet. The maximum velocity can be calculated from the following formula using the liquid velocity, v, interfacial tension, γ, liquid density, ρ, and the inner needle diameter, d_i:

$$v < 2\ (\gamma/\rho d_i)^{0.5}$$

Simple Dropping. The two main factors affecting drop size are the force of gravity trying to tear the drop from the needle tip, and the resisting product of the interfacial tension and the tip perimeter. Other forces interacting with drop formation are resistance power and inertial force, but these can be neglected in computations. The drop mass can be calculated from the equilibrium of the two main forces with the mass, m, gravity acceleration constant, g, external needle diameter, d_e, interfacial tension, γ, and the liquid density, ρ:

$$mg = \pi d_e \gamma \text{ and } m = \frac{\rho \pi d^3}{6}$$

The drop mass must however be corrected by a factor of 0.85 to get the true mass, because drops stretch out and leave a small portion behind when dropping from the needle. Even if very thin needles are used, it is very difficult to obtain droplets with a diameter of 1 mm or less (47,48).

Dropping with a Superposed Air Jet. The droplet size obtained from simply dropping can be reduced by superposing the drop forming process with an additional air jet. For

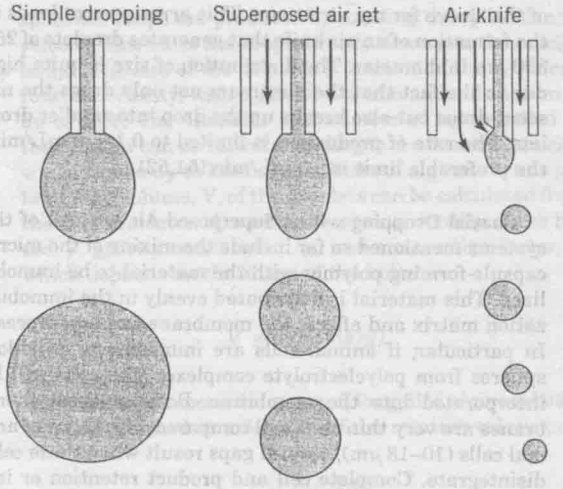

Figure 1. Monoaxial extrusion technologies based on simple dropping.

calculating the droplet mass, the dragging forces of the airflow have to be taken into account. The viscous drag force is the laminar effect of the fluid, and the kinetic energy dissipation term represents the impact of the turbulence on the drag. Assuming that the drop diameter is larger than the tip, that there are no lateral fluid effects, and that the nascent drop has a spherical shape, the mass, m, of the generated droplet can be calculated using the gravity acceleration constant, g, liquid viscosity, μ, fluid velocity, v, droplet diameter, d_D, external needle diameter, d_e, the surface tension, γ, and the density of the fluid, ρ (49):

$$mg = \pi d_e \gamma - (3\pi\mu v d_D + 0.055\ \rho v^2 d_D^2)$$

Depending on the immobilization material used, uniform droplets from 2 to 6 mm can be produced. Droplet production can be scaled up by use of several needles at once and has been successfully performed for the encapsulation of IgG-producing hybridoma cell cultures and yeasts (50). However, the increasing airflow influences the line of flight of the droplets, which becomes important if several adjacent needles are used simultaneously. As long as the superposed airflow is laminar ($Re < 0.3$, Stokes' area), droplets will fall in a perfectly vertical line. For turbulent superposed airflow producing periodical eddies ($1{,}000 < Re < 10^5$, Kármán vortex street), the diversion can be neglected up to $Re < 1{,}080$. Stronger turbulence may cause collisions of the falling drops but can be avoided if the distance between the needles is increased. At any rate, small satellite drops are formed and site distribution increases significantly.

This method uses an airstream that is concentric with respect to the droplet-forming needle. Excentric air sleeve positioning combined with a beveled droplet-forming needle facilitates the generation of very small droplets. The distal end of the needle is beveled at an angle of about 15 to 45°; the beveled end is disposed facing the central axis

of the sleeve for the airstream. This arrangement leads to the formation of an air knife that generates droplets of 25–300 μm in diameter. The distribution of size is quite high due to the fact that the airstream not only drags the nascent drops but also breaks up the drop into smaller droplets. The rate of production is limited to 0.1–3.0 mL/min; the preferable limit is 0.3 mL/min (51,52).

Coaxial Dropping with a Superposed Air Jet. All of the systems mentioned so far include the mixing of the microcapsule-forming polymer with the material to be immobilized. This material is distributed evenly in the immobilization matrix and affects the membrane-forming process. In particular, if animal cells are immobilized in hollow spheres from polyelectrolyte complexes, the cells will be incorporated into the membrane. Because these membranes are very thin (1–5 μm) compared with those of animal cells (10–18 μm), several gaps result when these cells disintegrate. Complete cell and product retention or immunprotection cannot therefore be achieved. Coaxial dropping can be used to overcome this problem. This system uses one needle for the membrane-forming polymer solution and a second one, which is fixed in the center of the first, for the cell suspension. Both needles are of the same length. The nascent drop formed at their ends has a core containing the cell suspension and is enveloped with the polymer solution. While falling, the polymer solution completely surrounds the cells that comprise the core. The membrane-forming process in the precipitation bath is not affected. The core may have a volume of up to 25% of the whole drop. The droplet diameter can be varied from 1 to 5 mm. All the equations of the previous section are still valid.

This system has been scaled up to a productivity of 4 L/H by the simultaneous use of 24 concentric needles. To date this system has been successfully used for the immobilization of hybridomas (53), insect cell cultures (54), and the treatment of rheumatoid disease in mice, where microcapsules have been used intraperitoneously in mice containing a hybridoma cell line secreting antirheumatoid monoclonal antibodies (55).

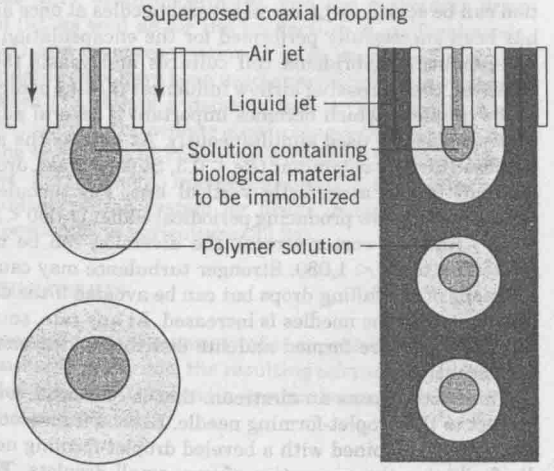

Figure 2. Coaxial extrusion technologies based on simple dropping.

Coaxial Dropping with a Superposed Liquid Jet. Superpositioning is not restricted to the use described in the last section; it can be used with any liquid immiscible with a drop-forming solution along with a precipitation bath. There are two main advantages to this method. Fluids still flow in a laminar fashion at higher velocities as compared with gases, and the viscous drag force is much higher. Both effects enable the production of hollow spheres from polyelectrolytes with a diameter ranging from 500 to 1,000 μm. This is considerably less compared with the previous system. Unfortunately this system is restricted to a productivity of 150 mL/h, and scale-up by use of multiple devices is problematic.

Electrostatic Extrusion. The application of a high static potential between the capillary and collecting solution has been used to improve droplet formation with respect to reducing the droplet size. The electric field force effectively pulls the forming droplet off the tip of the needle at a much lower mass (and hence size) compared with the simple dropping method. A series of small droplets (as small as

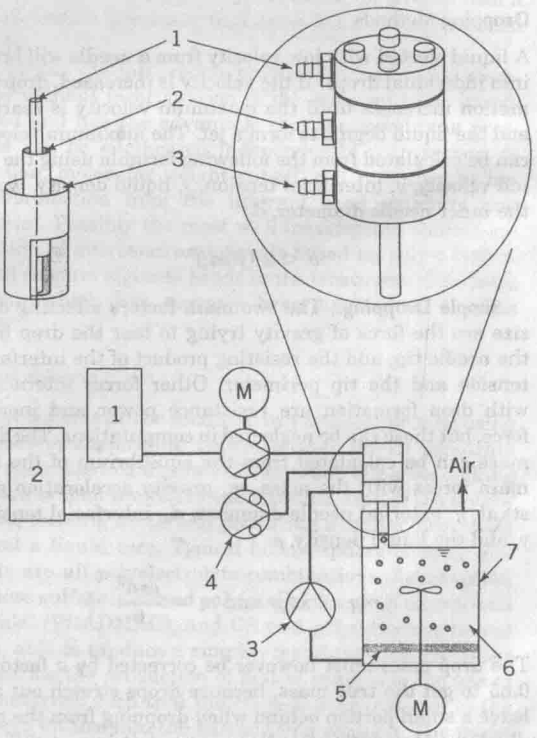

Figure 3. Schematic representation of the coaxial encapsulation apparatus (Torsten Steinau Verfahrenstechnik Berlin): (1) cell suspension, (2) cellulose sulfate solution, (3) inert carrier fluid, (4) multichannel peristaltic pump, (5) sieve for capsule retention, (6) polyDADMAC precipitation bath, and (7) stirred tank reactor.

26 μm in diameter) is generated with a comparably low standard deviation. The average microbead size can be easily adjusted from 2.5 to 0.2 mm by decreasing the concentration of the polymer solution, by using a higher-gauge needle, and by increasing the applied voltage. However there is a limit to how much the size can be reduced. In very high electric fields, droplets are no longer formed, and the solution pours from the needle in a steady stream. Charged molecules accumulate at the nascent drop's surface and counteract the surface tension. According to Lippmann's theory, the size of the drops can be determined with the electrostatic force, Fe, electric permitivity, ε, droplet diameter, d, the applied electrostatic potential, U, surface tension, γ, mass, m, and gravity acceleration constant, g:

$$mg = \pi d\gamma - Fe$$

$$mg = \pi d(\gamma - (k\varepsilon U^2/d))$$

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

The correction factor, k, is difficult to evaluate. It depends on system design and may be a function of the form of the pending drop and of the electrostatic potential. This system has so far been applied to the encapsulation of cells in alginate beads (56,57), pancreatic islets in coated beads to study the influence of diameter and membrane compactness integrity and immunoprotection (58), and to the production of protein-loaded ethylene vinyl acetate beads for controlled-release purposes (59).

Rotating Disk. A liquid can be sprayed into a precipitation bath by using a rotating disk or a dish rotating at a high speed. The liquid for dispersion emerges from an orifice at the center of the axisymmetric rotating disk. The liquid leaves the disk in the form of small jets or ligaments, which form drops prior to falling into the precipitation bath. Droplet diameters, d, can be determined from the surface tension, γ, rotation speed (per second) of the disk, w, disk radius, r, and the liquid density, σ (60,61):

$$d = 0.425\ (\gamma/(w^2 r\sigma))^{0.5}$$

This system can be improved by applying a well-designed wave on the liquid flowing on the spinning disk. The ligaments are broken into uniform droplets with a standard deviation lower than 5%. Typically, a disk of 1 cm in diameter rotating at 2,000 rpm forms about 60 ligaments, which then form droplets of 500 μm at a flow rate of 6 L/h. Equations describing this process are much more complex than those for classical jet rupture. Physical properties of the liquid, design of the rotating disk, and wave frequency and amplitude must be adjusted to achieve correct particle size. This method is currently being used for experimental research and agriculture (62).

Rotating Nozzles. Instead of a rotating disk, a perforated rotating cylinder can be used. This cylinder can be placed in a reactor, with the reactor serving as the precipitation bath and the culturing vessel. Monodisperse and spherical drops are produced by rotating the cylinder at a velocity that hurls the drops directly down into the reactor liquid. The size of the beads is a function of the cylinder's rotation velocity, with a size of 1–3 mm and a deviation of up to 10%. The diameter and number of holes drilled in the cylinder can be varied according to the desired capacity, which can be up to hundreds of liters of alginate beads per hour. The volume, V, of the droplets can be calculated from the hole diameter, d, surface tension, γ, density of the liquid, ρ, gravity acceleration, g, and a correction factor, k, which depends on the shape of the holes:

$$V = (2\pi d)/(k\rho g)$$

The variation in bead diameter, d, for gelled alginate beads can be expressed as a function of the rotation velocity, ω:

$$d = (1/\omega^2)^{1/3}$$

As has been observed for simple dropping, the diameter, d, has to be corrected by a factor of 0.84 to 0.95, depending on the rotation velocity used, because the drop leaves behind a portion of the pendant drop upon breaking. The landing radius of the drops on the liquid surface must be taken into account to avoid splattering of the drops on the reactor walls. The landing radius, r, is dependent on the velocity, v, of the drops when leaving the hurler, the height, h, of the holes above the liquid surface, and the acceleration force, g:

$$r = v(2h/g)^{0.5}$$

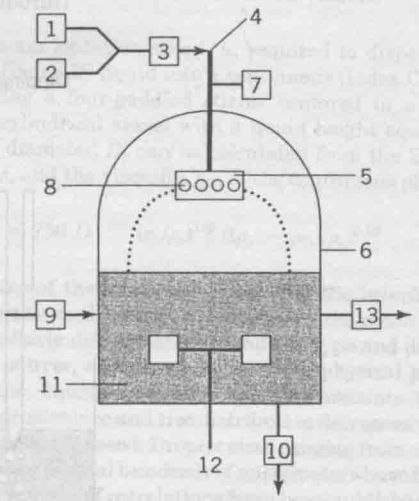

Figure 4. Schematic representation of the Biosphere (Landteknikk A/L): (1) biocatalyst solution, (2) polymer solution, (3) mixing chamber, (4) rigid tube, (5) perforated cylinder, (6) bioreactor, (7) velocity regulation unit, (8) nozzles, (9) feed pipe, (10) product outlet, (11) precipitation bath, (12) stirring device, and (13) reactor outlet.

Liquid-Jet-Based Methods

Dropping methods can be scaled up only by the parallel use of several needles, because drop formation depends on a relatively slow liquid velocity. Hence, dropping methods are limited to about 200 mL/h per needle. To overcome this problem, several methods for producing droplets from liquid jets have been developed, and several liters of microcapsules can be produced. The maximum productivity of these systems is limited due to the fact that the pressure needed to ensure flow increases with the second power of the liquid velocity, and with increasing liquid viscosity and decreasing orifice diameter. Pressure can be calculated using the following equations with pressure, P, resistance coefficient, χ, liquid density, ρ, liquid velocity, v, needle diameter, d, needle length, l, Reynold's number, Re, and the liquid viscosity, η:

$$\Delta P = (\chi \rho v^2/2)(l/d)$$

$$Re = v\rho d/\eta$$

and

$$\chi = 64/Re \text{ for } Re < 2{,}320 \text{ (laminar flow)}$$

or

$$\chi = 0.3164/Re^{0.25} \text{ for } 3{,}000 < Re < 10{,}000 \text{ (turbulent flow)}$$

or

$$\chi = 0.0054 + 0.3964/Re^{0.3} \text{ for } 20{,}000 < Re < 10^6 \text{ (turbulent flow)}$$

Dipping Jet. The dipping jet method is the simplest process for producing a large amount of microcapsules. It depends on the fact that a liquid jet at high velocity disintegrates into many drops when penetrating a fluid. Drop formation depends on irregular dynamic processes and leads to drops with a large size distribution (63,64). This method has been used to immobilize the fungi *Claviceps purpurea* in alginate particles for further production of alcaloids in a gas–solid fluidized-bed reactor. Researchers produced 110-L capsules with a mean diameter of 2.5 mm (65).

Vibrating Jet Breakage. Lord Raleigh influenced liquid jets with tuning forks in the late nineteenth century. He observed that vibration can force a liquid jet to disintegrate into equal drops if the wavelength of the vibration is greater than the perimeter of the liquid jet. He showed that the frequency, f, for maximum instability is related to the jet velocity, v, and the wavelength, λ (66):

$$f = v/\lambda$$

When a laminar-flowing jet is mechanically disturbed at this frequency, uniform drops are formed. The optimum wavelength, λ_{opt}, for breakup can be calculated with the needle diameter, d, dynamic viscosity, η, liquid density, ρ, and the surface tension, σ (67):

$$\lambda_{opt} = 2^{0.5}\,\pi d(1 + (3\eta/(\rho\sigma d)^{0.5}))^{0.5}$$

By considering that the jet will break into liquid cylinders with the wavelength λ, each of which will form uniform droplets, the diameter of the droplets, d, can be calculated:

$$d = (1.5\, d^2\lambda)^{1/3}$$

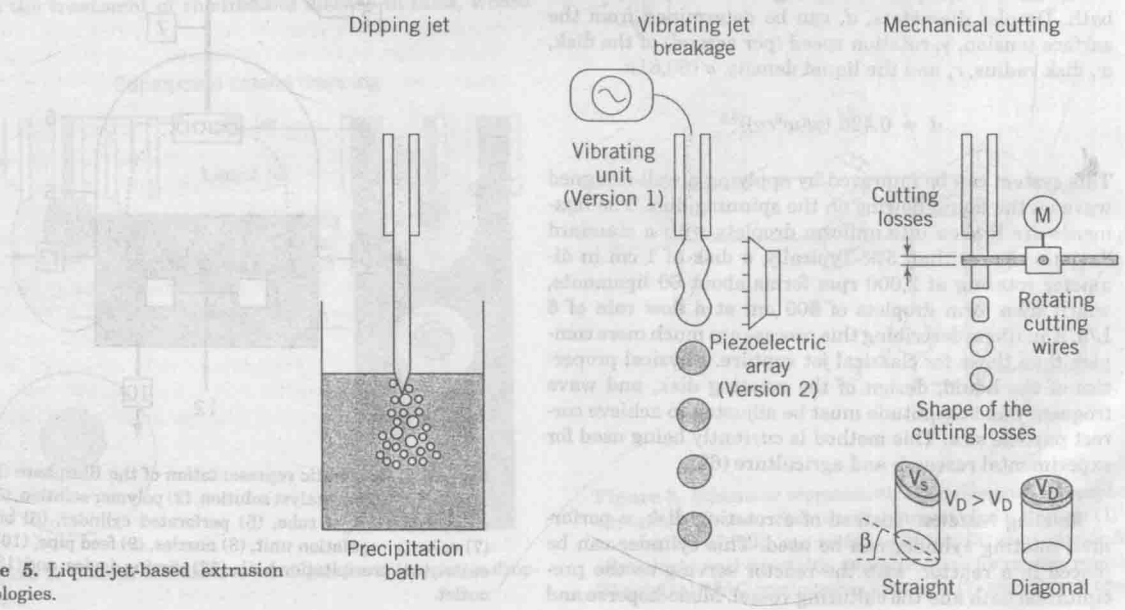

Figure 5. Liquid-jet-based extrusion technologies.

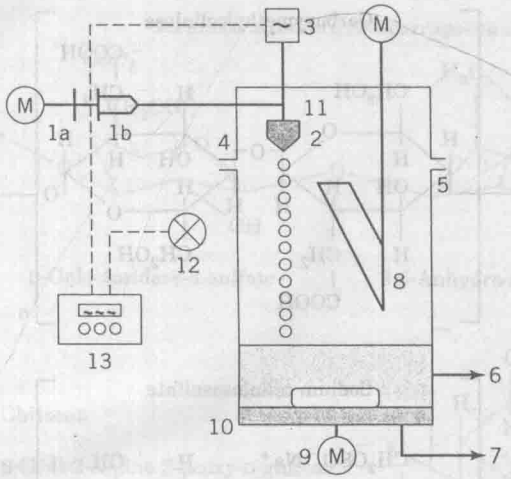

Figure 6. Schematic representation of the Encapsulator AP (INOTECH AG): (1a) syringe pump, (1b) syringe containing polymer solution and cell suspension, (2) vibrating nozzle, (3) vibration unit, (4) pulsation unit, (5) reaction vessel, (6) reactor outlet for capsule harvesting, (7) waste outlet, (8) bypass unit (used to eliminate undesired beads at the beginning and the end of bead formation), (9) magnetic stirrer, (10) sieve, (11) capillary, (12) stroboscope lamp, and (13) frequency generator.

Mechanical Cutting. The principle of the mechanical cutting encapsulation method is to cut a liquid jet with a rotating wire into a series of uniform liquid cylinders behind the cutting wire. Due to surface tension, the liquid cylinders form drops immediately as they fall down into the precipitation bath. The bead build volume, V, of the cylinder can be calculated from the cutting angle, β, which is a function of the liquid velocity, v, and the local velocity of the wire, w, at the cutting point. Other relevant parameters are the thickness of the wire, d_w, the number of revolutions, n, of the cutting wire, the number of wires, z, and the nozzle diameter, d.

$$V = 0.25\ \pi d^2((v/(nz)) - (d_w + d\ \sin\beta)/\cos\beta))$$
$$\text{with } \beta = \arctan(v/w)$$

Because the wire used for cutting cannot be infinitesimally small, and its size is also determined by the material, it slings away about 10–20% of the liquid jet; this can be considered lost material. This cutting loss, V_L, can be determined as follows:

$$V_L = 0.25\ \pi d^2(d_w/\cos\beta + d\ \tan\beta)$$

When the cutting device is arranged at an optimal angle, α, depending on the liquid jet's velocity, the the drop volume is affected, and cutting loss can be reduced further:

$$\alpha = \arcsin(v/w)$$
$$V = 0.25\ \pi d^2((v/nz) - d_w)$$
$$V_L = 0.25\ \pi d^2 d_w$$

The cutting loss can also be reduced by use of a thinner wire for cutting. However, if a stainless steel wire becomes thinner than 0.2 mm, distortion effects of the wire can be observed when using fluids of high viscosity. Therefore, special stabilized wires 0.06 mm in diameter have been designed; in combination with diagonal cutting, they reduce cutting loss to only 3.3% (68,69).

This method is especially suited to produce beads from high-viscosity polymer solutions, with high productivity rates of up to 20 kg/h for 1-mm beads. This method has been used to immobilize a variety of microorganisms for further use in wastewater treatment experiments.

Liquid–Liquid Emulsification

Microcapsules can be obtained from dispersion of one liquid into another followed by a membrane-forming process. Generally, dispersions can be obtained from static and dynamic mixers. However dynamic mixers are preferred for microencapsulation because of the need to stabilize the dispersion until microcapsule formation has taken place, otherwise coalescence leads to increasing drop size.

Dynamic Mixer. It is advantageous to use an impeller that disperses the lighter liquid in both the center and the periphery at the same impeller speed. The optimum impeller diameter, d, depends on the vessel diameter, D, and presence of baffles:

$$d = D/3 \text{ (impeller)}$$

or

$$d = 0.4\ D \text{ (impeller in baffled vessels)}$$

The minimum agitation speed, n, required to disperse a dispersed (index D) liquid into a continuous (index C) liquid by using a four-paddled stirrer centered in a flat-bottomed cylindrical vessel with a liquid height equal to the vessel diameter, D, can be calculated from the liquid densities, ρ, and the viscosity, η_H, of the continuous phase:

$$n = 750\ D^{-2/3}\ (\eta_c/\rho_c)^{1/9}\ ((\rho_c - \rho_D)/\rho_c)^{0.26}$$

The viscosity of the dispersed phase and the interfacial tensions can be neglected (70). Calculating the mean particle size is fairly difficult and depends on type and diameter of the stirrer, agitation rate, and the physical properties of the liquids and their relative amounts (71). Generally, droplet size and size distribution decreases with increasing agitator speed. Droplet sizes ranging from a few micrometers to several hundreds of micrometers have been reported. A variety of correlations have been published relating the Sauter mean diameter, d_{32}, of the droplets to the vessel geometry and the physical properties of the dispersion system. The most well known correlation is derived from the impeller diameter, d_I and speed, n, the Weber number, N_{We}, liquid density, ρ, interfacial tension, η, the dispersed phase volume fraction, φ, and two correlation factors, b and c (72):