Research on . . .

ANIMAL CELL CULTURE IN MICROCAPSULES

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THE SINGLE MOST successful biotechnology product to date, the monoclonal antibody, is utilized for the detection of drugs in the blood (such as cocaine) and in the early diagnosis and treatment of diseases such as cancer. In another important area, genetic engineering, the development of new techniques has allowed for the enhanced production of a variety of polypeptides and proteins such as human insulin and growth hormone. There are still many human biologicals, however, which are too complex to be produced by either yeast or bacterial systems. Animal cell culture is presently the only method for the synthesis of many of these complex biologicals.

The major market driving force behind biotechnology is economic potential. For example, current markets for monoclonal antibodies for use in cancer therapy are in the hundreds of millions of dollars. It has been projected [1] that by 1991 the world market for monoclonal antibodies will be about 1.2 billion dollars (US).

It has become apparent over the past two decades that conventional suspension cell culture is limited by relatively low cell densities. As a result, the concentration of the desired product is low and purification from the growth medium is difficult. A major focus, therefore, has been placed on attempting to find cell culture methods which can improve the concentration of cell products and enhance product recovery, thereby permitting cost-effective, large-scale production. The long-term objective of the work being undertaken in our laboratory is the use of membrane technology, such as microencapsulation, in animal cell culture for the enhanced production and recovery of monoclonal antibodies and recombinant proteins.

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HYBRIDOMAS AND MONOCLONAL ANTIBODIES

Antibodies are proteins produced by white blood cells (B-lymphocytes) to aid in the destruction of foreign antigens. Hybridomas result from the fusion of antibody-producing lymphocytes with their malignant counterparts (myelomas) and exhibit the genetic characteristics of both parent cells. After a screening process, hybridoma cell lines can, if they are subcultured at regular intervals, indefinitely produce antigenically specific and identical (monoclonal) antibodies of the lymphocytes while, at the same time, retaining the ability to proliferate like the myeloma cells. Typically, levels of antibody in tissue culture supernatants are 5 to 50 μg/mL of medium [2]. Monoclonal antibodies have been used in immunoaffinity columns for the efficient purification of proteins such as interferon. As diagnostics, they have been used as sensitive detectors of minute quantities of illegal drugs such as marijuana and cocaine in the blood. They are also being employed in the treatment of diseases such as leukemia and cancer. In the latter case, a chemotherapeutic drug was covalently attached to an antibody which has a high specificity for the tumor cells.

INSECT CELLS AND THE BACULOVIRUS EXPRESSION SYSTEM

Insect cell culture has received an increased amount of attention recently since these cells are hosts for a class of viruses, the baculoviruses, which has been shown to be an excellent vector for genetic engineering [3]. This is mainly due to the high expression rate of the baculovirus and its post-translational processing capabilities [4]. After a protein's amino acid structure has been synthesized, certain processing or post-translational modifications must be made in order to make the protein biologically active. These modifications include efficient secretion, proteolytic cleaving, phosphorylation, N-glycosylation and possibly myristylation and palmitylation. Procaryotes (bacteria), on the other hand, cannot perform many of these modifications.

SUSPENSION CELL CULTURE

Perhaps the most widely used of all available cell culture methods is suspension culture. With this technique, cells grow in suspension throughout the medium and are circulated by means of air sparging (which also serves to transfer oxygen to the cells) and/or mechanical agitation. Careful consideration, however, must be given to the type of system to be used for large-scale work. Several investigators, for example, have noted that, unlike bacterial fermentations, agitation and aeration must be carefully controlled since mammalian [5] and insect cell cultures [6] have been reported to be extremely shear-sensitive. For this reason, air-lift bioreactors have proven to be useful. Besides having lower shears, higher oxygen transfer rates may be obtained.

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The major drawbacks associated with suspension cell culture, however, are the low cell densities (10^6 cells/mL medium) and low product concentrations (for example 10-150 μg antibody/mL medium). As a consequence, the recovery of biologicals from the culture medium is difficult and expensive. In addition, the sensitivity of mammalian and insect cells to shear stress is of major concern. A major focus, therefore, has been placed on attempting to find cell culture methods which can improve the concentration of cells and cell products and thus permit cost-effective large-scale production while, at the same time, offer protection to the shear-sensitive cells.

IMMOBILIZED CELL BIOREACTORS

One of the most common of all cell immobilization techniques is gel entrapment. This involves the confinement of cells within porous polymer matrices such as calcium alginate, polyacrylamide gels, κ-carrageenan and chitosan. This method offers the advantages of increased stability and protection from shear forces for fragile mammalian and insect cells, allows for higher cell densities to be obtained and virtually eliminates the need for the separation of the cells from the medium [7]. However, with this technique, there is no physical barrier to separate the proteins in the growth medium from the proteins produced by the cells. In addition, the antibody must be recovered from relatively large volumes of medium. Bugarski *et al.* [8], for example, immobilized mouse hybridoma cells in calcium-alginate beads, obtained, in an 11-day culture period, cell densities of 4.3×10^7 cells/mL of alginate bead and antibody concentrations of 100 μg/mL of medium (Table 1). This represented a productivity of 9 mg of antibody (IgG) per litre of medium per day. The advent of hollow fiber bioreactors, a major advancement in cell culture technology, resulted in significantly higher cell densities and antibody concentrations. With this type of reactor, the cells are cultivated on the outside surface of hollow, semi-permeable fibers while medium passes through the interior of the fibers. Diffusion of oxygen and nutrients to the cells, the removal of wastes from the cells, and the retention of high molecular weight cell

TABLE 1
Comparison of Different Cell Culture Methods

Culture Method	Specific Antibody Productivity (mg/L of medium • day)	Antibody Concentration in Harvest Liquor[1] (mg/L of Harvest Liquor)	References
Suspension (Batch)	1	6.5	Phillips *et al.*, [21]
Suspension (Chemostat)	110[2]	240	Dean, *et. al.*, [10]
Alginate Gel Beads	9	100	Bugarski, *et al.*, [8]
Hollow Fiber	5.5[3]	150[6] 740[7]	Altshuler, *et al.*, [9]
Microcarrier (Verax)	600[2]	100	Dean *et al.*, [10]
Microcapsules	28[4]	1000-4000	Posillico [17]
Microcapsules			
multiple membrane	8.8[5]	500-5000	King [18]
single membrane	1.0[5]	200-900	King [22]

1. Harvest Liquor = liquid which must be processed to recover the antibody
2. Space Time Productivity
3. Total Antibody Produced/Total Medium/Culture Period
4. Based on 7 litres of capsules + 33 litres of medium
5. Based on 1 mL of Capsules + 30 mL of medium
6. Shell side
7. Fiber side

products can be controlled by the choice of the fiber membrane molecular weight cut-off, medium flow-rate, and pressure drop across the fiber membrane. Ideally, retention of the cell product in the volume outside of the fibers (shell side) is desired. Altshuler *et al.* [9], for example, produced an IgG antibody in such a device reporting IgG concentrations of 740 μg/mL in the 2.5 mL shell space and maximum IgG concentrations of 150 $\mu g/mL$ in the bioreactor reservoir (volume 150 mL). This compares favourably with a maximum concentration of 7.6 $\mu g/mL$ for the same cell type in suspension culture. Cell concentrations approached 1×10^7 cells/mL in the reactor shell as compared to 1×10^6 cells/mL in suspension culture.

A major problem encountered with hollow fiber systems, though, was the poor control of the membrane molecular weight cut-off and the resulting loss of product. A polysulfone fiber rated to nominally retain 90% of molecules with molecular weight greater than 10^5 was employed by Altshuler and co-workers. However, their data suggest that only about 10% of the antibody (MW 160000) was retained by the membrane over a four-day culture period.

Microcarriers have also been investigated as an alternative to suspension culture. Successful carrier materials, based on the adsorption of cells onto the surface of or into a pore structure, include ion-exchange resin, ceramic supports, stainless steel mesh, and polyacrylamide beads. Perhaps the most interesting carriers are the weighted microsponge beads produced by the Verax Corporation [10]. The major problem associated with microcarriers as with gel entrapment and suspension, is loss of cells from the matrix.

MICROENCAPSULATION OF ANIMAL CELLS

Viable cells may also be immobilized in microspheres which possess a semi-permeable membrane. The membrane offers protection to shear-sensitive cells and provides a surface to which anchorage-dependent cells can adhere. Perhaps the greatest asset of microencapsulation, though, is the ability of the semi-permeable membrane to retain a high percentage of the protein product within the capsule. This high product retention may greatly reduce downstream processing problems.

Over the past two decades, several enzyme and living cell microencapsulation procedures have been developed. These include the polyacrylate capsules of Sefton [11], the chitosan/alginate system of Rha [12] and McKnight [13], the alginate-polylysine (PLL) polyethyleneimine (PEI) system of Lim and Sun [14] and the alginate-PLL technique of Goosen *et al.* [15]. Sun's group [16] extended this work to streptozotocin-induced diabetic rats, and found that transplanted, microencapsulated islet cells were effective in reversing the diabetic state in animals for more than 12 months.

In 1986, Posillico reported [17], for the first time, the use of microencapsulation for the production of monoclonal antibodies in multigram quantities. They reported, however, that their cells appeared to grow preferentially near the interior surface of the microcapsule membrane and speculated that this could have been due to mass transfer limitations during the cell culture or to the presence of a viscous intracapsular alginate solution. We have been able to show [15, 18] that the physico-chemical properties of these alginate/polyamino acid microcapsules such as size, shape and membrane molecular weight cut-off, could be varied by: changing the molecular weight and concentration of the PLL used in the encapsulation procedure and by adjusting the alginate-PLL reaction time. A review of this technology was recently published [19].

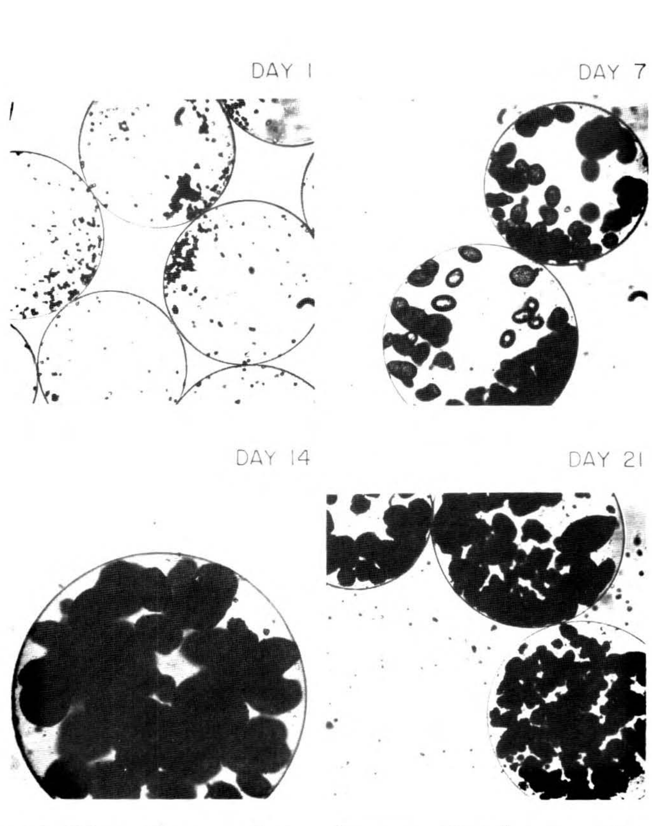

FIGURE 1. Tissue culture of encapsulated mouse hybridoma cells using a single alginate-PLL membrane. The initial cell density was 5×10^5 cells/mL of capsules and the final density was 2×10^7 cells/mL of capsules after about two weeks. The capsule diameter was 600 $\mu m \pm 60$ μm. [18]

CULTURE OF ENCAPSULATED HYBRIDOMA AND INSECT CELLS

The tissue culture studies in our laboratory with single-membrane alginate-PLL encapsulated mouse hybridoma cells confirmed similar work reported by Posillico [17] and Rupp [20]: hybridoma cells preferentially grow near the interior surface of the capsules, reaching a maximum cell density of about 2×10^7 cells/mL of capsules after about two weeks of growth. However, it was found that while the encapsulated hybridoma cells produced active monoclonal antibody, approximately two thirds of the capsule volume remained free of cells and was actually occupied by a viscous alginate core (Figure 1). This difference in the physical state of the capsule core may have been due to the fact that, in the present system, the capsule membrane molecular weight cut-off was lower (60000) than that reported by Posillico (80000). However, similar to our own observations, they found that only part of their capsule volume was occupied by cells. The presence of significant amount of intracapsular alginate (gel or liquid) would not only result in an inefficient use of capsule volume, but may also cause problems in the recovery and purification of the desired intracapsular protein product(s). With a modified, multiple membrane, capsule [18] on the other hand, significantly higher (300%) cell densities and product concentrations could be obtained (Figure 2). The entire capsule volume was eventually occupied by cells. This was presumably due to the lower viscosity of the intracapsular core.

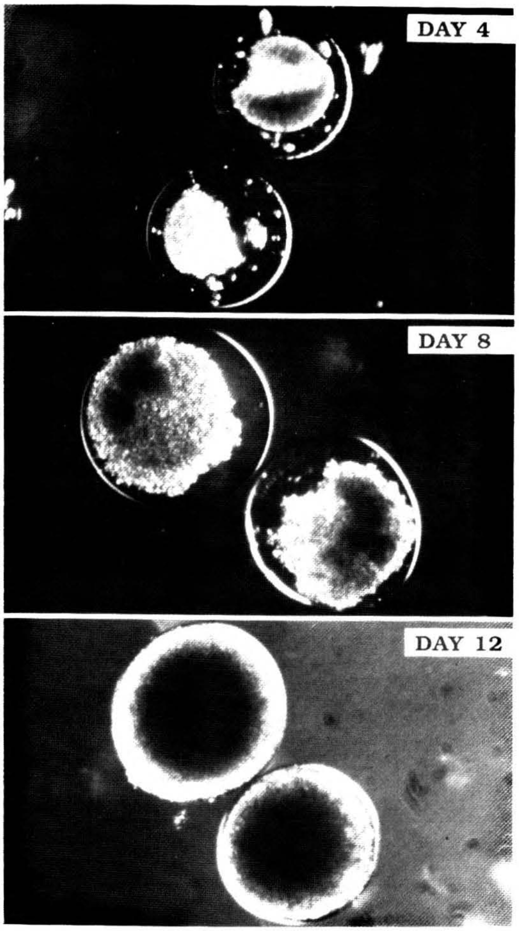

FIGURE 2. Hybridoma cells cultured in multiple membrane alginate-PLL microcapsules. Hybridoma cells were encapsulated with an initial cell density of 5×10^5 cells/mL of capsules. After about two weeks, the cell density had risen to 7×10^7 cells/mL of capsules. The average capsule diameter was 850 μm $\pm$ 85 μm. [22].

Various cell culture methods are summarized in Table 1. In terms of productivity, it appears that the Verax microcarrier system is superior. However, what is perhaps more important is the antibody concentration in the harvest liquor; that is, the liquid that must be processed to recover the antibody. In every case, except microencapsulation, this liquid is serum-supplemented medium. Virtually all of the harvest liquor antibody concentrations are equal except that of microencapsulation which is, at least, a factor of 10 higher. The result of this is that significantly less purification is required to recover the antibody. Other factors, however, such as process scale-up, equipment, raw materials and labour cost, and the mode of operation (*i.e.*, batch or continuous) must also be considered.

The culture of encapsulated insect cells in our laboratory proved to be more difficult. Insect cells, infected with a temperature sensitive baculovirus, could not be cultured in either single or multiple membrane capsules when the initial intracapsular alginate concentration was 1.4%. It can be postulated that the alginate may have inhibited oxygen or nutrients from reaching the insect cells or perhaps the cells were sensitive to the viscous alginate environment. Toxicity tests supported this observation. Only at alginate concentrations of 0.75% or less was cell growth observed. Cells encapsulated using single (low molecular weight cut-off) membrane capsules grew poorly (possibly due to some inhibitory effect of the alginate). However, infected cells would grow well in single (high molecular weight cut-off) membrane capsules but the membrane which formed was weak (causing the capsule to collapse) and often broke, allowing cells to escape into the medium. On the other hand, multiple membrane capsules were significantly stronger than their single membrane counterparts. Better cell and virus growth was obtained with the former capsules. This was possibly due to the lower intracapsular alginate content. High virus concentration (9×10^8 IFU/mL) were obtained with these microcapsules.

The growth of temperature-sensitive baculoviruses inside microcapsules appears to be a novel development. The ability to turn viral replication on by simply lowering the culture temperature has allowed, for the first time, the growth and concentration of virus inside of cell-filled microcapsules. Work is continuing on the production of recombinant proteins by encapsulated infected insect cells in an external-loop air-lift bioreactor.

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REVIEW: Levenspiel

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pressible Newtonians in Pipes, Compressible Flow of Gases, Molecular Flow, Non-Newtonian Fluids, Flow Through Packed Beds, Flow in Fluidized Beds, and Solid Particles Falling Through Fluids. The heat transfer section covers: The Three Mechanisms of Heat Transfer, Combination of Heat Transfer Resistances, Unsteady-State Heating and Cooling of Solid Objects, Introduction to Heat Exchangers, Recuperators, Direct-Contact Gas-Solid Nonstoring Exchangers, and Heat Regenerators. The book ends with a chapter called Potpourri of Problems.

The preface to the book indicates it is not for beginners. Levenspiel carefully states that the book is meant for practicing engineers and for those who have had an introductory course in transport phenomena. In keeping with that statement, the first paragraph of the text starts out with the First Law of Thermodynamics; the Second Law is covered in the second paragraph. This is definitely not a place for the raw beginner. Levenspiel quickly develops the macro-