# Introduction

At the dawn of recombinant DNA technology, cell culture was largely a research tool. The industrial use of animal cells was limited to the production of viral vaccines for human and veterinary applications. Some cell lines used for virus production could be grown in suspension cells and were cultured in flasks or stirred tanks. The majority of cell lines used were adherent and were mostly grown in roller bottles. Nowadays, industrial cell culture is operated in scales up to tens of thousands of liters. In spite of the dramatic increase in its industrial output over the last four decades, the basic design and operation of the bioreactor in cell culture processes have remained remarkably similar. The stirred tank bioreactor that has been the workhorse of bioprocess for microbial fermentation is also the reactor of choice for cell culture bioprocess. However, the adoption of the stirred tank for cell culture was not uniformly accepted in the early stage of cell culture bioprocess development. For nearly a decade until the early 1990s, much research and commercial development focused on exploring new bioreactors to overcome a number of hurdles cell culturing faced. The complex nutritional requirements of mammalian cells, especially their dependence on animal serum for growth and the resulting very high protein content in the medium, posed challenges in air sparging. The mechanical damage caused by agitation was another concern. What was unanticipated was the extraordinary capability of cells to adapt to suspension growth in highly turbulent flow conditions, and to adapt to serum-free, and even protein-free, culture media. The adoption of much leaner and simplified media helped expand

the bioreactor's operational parameter space, especially the reduction of protein content in the medium, which eased the use of air sparging for oxygen supply. By the early 1990s, the stirred tank that had been the workhorse for biochemical manufacturing for products such as antibiotics and amino acids became the bioreactor of choice. What has not changed since is the fact that the culture medium, constrained by cells' growth needs and intolerance to high osmolality, can support only relatively low cell concentrations and resultant low product concentrations, and that the accumulation of metabolites causes growth inhibition and limits productivity. These constraints are major factors in selecting the operating mode of a process.

This chapter will discuss the fundamentals of bioreactors. Despite virtually all industrial bioreactors being of the stirred tank type, some auxiliary equipment used in the process utilize a different type of reactor, and should be analyzed as such. Hence, an understanding of the basics of reactor performance is useful. For more in-depth discussion, readers are referred to a biochemical engineering textbook.[1] This chapter will also highlight a number of bioreactors that were developed in the exploration stage of cell culture processing. These examples are useful for their conceptual novelty and help to illustrate the limitations on their use as industrial bioreactors. Although these reactors are not commonly used in current processes, they might find specialized applications in other areas, such as tissue engineering and gene or cellular therapy.

# Basic Types of Bioreactors

Bioreactors can be generally categorized according to their mixing characteristics. The two extremes of mixing are complete, instantaneous mixing and a solid-like complete absence of mixing. The differences are best illustrated using a continuous flow reactor, although many bioreactors are used in batch fashion.

The two extreme mixing patterns characterize two types of idealized continuous reactors: the plug-flow (tubular) reactor (Figure 8.1) and the well-mixed stirred tank (Figure 8.2). In an ideal well-mixed bioreactor, the mixing is assumed to be so intensive that the fluid is homogeneous


**Figure 8.1.** Schematic of a tubular flow reactor.

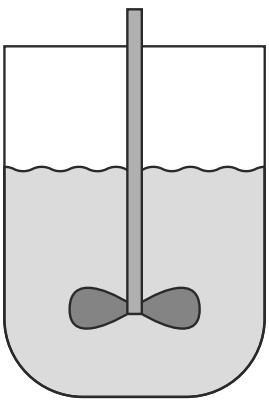


**Figure 8.2.** Schematic of a stirred tank reactor.

**Panel 8.1.** Idealized Bioreactor

- Plug flow reactor (no mixing)
- Well mixed reactor (instantaneously perfectly mixed)

throughout the reactor. When a new solution is added to the reactor, the solute is instantaneously and uniformly distributed.

A tubular reactor is the other extreme of an idealized bioreactor; it has absolutely no mixing (Panel 8.1). It is also called a plug flow reactor or piston flow bioreactor. As its name implies, a solution entering the bioreactor from one end moves downstream like a wall or a plug, and continues steadily forward until it reaches the exit.

## *Stirred Tank (Well-Mixed) vs. Tubular Reactor (Plug Flow)*

The distinction between a well-mixed continuous stirred tank reactor (CSTR) and plug flow reactor (PFR) is best illustrated by a comparison of their behavior after a step change in feed concentration. Consider a continuous reactor that has an inlet stream (feed) and an outlet stream (effluent) that are equal in volumetric flow rate; thus, the volume of the reactor is constant. Initially, both the fluid in the reactor and the feed stream are colorless. At time = 0, the feed stream is switched to a fluid containing a red dye at a concentration of $c_i$. We then observe how the concentration of the dye has changed at the outlet.

In a PFR, the red dye moves downstream like a sharp band, since there is no back mixing or diffusion to blur the sharp boundary between the colorful and colorless streams. After switching the feed stream to the red stream, it takes one holding time ($V/F$, see Figure 8.3b) for the red color stream to fill the reactor and reach the exit. When the dye begins to leave the reactor, its concentration is identical to that at the feed (Figure 8.3).

In the case that the reactor is well mixed (i.e., a CSTR), the color is distributed uniformly throughout the reactor as soon as the dye is added to the feed stream. Because the dye is uniformly distributed, the effluent will have the same concentration of dye as the reactor, regardless of where it is drawn from. Of course, for the purposes of our discussion, we

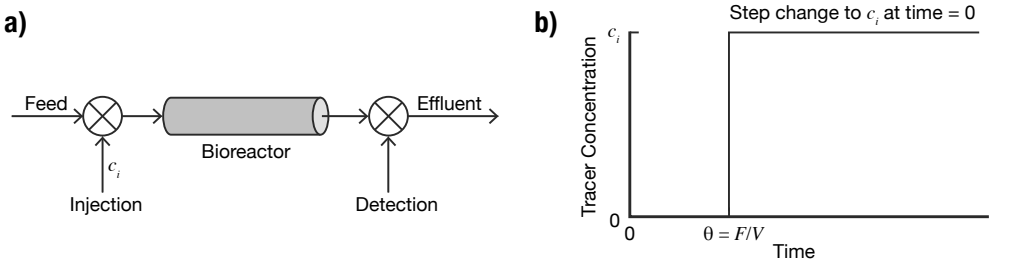



**Figure 8.3.** (a) A tubular reactor with a switch for tracer injection, and (b) the concentration profile of the tracer after the switch is turned on.

neglect the time delay caused by flow in the pipe leading into and out of the reactor. As more colored feed enters the reactor, the concentration of the color seen in the outlet will gradually increase. The balance equation for the concentration of the dye is shown in Panel 8.2 (Eqs. 8-1 and 8-2). Note that in this case, the concentration in the outlet flow is the same as in the reactor, because of the assumption of being well mixed.

By setting initial condition $c = 0$, one can solve the equation and plot the profile of the normalized concentration of the dye ($c/c_i$) over time (Eq. 8-3, Panel 8.2). *V*/*F* has units of time and represents the holding time (θ) (Eq. 8-4), the time it takes one reactor volume of feed to pass through the reactor. One can see that, after one holding time, the concentration of the dye in the reactor is 0.63 of that in the feed. It takes three holding times (3θ) for the concentration to approach (~98%) that in the feed (Figure 8.4). It is clear that a PFR has a very different mixing behavior from a CSTR. Of course, the reactors that are actually in use do not exhibit such ideal behavior, but many will have a mixing profile closer to one type than the other.

**Panel 8.2.** Material Balance of a Continuous, Well-Mixed Stirred Tank Bioreactor with a Step Change of Tracer Concentration

$$\frac{dV}{dt} = F_{\text{in}} - F_{\text{out}} = 0 \qquad \textbf{(Eq. 8-1)}$$

$$F_{\text{in}} = F_{\text{out}} = F$$

$$V\frac{dc}{dt} = F(c_0 - c) \qquad \textbf{(Eq. 8-2)}$$

Initial condition:

$$t = 0 \quad c = 0$$

Solution:

$$c = c_0\left(1 - e^{\frac{-F_t}{V}}\right) \qquad \textbf{(Eq. 8-3)}$$

Define holding time: $\theta = \dfrac{V}{F}$ **(Eq. 8-4)**

$$c = c_0\left(1 - e^{\frac{-t}{\theta}}\right) \qquad \textbf{(Eq. 8-5)}$$

## *CSTR and PFR with Reactions*

The discussion above considers only mixing behavior in the reactor, essentially just mixing tanks. In a bioreactor,

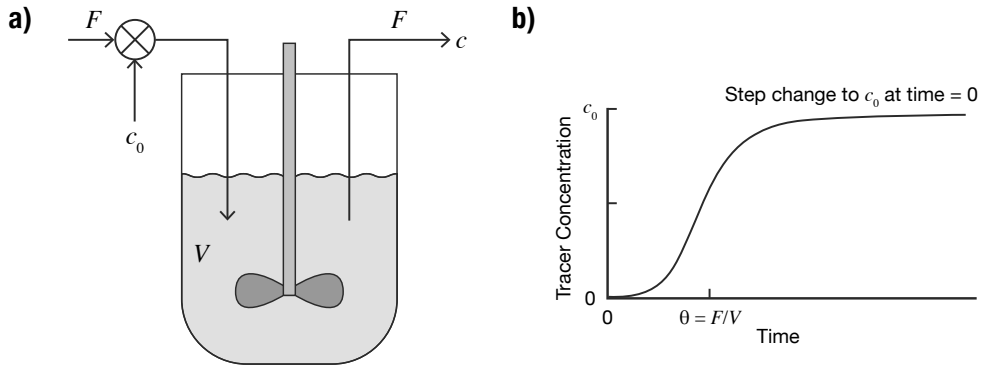



**Figure 8.4.** (a) A continuous stirred tank reactor with a switch for tracer injection, and (b) the concentration profile of the tracer after the switch is turned on.

**Panel 8.3.** Material Balance of a Stirred Tank Bioreactor with a Reaction at Steady State

$$V\frac{dc}{dt} = F(c_0 - c) - rV \qquad \textbf{(Eq. 8-6)}$$

$$V\frac{dc}{dt} = \frac{F}{V}(c_0 - c) - r \qquad \textbf{(Eq. 8-7)}$$

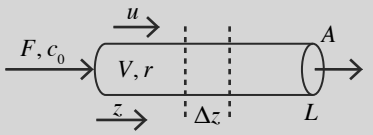
$$V\frac{dp}{dt} = \alpha_{p/c} r - \frac{F}{V} p \qquad \textbf{(Eq. 8-8)}$$

At steady state $\frac{dc}{dt} = 0$

$$\frac{F}{V}(c_0 - c) = r \qquad \textbf{(Eq. 8-9)}$$

$$\frac{F}{V} p = \alpha_{p/c} r \qquad \textbf{(Eq. 8-10)}$$

**Panel 8.4.** Material Balance of a Plug Flow Reactor




$$0 = Fc\big|_{z+\Delta z} - Fc\big|_{z} + r\Delta z A \qquad \textbf{(Eq. 8-11)}$$

$$Fc\frac{dc}{dz} = -rA \qquad \textbf{(Eq. 8-12)}$$

$$\frac{F}{A}\frac{dc}{dz} = -rA \qquad \textbf{(Eq. 8-13)}$$

$$u\frac{dc}{dz} = -r \qquad \textbf{(Eq. 8-14)}$$

consumption of nutrients and generation caused by product formation and growth take place; these processes (reactions) will cause the concentration profile to differ from that in a simple mixing tank. For a CSTR, the assumption that the concentration at the outlet is identical to that in the reactor holds. The balance of a reactant, i.e., how the amount of a reactant in the reactor changes with time, is thus the input of the reactant into the reactor, minus the output and the amount of the reactant consumed during the reaction (Eq. 8-6, Panel 8.3). For the product, it is then the input (usually nil) minus the output, plus the amount generated from the reaction. The effects of the reaction on the material balance equations are dealt with by adding a term to the material balance equation to account for the reaction (Eqs. 8-6 and 8-8, Panel 8.3). One balance equation is written for each of the reactants and products.

The equation describing the overall balance of the dye in a PFR differs from that for a CSTR. In a PFR, the concentration is not constant throughout the reactor, but is dependent on the position within the reactor. As the stream flows down along the reactor, the reactant concentration reduces as it is converted into product. Thus, the product concentration increases because the reactant and product concentrations both change along the reactor length. For a reaction that follows first-order kinetics (i.e., the reaction rate is proportional to the reactant concentration), the reaction rate decreases exponentially along the reactor length. At a steady state, the concentration at different positions in the reactor becomes constant. To account for the effects of position, we carry out a material balance on the dye over a very small section along the reactor (Eq. 8-11, Panel 8.4). The equation becomes an ordinary differential equation with the position as the only independent variable (Eq. 8-14).

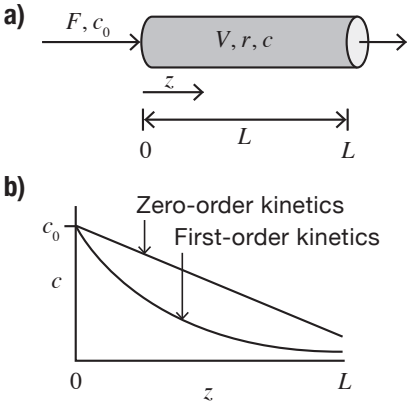



**Figure 8.5.** (a) Concentration profile of the reactant in a plug flow reactor at steady state. (b) The reactant concentration decreases along the reactor as it is converted to the reaction product.

**Panel 8.5.** Reaction Kinetics and Reactor Mixing Behavior

- The same reaction in a PFR or a CSTR has a different kinetic profile:
  - In a PFR, as the feed moves downstream, the reactant(s) is consumed and the product(s) is produced. The reactant and product concentrations change with the position in the flow direction.
  - In a CSTR, the concentrations of the reactant(s) and product(s) are homogeneous. The concentrations at the exit are the same as in the reactor.

It can be seen that, in the presence of a reaction, the concentration of the reactant decreases as the feed stream moves downstream, while the product concentration increases (Figure 8.5). For bioprocess applications using a PFR, the nutrient level will decrease while metabolite concentration increases along the bioreactor. At a certain point, the nutrients will become depleted, and a reactor longer than that length becomes unproductive.

## *Implications of Growth or Reactions Occurring in the Reactor*

Plug flow bioreactors are intrinsically more difficult to scale up than mixing vessels, as the concentration gradient of essential nutrients will inevitably become limiting in the downstream region of the reactor (Panel 8.5). One way to overcome nutrient limitations is to increase the nutrient supply rate by using a higher concentration in the feed or by operating at a higher flow rate. There are, however, practical limits on both nutrient concentration and flow rate. For example, nutrient concentration is limited by its solubility, and a high flow rate requires a higher capacity pump to overcome a higher pressure drop across a larger reactor. These kinds of limitations are especially true for growth of aerobic organisms, because oxygen solubility in water is very low and becomes quickly depleted unless supplied continuously. Thus, the size and scalability of a PFR reactor is somewhat limited. In a CSTR, all cells in the reactor encounter the same environment. The nutrients that feed into the reactor are distributed uniformly, albeit at abundant or suboptimal levels. Since all animal cells grow aerobically, virtually all industrial reactors for biologic manufacturing are of stirred tank types. However, plug-flow type

devices are used in various auxiliary operations such as the cell settler, where it is used for concentrating cells for recycle into the reactor.

### *Heterogeneous Reactor: High Solid Content*

A bioreactor typically encompasses three phases: a liquid phase of dissolved nutrients and metabolites; a solid phase consisting of insoluble nutrients, cells (which can be regarded as "solid"), and sometimes cell support materials; and a gas phase (bubbles for aeration). Since culture samples taken from the liquid phase do not contain gas phase, and cell mass represents a very small fraction of the culture liquid volume, the content of a cell culture bioreactor is often treated as homogeneous, as if having a single liquid phase. Cells, like nutrients, are treated as part of the liquid phase, and no special consideration is given to the volume taken up by cells in establishing material balance equations.

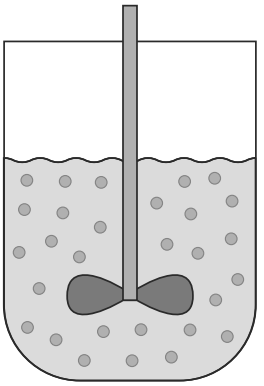


**Figure 8.6.** A microcarrier bioreactor. Cells attach to the surface of microcarriers, which are suspended in the culture medium. It is a heterogeneous reactor with a solid phase (microcarriers) and liquid phase.

In microbial fermentation and plant cell culture, cells may make up a large fraction (up to 50%) of total reactor volume. The reactor content is therefore partitioned into cell mass and culture medium, each occupying some volume. The cell and nutrient concentrations calculated from medium volume or total reactor volume have different values.

Some cell culture bioreactors contain a significant volume fraction of solid phase such as microcarrier beads (Figure 8.6). Such heterogeneous bioreactors must be treated as two separate phases in material balance. In a microcarrier bioreactor, for example, microcarrier beads often constitute 10–30% of the culture volume. In this circumstance, even cell concentration needs to be well defined. For example, it must be specified whether $10^7$ cells per milliliter is with respect to total culture volume or to liquid volume.

## Tissue Culture and Disposable Cell Culture Systems

Arguably, the oldest bioreactors for protein and virus production are animals themselves or their tissues. The application of virus vaccines produced in animals dates back two hundred years to when Edward Jenner used cow pox to inoculate humans for protection against small pox. Many viral vaccines developed in the first half of the twentieth century employed animal or animal tissues as production vehicles. Even today, many viruses, including the vast majority of influenza viruses used in vaccination, are produced in embryonated chicken eggs. The

production of biologics is carried out in a wide variety of bioreactors, from tens of thousands of chicken eggs in a viral vaccine factory to multiple stirred tanks of tens of cubic meters in size. They cover applications from treating a single patient a limited number of times to providing medicine to tens of thousands of patients for the treatment of a chronic disease. In this section, we will describe reactors used for large-scale cultivation of mammalian cells.

## *Disposable Culture Systems*

The classic Roux culture flask, the narrow-mouthed, rectangular glass flask, started to be replaced by disposable plastic T-flasks four decades ago. The takeover was complete by the early '90s. Today, virtually all laboratory cell culture in the western world today is carried out in disposable flasks, including T-flasks, roller bottles, and multiple flat panels. For small quantity production, such as in the case of growing cells for autologous therapy, flasks are still commonly used for both attachment-dependent and suspension cells.

**Panel 8.6.** Single-Use Bioreactor

- Simple, low-capital investment in plant construction, fast to implement, ease of equipment validation
- A variety of systems available with vastly different mixing mechanisms
- Less well-characterized in fluid dynamics, mass transfer
- Suitable for seed culture, scales up to hundreds of liters
- Possible faster turnaround
- Lacks the strength of steel; some operations that are routinely performed in steel vessels, such as pressurized liquid transfer, are not easily undertaken in single-use vessels

In the last two decades, the disposable culture system has gone beyond the traditional bottle- and flask-based systems (Panel 8.6). Instead of relying on employing more units to increase production, the new disposable systems aim to increase reactor volume and reduce the requirements of labor and auxiliary equipment. These disposable systems have been widely used in the seed train or cell expansion stage after thawing from cryopreserved vials. As discussed in Chapter 1, even thousand-liter disposable reactors are available for manufacturing.

### *Roller bottles, flasks*

Roller bottles are cylindrical screw-capped bottles with a total volume ranging from 1 to 1.5 L, and are suitable for a culture volume of 0.1 to 0.3 L. The large surface that is exposed to air allows for oxygen diffusion into the medium. Adherent cells can attach to the inner wall of the bottle, while suspension cells simply grow unattached (Figure 8.7). Stacks of bottles are placed on a rack and rotated at 1 to 4 rpm. For small-scale operations, roller bottles provide many advantages for the cultivation of adherent cells. The system is relatively inexpensive to set up and allows for rapid adjustment of production throughput in response to changing needs. Furthermore, complete replacement of medium (e.g., from growth medium to product medium) is relatively straightforward.

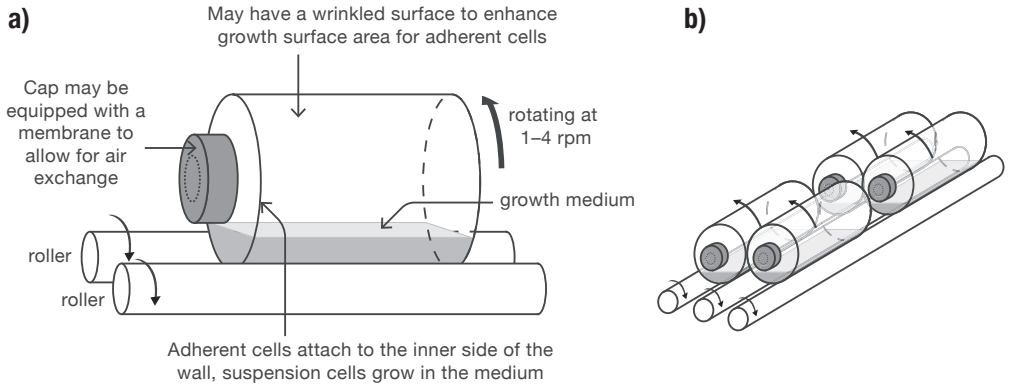



**Figure 8.7.** A roller bottle for cell culture.

Roller bottles are particularly useful if a serum-containing medium needs to be replaced by a serum-free or protein-free medium for the production phase. The transparent plastic wall allows visual or microscopic examination of the culture status. Most microbially contaminated bottles can be readily identified and discarded before mixing with others.

For large-scale production of biologics, however, there are numerous drawbacks to roller bottles. In-line and on-line environmental monitoring and control is virtually impossible or at least impractical. Aseptic bottle handling for inoculation, protease-treatment for cell detachment and expansion, medium exchange, and product harvest are labor intensive and must be performed by skilled technicians to ensure a low failure rate. A batch of a size suitable for manufacturing purposes may require hundreds or even thousands of bottles, and the large number of manual steps involved dramatically increases the risk of microbial contamination.

Despite these significant drawbacks, roller bottles are widely used in the production of viral vaccines. In some cases, roller bottles are still in use because the legacy product was approved by regulatory agencies decades ago and a process change would incur significant costs for regulatory approval and could even result in product comparability issues. In other cases, roller bottles are selected because a roller bottle process is easy to implement and the production capacity needed is manageable. However, when faced with the need to increase production, e.g., due to market expansion, scale-up of a roller bottle process is challenging.

Notable examples of industrial roller bottle processes include the production of erythropoietin (EPO) using recombinant Chinese hamster ovary cells and the production of live attenuated chickenpox (varicella) vaccine and herpes zoster (shingles) vaccine using the adherent secondary human lung fibroblast cell strain, MRC-5. In some manufacturing facilities, a fully automatic, robotic roller bottle handling system is available. However, a robotic system does not handle a large number of

bottles in parallel, thus prolonging the duration of handling when a large number of roller bottles are to be processed. The virus inoculation step can be stretched for many hours, from starting the first bottle to finishing the last bottle, possibly making the production conditions less optimal.

Roller bottles are typically incubated in a temperature-controlled environment in an industrial process. Usually no air exchange is necessary in a days-long process. Some roller bottles have a gas-permeable membrane for gas exchange for use in a $CO_2$ cell culture incubator. Recently, variations of T-flasks and roller bottles have been used in the cell therapy community. In the cultivation of T cells and NK cells, stationary culture has been found to be preferred, possibly to allow for minute factors to accumulate in the cells' surrounding microenvironment. These culture devices install a gas-permeable membrane at the bottom that allows cells to settle on it. Even blood bag-like devices with high gas permeability are available commercially.

### *Multiple plate systems*

Systems consisting of multiple parallel plates enclosed in a chamber are widely used in laboratories and in industrial production of viral vectors, vaccines, and cells for therapy. These parallel plates, which are stacked inside a transparent plastic box, increase the available surface area for adherent cells to attach to and grow on. A 40-tray Nunc Cell Factory® (NCF) has a capacity of 25,280 $cm^2$ and an ~8 L liquid volume. One notable example of NCF use is the production of the multivalent Rotavirus vaccine using Vero cells, a continuous African green monkey kidney cell line. Another multiple plate system, CellCube® Module, consists of nine-inch square polystyrene plates stacked vertically in a resin case with 1 mm spacing. The space between stacks is completely filled with medium. Continuous medium circulation is used for oxygen and nutrient supply. CellCube has been used in the cultivation of MRC-5 cells (human fibroblasts derived from the lung) for the production of both attenuated and inactivated hepatitis A virus (HAV) vaccines. These disposable bioreactors have also been used in the clinical production of cells for immunotherapy and gene therapy vectors.

### *Bags and moderately scalable disposable culture wares*

Blood bags were used for the cultivation of cells for cell therapy three decades ago. Small bags are placed in incubators for temperature control. Typically, the bag is not filled up with medium; instead, an air space is left in the bag for oxygen supply. For larger, single-use bag systems, mechanical mechanisms provide mixing and oxygen transfer. Wave™ consists of pre-sterilized, disposable Cellbags resting on a special rocking platform.[2] The rocking motion of the platform induces waves in the culture fluid, which provides mixing and enhances oxygen transfer (Figure 8.8).

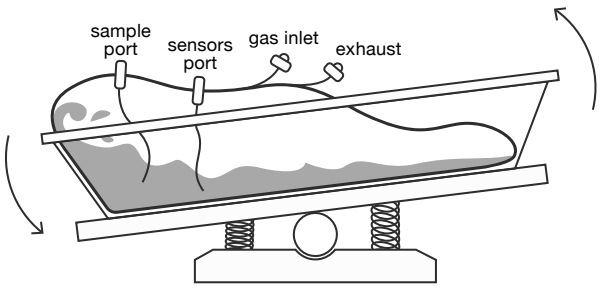



**Figure 8.8.** Schematic of a wave bag cell culture system.

This and other simple disposable systems are basically the combination of a culture medium container and an external mechanism to move the container and provide mixing. Their ability to increase in size is limited by the capability to supply oxygen and the ability to move a large mass of liquid.

Disposable systems provide opportunities for manufacturers to offload to specialized disposal suppliers the work related to equipment preparation, validation, and maintenance. In order to allow for further scale up, direct aeration into the liquid phase must somehow be introduced. Single-use plastic equivalents of existing stainless-steel bioreactor vessels emerged to fill this niche. Such a reactor consists of a reusable stainless steel outer support container and a single-use cell chamber with a working volume of up to 1000 L, which can be integrated into a bioreactor control system. A manufacturing plant consisting of these modest-scale, single-use reactors is becoming an alternative to a conventional stirred tank made of stainless steel with fixed piping, auxiliary equipment, etc. Because of the material of construction, its operation faces limitations. For example, it cannot be pressurized to quickly transfer its contents to other process units. It is also challenging to scale it up much beyond its current size, unless the mechanical strength of its materials is reinforced to withstand the higher power input at an affordable cost, while still mitigating the environmental issue of employing disposable plastic.

# Adherent Cell Support Systems

## *Suspension vs. Adherent Culture*

For processes whose production scale is relatively small, a disposable system provides some advantages. For protein therapeutics needed in large quantities, the use of disposable systems may be limited to inoculum preparation. Large-scale operations therefore continue to employ fermenters or other bioreactors.

The majority of cells used for protein production via rDNA are suspension cells grown in stirred tank bioreactors. However, many cell lines used for the production of viruses for vaccine and gene therapy applications are adherent. For the cultivation of these cells, a supporting surface, sometimes called a substrate, is needed. Many disposable systems have been commercially developed to provide a large amount of cell growth surface area in a single structure.[3] These systems are invariably limited in

their scale of operation. For applications that require a very large quantity of cells, it is necessary to employ support for cells to adhere to and grow on in a bioreactor.

## *Microcarriers*

Microcarriers are small particles that are large enough to support the growth of adherent cells. In a stirred tank bioreactor, cells can be cultivated on the surfaces of the suspended microcarriers, thus allowing the process to be scaled up.[3] They fall into three general types: conventional solid or microporous microcarriers that provide external surface area for cell attachment and growth, macroporous microcarriers that also allow cells to grow in pores in their interiors, and microspheres that facilitate the formation and growth of cells as aggregates.

An advantage of microcarrier culture is the ease of separating cells from the culture medium. Many microcarrier cultures require medium exchanges during cultivation to remove lactate, ammonia, and other metabolites and to replenish nutrients, or to remove serum in the medium before virus infection. This can be accomplished by simply allowing cell-laden microcarriers to settle, such that the spent medium can be withdrawn from the top and replenished. In large-scale operations, continuous or semi-continuous perfusion is accomplished by withdrawing the medium through a coarse screen or from a settling tank to retain microcarriers in the reactor.

### *Solid and microporous microcarriers*

Microcarriers were first used for cell culture by Van Wezel in the 1960s. They changed the cultivation vessel of adherent cells from a two-dimensional surface to a three-dimensional, scalable reactor. To reduce the power required to suspend the cell-laden microcarriers and reduce the mechanical damage of agitation, microcarriers typically have a diameter range of 100–300 μm and a specific density of 1.02–1.05 g/cm$^3$ (Panel 8.7). This diameter range provides good growth surface area per reactor volume. Even at a moderate microcarrier concentration occupying

**Panel 8.7.** Desired Characteristics of Microcarriers

| | | |
|---|---|---|
| Density | ~1.02 g/cm$^3$ | Only slightly higher than water for easy suspension and settling |
| Diameter | 150–200 μm | Carriers with a small diameter that still allows for cell spreading (more surface area per bead volume, slower settling velocity) |
| Porosity | From solid to nearly 90% | Prefer solid for use as inoculum (easier bead-to-bead transfer) |
| Surface properties | ECM coating, slight positive charge | Positive charge enhances initial cell attachment |

8–15% of the culture volume, a significantly larger surface area per reactor volume can be achieved than is possible with roller bottles.

Microcarriers have been fabricated from many different materials, including dextran, gelatin, polystyrene, glass, and cellulose. Many that were once commercially available are no longer on the market because microcarriers constitute only a niche market for some vaccines and a limited number of recombinant proteins. In general, microcarriers have a wettable surface and are sometimes coated with collagen or other adhesion molecules to enhance cell adhesion and spreading. The most widely used microcarriers, which are based on dextran, are derivatized with charged molecules or denatured collagen. These carriers are microporous. In its dry form, the bead is very small. Upon hydration in the medium or isotonic buffer, the bead diameter increases many times and ~90% of the bead volume is water. Hence the relatively low specific density. Normal diploid fibroblasts can grow well on microcarriers and exhibit normal morphology or even the typical parallelly aligned pattern seen when grown on Petri dishes (Figure 8.9). Continuous cell lines grown to high densities sometimes even form multiple layers (Figure 8.10).

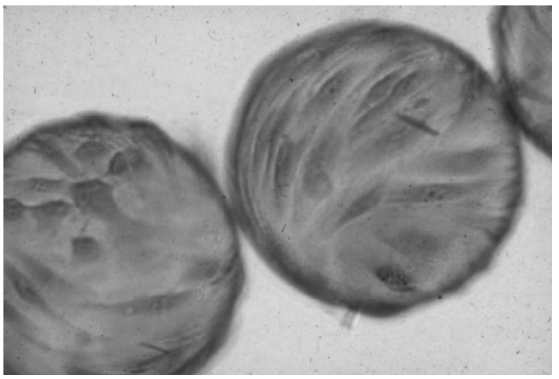


**Figure 8.9.** Human foreskin fibroblast FS-4 grown on charged dextran (Cytodex 1) microcarriers.

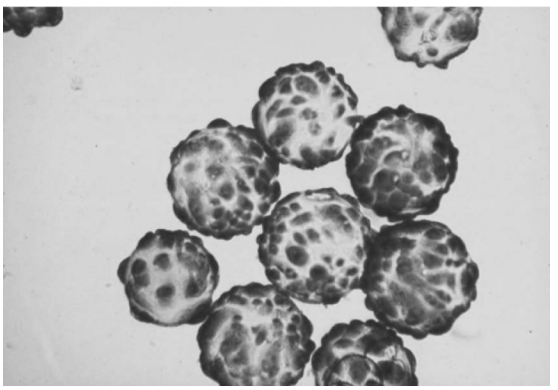


**Figure 8.10.** CHO cells grown on charged dextran (Cytodex 1) microcarriers.

Other types of commonly used microcarriers are made of polystyrene. Their specific density and settling velocity in water is higher than the dextran-based microcarriers. On a unit weight basis, polystyrene microcarriers have much less surface area than dextran carriers. While dextran beads are transparent under a microscope, easy for microscopic observation of cell morphology, polystyrene beads are refractive and therefore not convenient for cell observation. Additionally, the surface of polystyrene beads is hydrophobic, making them unsuitable for cell attachment. Thus they are subjected to irradiation, plasma treatment, or another chemical modification to increase the wettability of the surface, or are derivatized with cell adhesion peptides to facilitate cell attachment.

A wide variety of cell types have been grown on microcarriers, including fibroblasts, epithelial cells, hepatocytes, neuroblastoma cells, and

endothelial cells from various species. Overall, microcarrier culture allows a very large number of adherent cells to be grown in a stirred tank bioreactor. Depending on the cell type, a cell concentration of 2–10 x $10^9$ cells/L is achievable. However, in very large-scale operations it is not feasible to inoculate cells from conventional flask or roller bottle cultures; the inoculum will have to be from a seed microcarrier culture. For cell therapy applications, it is also necessary to detach and recover cells. Detaching adherent cells from microcarriers requires extensive optimization to achieve a high recovery yield. The selection of a microcarrier type will influence the ease of cell detachment.

### *Macroporous microcarriers*

Macroporous microcarriers contain large interconnected internal pores (Figure 8.11). The void space inside allows cells to be cultivated on internal and external surfaces. Cells in the interior are less susceptible to mechanical damage caused by agitation and gas sparging. On the downside, cells in the interior of microcarriers are more likely to be subject to oxygen limitation due to the long diffusional distance, especially since most macroporous microcarriers have a larger diameter (500 μm–2 mm) than standard microcarriers.

**Figure 8.11.** Scanning electron micrograph of a collagen-based macroporous microcarrier.

Macroporous microcarriers are made of various materials, including gelatin, collagen, and plastic. Many cell lines have been successfully grown on macroporous microcarriers, such as Vero, HepG2, CHO, and HEK293 cells. The final cell concentration achieved tends to be higher than that obtained with an equivalent volumetric concentration of conventional microcarriers. In some cases, however, the growth kinetics are slower because the penetration of cells into the interior may be slowed or even retarded by the restrictive openings of the pores (Figure 8.12).

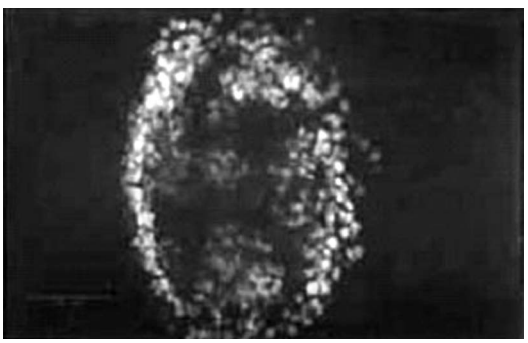


**Figure 8.12.** Confocal microscopic section of HepG2 cells grown in a macroporous microcarrier.

### *Microsphere-induced cell aggregates*

Most anchorage-dependent cells do not develop normal morphology and do not multiply on surfaces with an excessively high curvature. When cultivated on the surface of a small cylindrical rod

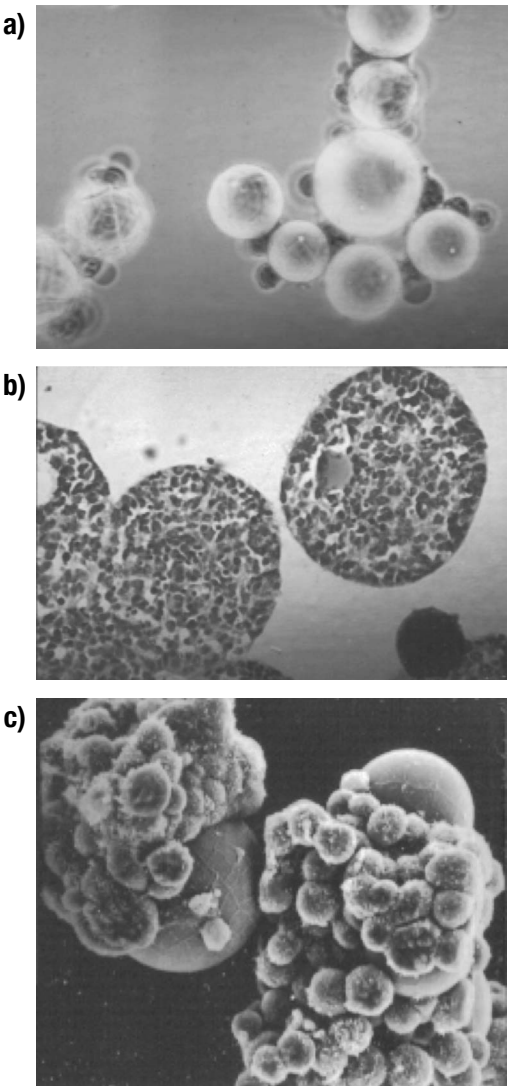



**Figure 8.13.** Microsphere-induced cell aggregates. (a) CHO cells attaching to a microsphere 1h after inoculation. (b) A thin section of HEK293 cell aggregates. (c) Scanning electron micrograph of HEK293 cell aggregates.

with a sufficiently large diameter, fibroblastic cells will grow in random orientations. If the diameter is reduced to a level with a high curvature, cells will begin to align in the longitudinal, rather than radial, direction. Similarly, if the diameter of a microcarrier is too small, many cells will not spread out on the surface and grow. However, many continuous cell lines can multiply even if they are not spread out like they would be on a flat surface. When these cells are grown on very small microcarriers, they agglomerate to form aggregates and continue to grow to a high density. These microspheres, usually possessing a diameter of about 50 μm, provide the initial surface for cell agglomeration and proliferation. These agglomerated microspheres become aggregates as the cells grow. If the aggregate diameters become too large, dead cells can be seen in the interior as a result of transport limitations. Many cell lines, including BHK, CHO, HEK293, and swine testicular cells, have been cultivated as aggregates using microspheres (Figure 8.13).

## *Cell Aggregates*

Some cells can be grown as aggregates when cultivated in shaker flasks or stirred vessels without any surface support. These include not only continuous cell lines like BHK and HEK293, but also some primary tissue isolates and stem cells. For example, pluripotent stem cells can be grown as aggregates called embryoid bodies and increase in cell number and differentiate. Hepatocytes isolated from liver can form three-dimensional spheroids that retain differentiated properties but have limited capacity for cell expansion. These cells form cell-cell interactions in a short time when placed in a suspension, and can then grow without cell-substrate adhesion (Figure 8.14). It has been shown that high $Ca^{2+}$ concentrations promote HEK293 cell aggregation, while low concentrations elicit partial dissociation. Some cells do not form aggregates quickly when placed in

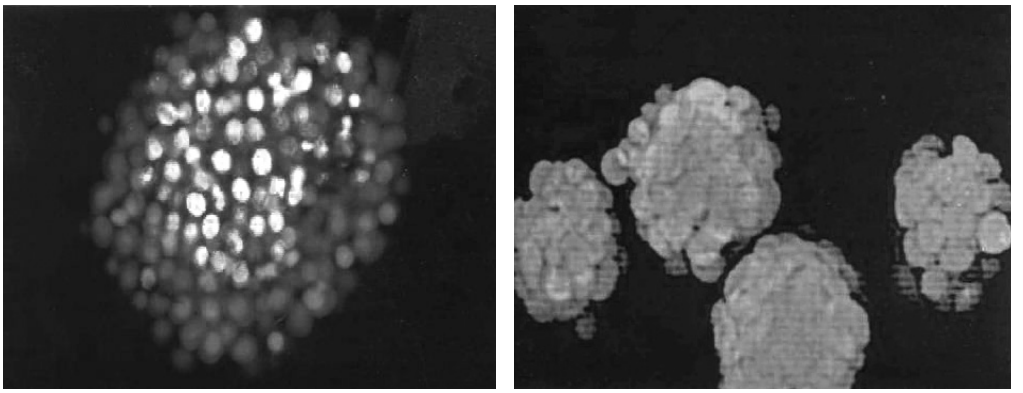


**Figure 8.14.** Confocal microscopic section of HEK293 cells grown as aggregates.

suspension. These cells, e.g., embryonic stem cells, can be forced to form aggregates by placing them in round-bottom, low-attachment wells or in suspended medium drops (hanging drops) for about two days. Upon forming aggregates, they can be cultured in stirred tanks.

Aggregate cultures have advantages similar to microcarrier cultures. They can be cultivated in conventional stirred tank reactors with environmental controls. Upon the cessation of agitation, particles will settle relatively rapidly, permitting easy medium replenishment or perfusion. A downside of aggregate cultures is the controllability of the aggregate size. Excessively large aggregates often have a lower viability that may influence product quality if the product is degraded or otherwise modified by enzymes released by lysed cells. For some applications that require dispersed cell suspension rather than cell aggregates, dissociation of highly viable cells from aggregates will require some optimization effort.

# Bioreactors Developed for Cell Culture

At the dawn of recombinant DNA technology, cell culture faced great challenges. The traditional processes for virus vaccine production did not support the productivity and product quantities required for new protein biologics. Cells were grown in media containing high levels of protein, for which supplying oxygen by direct air bubbling was difficult. Cells were sensitive to mechanical stress incurred by impeller agitation, and they produced large amounts of lactate, ammonium, and other metabolites that inhibited cell growth. To overcome those hurdles, much effort was devoted to cell culture technology development and numerous innovations were developed. In the end, the problems were resolved by means other than adopting new types of manufacturing bioreactors. With the advances in our understanding of cells' nutritional needs, serum was eliminated from media and the protein content drastically reduced, making direct sparging of air manageable. Over time, cells were generally

adapted to sustain the mechanical agitation of a stirred tank bioreactor. The problem of low cell concentration due to metabolite accumulation was overcome by adopting fed-batch or perfusion culture and managing the accumulation of inhibitory metabolites. In the end, the prevailing bioreactor used in cell culture was, and remains to this day, the stirred tank. It is instructional to briefly review some of those earlier innovations in order to glean the lessons learned. Furthermore, some bioreactors may find applications in tissue engineering or cell therapy applications.

## *Stirred Tank Bioreactors*

Stirred tanks have been the workhorse for microbial fermentation since the 1950s and began to be used in cell culture for vaccine production using BHK cells in 1960s.[4] The basic configuration of the stirred tank bioreactor for mammalian cell culture is similar to that for microbial fermentation. While the Rushton type impeller is the norm in microbial fermenters, mammalian cell culture fermenters mostly employ axial flow type impellers (see Chapter 12). The difference reflects the different purposes of agitation in microbial fermentation versus cell culture. In microbial fermentation, agitation is needed at a high power input to disperse air bubbles and to increase oxygen transfer efficiency, whereas in mammalian cell culture the primary purpose of agitation is to maintain relatively uniform suspension of cells or microcarriers.[5] The power input per unit volume of bioreactors is also substantially lower. In general, the mixing time in a mammalian cell culture bioreactor is substantially longer than that in a microbial fermenter of similar scale. The oxygen transfer capacity in a cell culture bioreactor is also substantially lower than that in a microbial fermenter. However, the typical oxygen demand in a mammalian cell culture is 10 to 50 times lower than that in microbial fermentation.

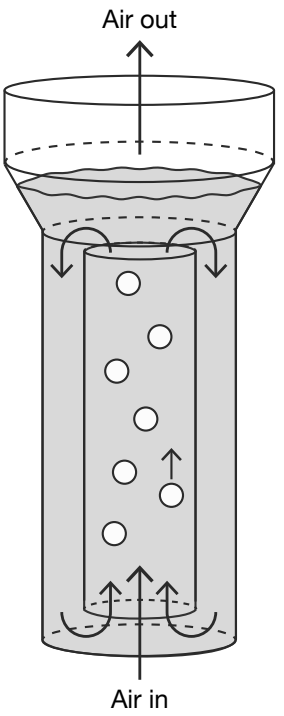



**Figure 8.15.** An airlift bioreactor.

## *Bioreactors with Alternative Mixing Mechanisms*

### *Airlift bioreactor*

An airlift reactor uses large air bubbles to drive the liquid in a segregated region in the bioreactor upward, which then induces the liquid to move downward in a second region, thus creating an internal circulation loop that suspends cells, provides mixing, and supplies oxygen. Airlift reactors are used to grow mammalian, insect, and plant cells. A common configuration has a cylindrical internal draft tube that is concentric to the reactor wall above the air sparger (Figure 8.15). The air bubbles drive the liquid upward because of the upward momentum generated by the

air flow and the lower effective fluid density in the draft tube. Medium flows upward through the sparged section (riser) and downward in the bubble-free section (downcomer). This method of generating circulation has a low energy requirement compared with stirred-tank reactors. Airlift bioreactors for cell cultivation are considered to be low-shear devices because there is no mechanical agitation. They have been used successfully with suspension cultures of BHK21, human lymphoblastoids, CHO, hybridomas, and insect cells. Airlift bioreactors are effective when the cell concentration is low and a small air flow rate is sufficient to provide mixing and oxygen. Without mechanical agitation to break up air bubbles, the bubble size is large and oxygen transfer efficiency is lower at a given air flow rate per reactor volume. As cell concentration increases in a fed-batch culture, a higher aeration rate has to be used. Airlift bioreactors are primarily used in laboratories for insect or plant cell cultures.

### *Fluidized bed bioreactor*

Fluidized bed reactors have long been used in chemical heterogeneous catalysis. The fluid stream carrying the reactant (often gas phase) enters through a flow distributor at the bottom of the reactor at a sufficiently high velocity to suspend the solid catalyst particles. The reactant enters the catalyst, and product diffuses out into the fluid, where it is carried out at the top of the reactor. A separator prevents the catalyst particles from being blown out. The main advantage of a fluidized bed is the high heat transfer efficiency between the fluid and the catalyst surface. When applied to cell culture, cells must be immobilized on particles, typically macroporous microcarriers, so that the particle can be separated from the exiting liquid phase and be retained in the reactor (Figure 8.16). However, unlike in chemical catalysis, mass transfer and heat transfer at the external surface of the microcarrier are not rate controlling. Rather, the limiting factor is often the accumulation of metabolites in the medium. Furthermore, the consumption of the nutrient is relatively low; one single pass of medium through the reactor would consume only a small fraction of the nutrient. Hence, the bulk of the medium is recirculated continuously to suspend the particles. The recirculating medium also supplies the oxygen by passing through an

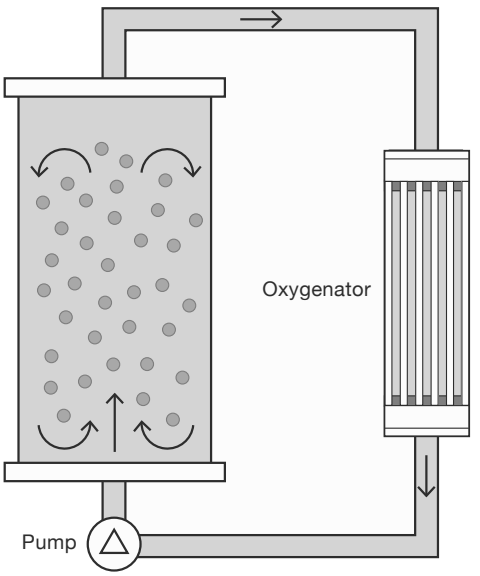



**Figure 8.16.** Schematic of a fluidized bed bioreactor. The carrier-free culture fluid is pumped into the fluidized bed. The heavy carriers recirculate in the reactor. The culture fluid exits from the reactor and is re-oxygenated before returning to the reactor.

oxygenator before returning to the reactor. In order to increase productivity, the microcarrier concentration is high, which in turn requires a high medium recirculation rate to supply a sufficient amount of oxygen. To sustain a high flow rate, the density difference between solid phase and liquid phase must be sufficiently large to allow for the retention of particles. In the fluidized bed bioreactors for cell culture commercialized in the 1980s, the carriers were weighted down by iron particles to increase the density difference between fluid and carrier. This allowed the particles to be retained in the reactor at the flow rates required to supply sufficient oxygen for cell growth.

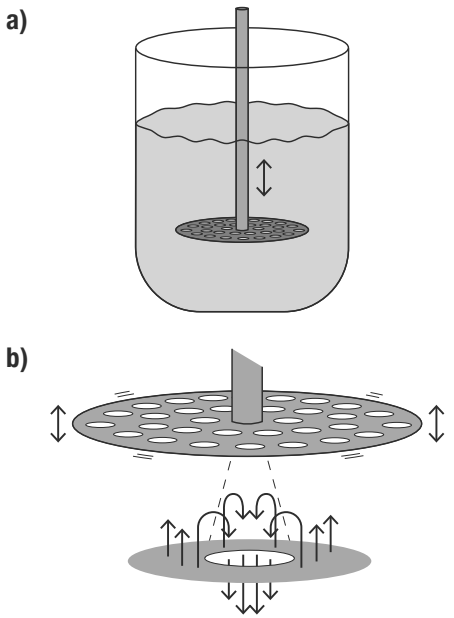



**Figure 8.17.** (a) A vibromixer bioreactor and (b) its fluid flow pattern.

### *Vibromixer*

A vibromixer uses a perforated disk as the mixing mechanism rather than a conventional impeller. The disk vibrates in a vertical direction (perpendicular to the plane of the disk) at a high frequency, causing liquid to circulate through the perforated holes and provide mixing (Figure 8.17). The vibromixer was used for the cultivation of suspension cells for virus production. It has since been replaced by the stirred tank. However, it is still used to provide mechanical agitation to suspend microcarriers at high concentrations after trypsinization, in order to facilitate the detachment of cells from microcarriers.

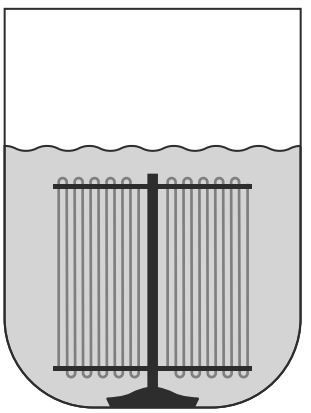


**Figure 8.18.** A stirred tank bioreactor with polymer tubing for oxygenation and mixing.

### *Membrane stirred tank*

The membrane stirred tank was developed by Professor Jürgen Lehmann in the 1980s to overcome the problem of supplying oxygen in a high-serum medium (Figure 8.18).[6] The bioreactor had a rotating shaft with a number of extended upper and lower arms that rotated with it. Pieces of long microporous polypropylene tubing for supplying oxygen were wrapped around the rotating upper and lower arms. Air flowed through the tubing and exited above the surface of the medium without making direct contact with the medium. By adjusting the air pressure in the polypropylene tubing, the micropore could be expanded to allow the gas that is near bubble-bursting pressure to be in direct contact with the medium, thereby providing bubble-free aeration. The rotation of the tubing also provided gentle

agitation to microcarriers or suspended cells. The design illustrates the challenge of supplying oxygen in a high protein content medium.

## *Membrane Bioreactors*

The primary aim of all membrane reactors is to keep cells in a compartment that is isolated from mechanical stress caused by agitation, liquid pumping, or air sparging. The reactor, depending on its configuration, may also allow the product to accumulate in that compartment. All are plug-flow type reactors and rely on medium recirculation to supply oxygen to the cell, thus limiting the scalability of the reactor.

### *Hollow fiber bioreactor*

A hollow fiber reactor consists of a bundle of microporous capillary fibers encased inside a cylindrical casing, thereby providing two different spatial compartments: the tube (fiber lumen) side and the shell (extracapillary) side. The culture media is pumped into the fiber lumen from one end, and exits from the fiber's other end (Figure 8.19).[7] As the medium passes through the fiber lumen, oxygen diffuses through the fiber wall into the shell side and supplies oxygen to the cells. Hollow fiber bioreactors are used primarily for the cultivation of suspension cells that grow in the extracapillary space on the shell side. The extracapillary space also has a medium flow loop that allows for additional medium supply, especially high molecular weight components of the medium, to the cell compartment and for harvesting the product. This basic configuration is somewhat similar to the hollow fiber cartridge used in kidney dialysis.

Each hollow fiber consists of a porous polymeric layer that provides mechanical support and is coated with a thin membrane that selectively allows molecules to pass through based on their size. In most cases, an ultrafiltration membrane is used. The molecular weight cut-off (MWCO) of the membrane differs according to the specific application, ranging from a few thousand to a hundred thousand daltons.

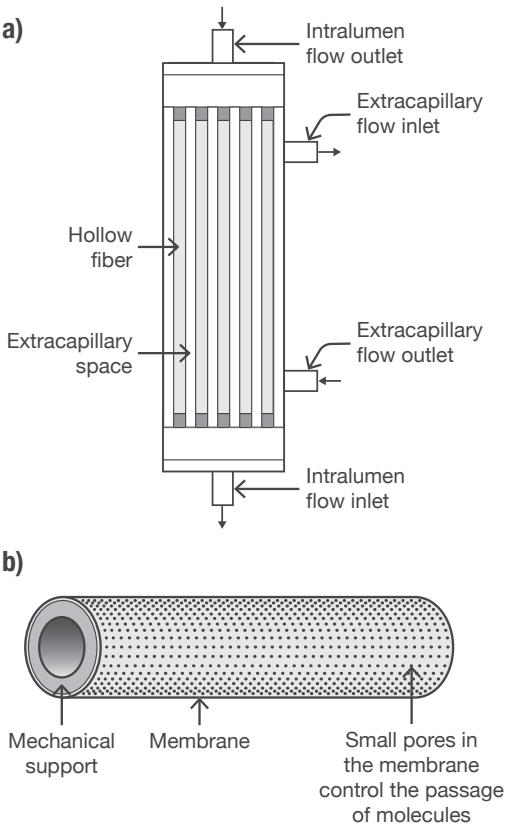



**Figure 8.19.** (a) A hollow fiber bioreactor and (b) a single fiber. Thousands of such fibers are encased in the reactor.

Supply of low-molecular-weight nutrients and oxygen to the cells and the removal of waste products occurs by diffusive transport across the membrane between the lumen and the shell spaces. The ultrafiltration membrane prevents free diffusion of secreted product molecules through the membrane, instead allowing them to accumulate in the extracapillary space to a high concentration. The medium recirculating in the lumen side is passed through an oxygenator before returning to the reactor. Although the use of hollow fiber bioreactors is now infrequent, they are still an easy and convenient tool for the production of small quantities of antibodies for research or diagnostic applications.

### *Multiple membrane plate bioreactor*

The scaling up of a hollow fiber system is limited by the ability to extend the axial length of the fiber due to oxygen transfer limitations. The use of a very high flow rate to supply more oxygen is limited by the capacity of the pump and the mechanical strength of the membrane. Expanding the cartridge diameter to increase the capacity eventually causes an uneven flow distribution to the fibers. Furthermore, major resistance to oxygen transfer resides in the diffusion in the extracapillary, inter-fiber space (i.e., from the fiber wall to where the cells are). One more hurdle is in achieving an even distribution of cells upon their inoculation into the tortuous extracapillary space. In principle, one can use two different fibers to supply oxygen and medium separately but simultaneously, using one set of fibers for gas phase and another for medium. However, mixing different hollow fibers in a cartridge poses a major challenge in manufacturing. An alternative configuration can be made by using multiple flat membranes that allow for more even cell distribution in the cell chamber and better control of the oxygen diffusion distance to the cells. However, this seemingly versatile reactor also suffers from practical manufacturing complexities and is not used in large-scale operations. Overall, membrane reactors constitute only a very small fraction of all reactors used in cell culture. If one wishes to employ a membrane bioreactor for cell cultivation, the commercially available kidney dialysis hollow-fiber cartridges are most assessible.

## *Cell Support Systems that Shield Cells from Mechanical Stress*

In addition to microcarriers developed for adherent cells, cell support systems were developed for suspension cells. The goal was to find a way to protect cells from possible damage caused by mechanical agitation and bubble aeration while retaining cells (along with the cell support mechanism) in the reactor during medium exchange. These systems invariably entailed encasing each cell in some form of bead. While these approaches dealt with the obstacles of the day, they also had limitations.

Many of the materials utilized prevented them from being used in stirred tanks unless the scale was small. Most methods generated particles of a fairly large size—in the range of millimeters—that required a large liquid pumping capacity to keep the particles in suspension. The cell mass was typically a very small fraction of the bead and an even smaller fraction of the reactor volume. Some of the systems later found applications in tissue engineering, for example, as a method of entrapping beta cells for insulin secretion. However, they often face the problem that only a small fraction of the total volume of implanted beads are active cells.

### *Agarose*

Agarose entrapment of cells can be easily accomplished by adding cells in agarose suspension drop-wise into an immiscible oil phase, where it is allowed to gel. The beads formed usually have a large diameter of millimeters. To control the particle size, the cell suspension can be injected through a concentric double tube. The cell suspension flows into the inside tube and is ejected through an orifice into an air stream that flows in from the space between the tubes. The air jet stream carries the ejected cell suspension drop-wise into a chilled oil phase that allows the agarose to gel. In another method of cell entrapment, the cell-agarose suspension is allowed to drop onto the central region of a fast-rotating disk. The centrifugal spinning force causes the droplets to form and be dispensed on the outside of the disk. The cell-entrapped beads can then be packed in a column or suspended in a stirred tank for cultivation. Cells entrapped in agarose beads grow into clusters and produce the product.

### *Microencapsulation*

In microencapsulation, cells are first entrapped in a polymeric matrix, such as alginate or chitosan, to form gelled spheres. Those cell-laden beads are then coated with a polymeric film that constrains the diffusion and release of the product molecules out of the microcapsule while allowing nutrients to diffuse through.[8] A polymer matrix commonly used for cell entrapment is calcium alginate. Cells are suspended in sodium alginate and added dropwise into a solution with a high calcium chloride concentration that causes the alginate to form a gel. The alginate beads are then coated with polylysine or other polymers to increase the mechanical and chemical stability of the beads (Figure 8.20). The alginate gel inside the beads coated with the polylysine membrane can be liquefied through treatment with a calcium chelator such as EDTA or citrate.

Large-scale application of microencapsulated cells is rare, since the beads are not mechanically strong enough to withstand the mechanical mixing encountered in scales beyond the pilot plant. However, the microcapsule provides a means of immunoisolation of transplanted cells or tissues, and could be suitable for some tissue engineering applications.

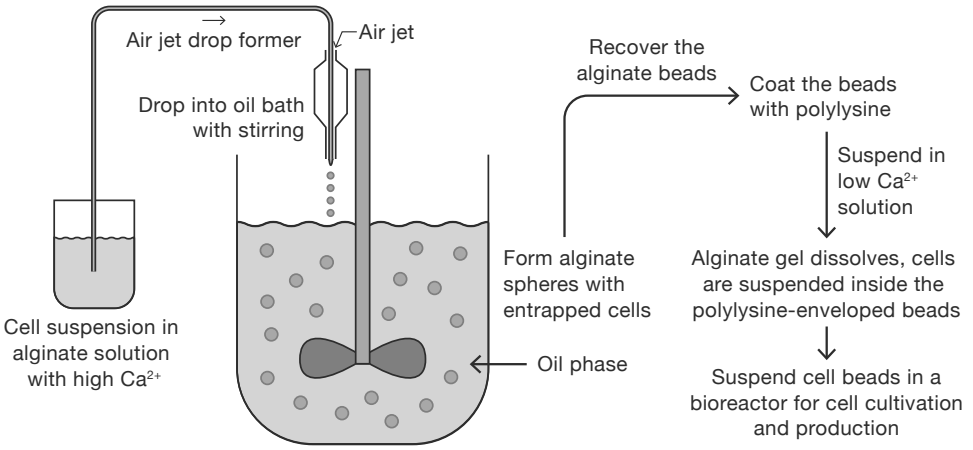



**Figure 8.20.** Preparation of a microencapsulation system for cell culture.

# Concluding Remarks

This chapter highlighted the key differences between well-mixed and plug-flow bioreactors. Because of the low solubility of oxygen and the very limited oxygen-carrying capacity of media, virtually all industrial manufacturing of cell culture products uses stirred-tank bioreactors. When a stirred tank bioreactor is used for growing adherent cells, a supporting surface for cell attachment is provided through the use of microcarriers or macroporous microcarriers. Alternatively, adherent cells may be grown as aggregates in suspension. Microcarriers and a number of the cell culture bioreactors developed three decades ago were the product of intensive efforts to bring about cell culture manufacturing. Today, the time-tested stirred tank bioreactor for suspension cells remains the bioreactor of choice. The durability of the stirred tank bioreactor is due to its simplicity as well as advances in other aspects of cell culture, including cell adaptation and medium improvements. Even as disposable devices become more widely used, the basic type of disposable device still relies on extensive liquid mixing. With an increase in scale, even the disposables tend to take the form of the stirred tank. Some innovative bioreactors developed at the dawn of cell culture bioprocess may now be finding new applications in tissue engineering and cell therapy. As these new technologies advance and the demand for those specialized cells grows, we may find some lessons that can be learned from earlier bioreactor development efforts.