Development of high-density cultivation systems by bioencapsulation

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Encapsulation of living cells within the confines of an enveloping material permits very high local cell densities to be achieved. Methodologies for bioencapsulation include liquid-based jet methods, drop techniques and emulsification. This review addresses the selection of suitable encapsulating material, enumerates polymers used in capsule formation and considers the significance of encapsulating eukaryotic cells. The importance of cell death as a factor limiting productivity of cell culture methods is emphasized.

ENCAPSULATION is the process of making capsules using certain controlled chemical reactions. Bioencapsulation takes advantage of this fundamental principle and effectively involves putting a living core inside a membrane which is essentially inert, non-toxic to cells and stable to conditions inside large-scale biochemical engineering reactions such as sparging or agitation.

Exploitation of the principles of encapsulation has already been done in the field of drug delivery. This field is relatively new in cell biology, but considerable information is available regarding encapsulation of prokaryotes. This review concentrates more on the scientific and economical importance of encapsulation, with reference to eukaryotic cell cultures.

Bioencapsulation or encapsulation of living cells in general[1], involves immobilization of bioactive material in gel beads or capsules. Thus, encapsulation consists of putting a living core, for example, an insect cell, a hybridoma cell or a liver cell inside a shell or an envelope. Encapsulation focuses on maintaining the solution environment, rather than maintaining the physical or chemical forces required for immobilization. Cellular encapsulation was first done by Lim and Sun[2] and its applications were later highlighted by Jarvis and Grdina[3].

Bioencapsulation of living cells is an attempt to separate the loci of viable cells from the nutrient medium, to reach high local cell densities. Immobilization of cells literally means reduction in the mobility of the cells. The concept of encapsulation is becoming increasingly important in biotechnology and its related fields due to its unique features, which facilitate continuous operation and maximum product output at low cost[4]. Further research in encapsulation is focused more on cell culture and its related fields. Suspended cells (vertebrate and invertebrate) are subjected to shear inside the reactors and cause a variety of changes in the chemical environment. This aspect is of great importance as these cells lack the cell wall present in microorganisms and are of large size and the normal repair mechanisms are reduced[5]. Growth of these types of cells in reactors without the proper precautions may result in loss of viability and a general or eventual decrease in final output of the product due to the release of certain enzymes like proteases. The main requirements to be satisfied with cultures of these types of cells are: (a) Cells inside of the immobilization matrix are protected from the shear forces in the medium necessary for agitation and sparging; (b) Mass transfer characteristics depend on the immobilization matrix and particle size only, which can be adjusted to the needs of the culture; (c) Easy scale-up for large-scale production.

Cells, which can grow freely or without the presence of any support have been termed as anchorage-independent cells, and cells which require the presence of a support for the growth are termed as anchorage-dependent[4].

Advantages and disadvantages of encapsulation

The advantages of encapsulation are: (i) Continuous operation of the system at high dilution rates, with no danger of the cells being washed out, resulting in high-localized cell densities; (ii) Faster reaction rates at high density, as the number of viable cells per unit volume is greatly increased; (iii) Higher yield of the product; (iv) Easy rheological control.

The main disadvantage is the interfering reactions that may decrease the product output. These reactions may occur due to the chemical nature of product which may react with the immobilizing material if the proper chemical nature of the material is unknown or due to the possible variations in pH that can occur, depending on the demands of the cell line currently being cultured and

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in the reactor during the course of production of the desired product.

Methodologies involved in bioencapsulation

All bioencapsulation techniques require a core material and an enveloping solution. The core material is the suspension of the cell in a suitable solvent. The shell or the envelope can be achieved by the following ways: (1) Change of phase and polyneutralization; (2) Change of hydro- or lipophilicity of the phase; (3) Change of temperature; (4) Change of pH.

Bioencapsulation can be carried out by the simple drop technique, which has been widely used. This method follows Tate´s law

$$mg = pd_e g \quad \text{with} \quad m = rpd\,3/6,$$

where m is the mass of the falling drop; r is the density of the dispersed phase; g is the gravity constant; d_e is the external tip diameter; and p is the liquid interfacial tension. The correction factor given is 0.85. The drops fall into a reservoir of coagulating solution, which should be preferably non-toxic. The drops fall by gravity. Other methods which may be used are: liquid-based jet methods and emulsification.

Liquid-based jet methods

These methods primarily depend on a very high velocity liquid flow and in principle have a higher productivity than drop-based methods. The methods using the above said principles are described below.

Dipping jet method: This is a process which is based on the fact that a liquid jet disintegrates into droplets when penetrating a fluid of high velocity. The undesired feature is that the drop formation depends on irregular dynamic processes and leads to drops with a considerable large size distribution (1–4 mm diameter).

Mechanical cutting: This involves cutting of the liquid jet with a wire, leaving behind a series of uniform cylinders which then form spheres. The disadvantage of this method is that the wire used for cutting cannot be infinitesimally small; it slings away about 10–20% of the liquid jet which can be considered as a lost material.

The only method which has been tried out with insect cells is 'vibrating jet breakage'. This is based on the fact that vibration can force a liquid to disintegrate into drops. Although this process is most advanced for construction of beads, construction of hollow spheres with an intact membrane is not possible using this technique.

Emulsification

Encapsulation by emulsification is not suitable for viable cells as encapsulation by emulsification is based upon the reduction in the interfacial tension between the viable cell in the capsulation medium and the dispersal medium. This may result in disruption of the cell structure as these cells lack a cell wall and are therefore fragile. Additionally, high internal shear forces are produced by static or dynamic mixers to stabilize the emulsion prior to bead-forming process. The entire work has been done using the simple drop method.

Selection of suitable encapsulating material

The materials used for this type of immobilization (encapsulation) should possess the following characteristics: chemical inertness to the core; flexibility of its physico-chemical properties according to the need; easily available and economical for scale-up; ease of downstreaming of the product; optimal mass transfer characteristics.

Materials used for encapsulation include polycations, polyanions, cross-linking agents and divalent ions. Polycations include poly-DADMAC, polyethyleneimmine and poly-L-lysine. Polyanions include cellulose sulphate, alginate and carrageenan.

In the present work, sodium cellulose sulphate–polyethyleneimmine and sodium alginate–calcium have been used for encapsulating insect cells, hybridomas and liver cells respectively. Ideal encapsulating agents/systems should possess the characteristics given in Table 1.

There is no universally acceptable encapsulating agent. Alginates have been used to encapsulate insect cells. Alginates have been specially treated with poly-L-lysine to prevent leakage of the cells. Alginates have also been used to encapsulate hybridoma cell lines with varying degrees of success.

In addition, cellulose sulphate has also been used. Cellulose sulphate is a polymer whose production has been designed and developed at Bioprocess Engineering Department, TU-Berlin. It has already been successfully used for the production of polyhedra by the culture of

Table 1. Characteristics of an encapsulating agent/system

Capacity to load high biomass
Provide to the cell, optimal access to the nutrient
The procedure of immobilization should be essentially non-toxic and provide maximum surface area to volume under use
The size distribution should be uniform and should be mechanically stable
It should be reusable or inexpensive, autoclavable and facilitate separation of the product from the media
It should be suitable for both suspendable and anchorage-dependent cells

Spodoptera frugiperda using cellulose sulphate–poly-DADMAC encapsulation system[6].

Chemistry of polymers used and preparation of capsules

Alginic acid monosodium salt

Purified alginic acid consists of three kinds of polymer segments obtained from the brown algae, *Macrocystis pyrifera*. Mild acid hydrolysis of this salt reveals the presence of three different blocks in the polymer chains. One chain consists of D-mannuronic acid units, one consists of D-guluronic acid units and the last segment is made up of alternating D-mannuronic and D-guluronic acid subunits[7–9]. The process of bead formation is essentially an ionotropic gelation, with the necessary ion required being calcium[10]. The bead formed as a result of reaction between alginic acid and calcium ion is basically a gel whose hardness is more than a gel due to its prolonged reaction with calcium ion (also depends on the concentration of the divalent ion). The typical structure of the gel can be described using the 'egg-box model', in which polyguluronate segments associate to form aggregates into which the calcium ions fit[11,12].

Secondary stabilization of the structure occurs due to ionic bonding between two carboxyl groups due to calcium ion[10].

Carrageenan (κ-carrageenan)

Carrageenan is a general name for polysaccharides extracted from certain kinds of algae, which are built up in contrast to agar from D-galactopyranose units only. It is obtained from the red seaweeds belonging to the family Rhodophyceae. Chemically, it is composed of an alternating copolymer of 3-linked beta-D-galactopyranose and 4-linked 3,6-anhydro-alpha-L-galactopyranose[13].

Sodium cellulose sulphate

Cellulose sulphate is a derivative of cellulose, consisting of a sulphate ion substitute at a carboxyl group at every third position[14].

Polyethyleneimmine

It is a water-soluble cationic polyelectrolyte comprising repeated ethyleneimmine units. It is reactive with acids, acid halides, isocyanates, alkyl halides, epoxides, carbonyl compounds and sulphonic acids. It reacts with cellulose sulphate and carrageenan, resulting in the formation of surface membranes. The reaction is described as mainly polyneutralization, as cellulose sulphate and carrageenans are polyanions[15].

Process of encapsulation

Encapsulation is based on controlled reaction between polyelectrolytes[16] or a simple reaction involving valence satisfaction[17]. Commonly used polymers for encapsulation include sodium cellulose sulphate, sodium alginate and carrageenan. The precipitation bath contains either poly-DADMAC or polyethyleneimmine. In case of alginates, calcium ions are used as the precipitating substance[18]. The precipitating bath is essentially toxic to cells (with reference to polyelectrolytes) during the initial stages of membrane formation of the capsule, with the initial cell densities required to obtain a viable cell count inside the capsule being very high.

Encapsulation has a wide variety of applications. Some of the applications are high-density cultivation of cell lines[19], drug delivery systems[20], waste water management[21] and production of secondary metabolites by prokaryotes[22].

We will now deal with the area of cell culture only. Encapsulation of cell lines has been projected as a method to obtain high cell densities. Tables 2 and 3 give a brief projection of the various processes used for cell culture and the cell densities obtained.

In suspension cultures, insect cells and hybridomas obtain cell densities less than those mentioned above. This is also true with primary hepatocyte cultures[23–25]. From Tables 2 and 3, it is clear that encapsulation systems tend to support high cell densities than the other conventional cell culture methods and immobilization techniques.

Significance of encapsulating insect cells, hybridomas and liver cells

Sf 21 cells have been described as super vector for expression of heterologous proteins. They are also used for the production of baculovirus[26]. By controlling the multiplicity of infection of baculovirus in insect cells inside the capsule, it is possible to produce baculovirus continuously and for its commercial large-scale use as a biopesticide.

Hybridomas have been encapsulated with varying degrees of success. The longest period of hybridomas surviving inside capsules is 30 days[27]. The production of antibodies can be obtained continuously with minimal labour when compared to individual cell culture processes. Table 4 gives the literature available regarding encapsulation of hybridomas. Cell densities obtained in these systems are very high compared to cell densities in suspension culture[28].

Table 2. Reactors and cell lines reported in literature for suspension culture

Bioreactor	Working volume	Cell line	Medium	Aeration	rpm	Growth rate	Maximum cell density	Reference
Airlift	21 l	–	IPL-41 + FBS + PF68	DO controlled at 20%	–	0.027	5×10^6	52
Spin filter perfusion	12 l	SF21	IPL41 + FBS	Submerged with DO controlled at 93%	–	–	–	Ibid
Spin filter perfusion	14 l	SF9	SF900	Submerged	150	0.034	1.45×10^7	Ibid
Helical ribbon impeller STR	11 l	SF9	TNM-FH + FBS IPL-41 SFM	Surface with O_2	60–75	0.028–0.031	5.4×10^6	Ibid
STR with marine impellers	4 l	SF9	TNM-FH + FBS IPL-41 SFM	Surface with O_2	100	0.030–0.035 0.029–0.032	4.7×10^6	Ibid
Airlift	10 l	SF21	IPL-41 + FBS	DO controlled at 20%	–	–	3×10^6	Ibid
STR	5–10 l	*M. brassicae*	Serum containing SF900 II	Silicon tubing	NA	0.035–0.039	3×10^6	Ibid
STR with marine impellers	2 l	SF9	SF900 II	Submerged with DO controlled at 50%	130	0.033	$6.8–8.0 \times 10^6$	Ibid
High aspect rotating wall vessel	50 ml	SF9	Ex-cell 401	0.6 l O_2/min	12	–-	1×10^7	53

Table 3. Immobilization systems for invertebrate cell lines

Type of immobilization	Cell density inside the matrix (cells/ml)
Encapsulation	8×10^7 (by use of alginates)[54] 2×10^8 (by use of cellulose sulphate)[6] 1×10^8 (porous alginate beads)[55] 2.1×10^7 (ref. 56)
Macroporous entrapment	1.5×10^7 (cellulose foam) 1.2×10^7 (polyurethane foam) 6×10^6 (Fibra cell carrier)
Microcarriers	2×10^7 (Verax microspheres)

Table 4. Encapsulation systems used for hybridomas based on alginate–calcium and its modifications

System of encapsulation	Reference
Alginate–calcium	57
Alginate–calcium	58
3-D alginate matrix	60
Porous carrier (alginate, bovine serum albumin and sodium bicarbonate)	61
Alginate–agarose	61
Alginate–poly-L-lysine–alginate	62
Alginate–polyethylene glycol	57

Liver cells have been grown more on three-dimensional collagen modules, rather than simple encapsulation systems[29]. The growth response of hepatocytes is always diminished in primary monolayer cultures even in the presence of mitogens, due to a loss in their gene expression patterns[30]. Liver cells are conventionally grown to high density using hollow fibre modules and by use of porous matrices. One report of a rat liver cell line immobilized in alginate capsules is available and it has been used for bile duct studies[31]. Encapsulation is more simple and economical than collagen modules.

Discussion

The present review highlights the importance of encapsulation, with specific reference to cell cultures. It is clear that high-density cultivation of cell lines is possible with the process of encapsulation. This process has a great commercial potential either for production of biopesticides or antibodies or artificial liver support systems. It also provides a method of cultivation of cell lines with certain physiological property such as anchorage-dependence, for a longer period of time than in monolayer culture. This type of cultivation system offers new possibilities in culture of sensitive cells. Due to protection obtained by encapsulation, the cells can tolerate a higher level of mechanical stress, which will extend the range of different reactors suitable for fermentation. A higher and more constant production of biomolecules is possible by continuous perfusion, using an encapsulated system. New reactors like packed beds can be used, creating a high catalytic activity in a small volume. The extremely high concentration of cells also creates a need for improvement of the medium to fully exploit the potential of immobilized cells.

Prospects

Cell death is a critical factor limiting the productivity of cultured animal cells. Substantial research has been

devoted in recent years to elucidate the mechanism of cell death. Cell death occurs due to the following reasons: (1) Severe hydrodynamic shear and stress[32]; (2) Elevated levels of toxic byproducts such as lactate and ammonia[33]; (3) Limited availability of oxygen, nutrients and serum[34]. Cell death occurs mainly due to two established processes, namely necrosis and apoptosis.

Necrosis is a passive pathological event that occurs under severe trauma, causing sudden and significant cellular damage. It can be caused by complement attack on cell membranes or by severe environmental insults[35]. The etiology of the onset of necrosis is swelling of the cell and mitochondria followed by breakdown of plasma membrane[36]. Cells then release their cytoplasmic contents to the extracellular fluid, causing damage to the neighbouring cells and generating an inflammatory reaction. There is no evidence for a specific signalling pathway that causes necrosis. The degradation of DNA is non-specific[36].

Apoptosis is an active intrinsically-controlled process in which the cell actually commits suicide in response to particular modifications in its environment[37]. Under the normal physiological condition, this serves as a finely-regulated mode to eliminate cells that are old or harmful. A specific sequence of events, requiring the active participation of the dying cells, leads to its inactivation and removal by phagocytosis, without the accompanying inflammatory reaction. During embryonic development, apoptosis acts as a homeostatic regulator of tissue mass and architecture. A characteristic feature of apoptosis is that the cell extrudes water from the environment in an energy-requiring process, which is dependent on the active participation of the mitochondrion. High levels of transglutaminase activity can be detected, which results in the cross-linking of cytoplasmic and nuclear proteins, converting the cell into a rigid body[38]. Sustained elevation of cytosolic calcium concentration stimulates calcium-dependent degenerative enzymes such as cysteine proteases[39]. They cause collapse of the cytosolic structures. Nuclear condensation is accompanied by double-stranded endonuclease cleavage of cellular DNA at the linker regions between the nucleosomes. Fragments of DNA (ladder DNA) having 180–200 base pairs can be detected[39], the cell eventually lyses and loses its exclusion property to membrane dyes.

Metabolic engineering approaches have been taken to reduce the effects of necrosis and apoptosis[40]. Many of the inducers of apoptosis have been recognized[41]. Efficient steps have been taken to improve the local cell densities. But the cell densities achieved in conventional methods and certain innovative methods have certain disadvantages, as they have a limiting factor during the long duration of cultivation of cells. These limiting factors are similar to those which cause apoptosis and necrosis. Encapsulation of cell lines helps to avoid these problems. In all the long-term experiments conducted, there was no significant decrease in cell densities during the experimental period. Further, selection of the encapsulating material to a particular cell line or selection of a particular cell line to an encapsulation material helps in reducing the process of cell death to a minimal level. The long-term cultivation inside capsules reduces the labour and also helps further in material management. The continuous production of baculovirus is very promising, as large-scale production of virus (*in vitro*) can be done using small amount of material and resources when compared to production of virus by culture and growth of insect larvae, which requires greater finance and labour[42]. The same property can be used for the continuous production of recombinant protein bodies using genomic inserts of materials transcripted in baculovirus, as it is the best recombinational vector after yeast artificial chromosomes (YAC). Growth of cells inside cellulose sulphate capsules occurs in the immediate vicinity of the capsule membrane, but in case of alginates it occurs inside a complex hydrogel network; cells form spindle-shaped colonies inside these networks. Polyethylene glycol increases the number of hydrogel networks, thereby increasing the cell density.

Non-apoptotic growth of hybridomas inside capsules has its own advantages, as this will cause an increase in the productivity of cell-related products. One more advantage is the optimal performance of the micro-filtration or ultrafiltration equipment, which gets essentially blocked during apoptosis due to release of intracyto-organelles[43]. Moreover, there is an increase in the protein productivity in absence of apoptosis, as the proteases released during apoptosis may lyse or degrade the secreted proteins. One more advantage of growing hybridomas inside capsules is that they can directly be used for immune therapy, as alginate produces only slight immune response in the absence of serum proteins[44]. The growth of certain hybridomas inside capsules made of cellulose sulphate and other cationic polymers is available in the literature, but there is no information available using polyethylene-immine[45].

Liver cells are capable of undergoing necrosis and to a certain extent, apoptosis. Their long-term growth inside capsules of alginates and cellulose sulphate is of tremendous importance in developing *in vitro* models for phase 1 and phase 2 reactions in pharmacology. The data presented demonstrate that hepatocytes can continue to function and proliferate in a three-dimensional system such as microcapsules. This suggests that such cultures developed here may be the basis for mass cell culture or tissue-like growth required for development of extracorporeal bio-artificial liver. Three-dimensional growth allows access to nutrients on a large scale. This is important for the transition from monolayer to three-dimensional cultures. The capsules may be further cultivated in perfusion chambers or dialysis-based reactors, which allow continuous exchange of medium and oxygen for long-term growth. The

growth of cells inside capsules in T75 flasks indicates that this system does not require complex bioengineering techniques for obtaining growth, it can be achieved in a simple manner.

However, the presence of perfusion in the medium containing capsules will help in achieving higher growth rates on a large scale and this cannot be neglected. The results also demonstrate that human liver cells in three-dimensional culture environment can enter proliferation. This provides a new beginning for exploration of such three-dimensional culture methods and to maximize their potential. Providing the liver's synthetic function for brief but critical periods particularly in acute hepatic failure has been the goal of developers of extracorporeal liver systems[44]. Hollow-fibre cartridges with liver cells have been used on a large scale in the experimental stage[47]. The cell densities and the long-term survival of cells inside capsules provide a new and rapid hope and solution to the development of such devices. The following are the features of an ideal liver-assist device[48,49]: (a) significant metabolic capacity; (b) continuous treatment; (c) simplicity of use; (d) biocompatiblity; (e) availability, consistency and economy.

Many of the above-said features can be achieved using an encapsulated liver-assist device. A natural solution which was available for developers was a hollow-fibre bioreactor which could support 200 g of liver cells in 10,000 hollow fibres[50]. The disadvantage of this system was its consistency and that the cells have to be harvested once the extracapillary space of the fibre had become confluent. A simple reactor filled with high-density capsules containing liver cells in a serum-free medium assisted with haemodialysis and plasmaphoresis kit, will provide with a solution. The capsules can also be replaced continuously without stopping the entire device. The problems of serum-dependence associated with many liver cell lines can be avoided since the capsules, as demonstrated in all the experimental studies, can be grown in serum-free medium. This also reduces hypersensitivity, avoiding the problem of immunotoxic reactions.

Economic importance of bioencapsulation

Economic importance of bioencapsulation compared to other conventional methods was shown by research[51] that encapsulated insect cells are more suitable for the continuous production of baculovirus. With a multiplicity of infection of 10^9 and 10^{10}, large-scale production of virus was successful. The cells inside the capsules were alive for more than 30 days after infection and could produce virus continuously. The equipment needed for such production would be minimal and inexpensive compared to *in vivo* production of virus in insect culture. This requires large manpower, material input and it risks the chance of the insect larvae being infected by disease. Such risks are minimal in encapsulation-propagated production of baculovirus, where there is little risk of naturally-occurring disease to insect cells.

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