Reprinted from *American Biotechnology Laboratory* July 2004

New Developments in Hollow-Fiber Cell Culture

by John J.S. Cadwell

With the growth and development of genetic engineering and hybridoma technologies, cell culture has taken on a whole new life. Genetically engineered mammalian cells produce and secrete proteins of scientific and therapeutic importance. Hybridoma technology, the science of fusing immune mouse cells to immortal cancer cells, makes it possible to produce monoclonal antibodies in quantity from cells grown in culture. To produce these proteins in the quantities required for commercial use, it became necessary to scale up cell culture efforts.

Many high-cost capital and equipment-intensive methods are available for the production of these proteins in commercial scale. The "trickle down" of these advanced molecular biology techniques to the average research laboratory means that virtually any graduate student can produce a recombinant protein or monoclonal antibody. Equipment readily available at large pharmaceutical companies are not practical in the academic research or start-up biotechnology company laboratory. The search for a simple, cost-effective cell culture system has resulted in the birth of a new area of biotechnology: the use of bioreactor systems to grow cells on a large scale. A bioreactor based on hollow-fiber bundles is the ideal method for culturing 10^9 to 5×10^{10} cells in any laboratory. This article describes the classic applications for hollow-fiber systems as well as some newly developed ones.

Hollow fibers are small, cylindrical filters, shaped like drinking straws, and as small in diameter as a human hair (200 µm) (see *Figure 1*). Large bundles of these fibers can be potted into cylindrical housings in such a way that any liquid entering from the ends of the cartridges (end ports) will go through the insides of the fibers, while access to the area outside the fiber (extracapillary space, or ECS) is provided by side ports on the outside of the housing. In general, cells are placed on the outside of the fiber where they can attach and grow, while cell culture medium is continuously circulated through the interior of the fibers to provide nutrients and oxygenation. Because of the characteristics of the filter, smaller molecules such as glucose and lactate can cross the fiber freely, while larger molecules such as proteins cannot cross the fiber. If there are any cytokines or autocrine factors that either potentiate or inhibit cell growth, their effects on the cells can be controlled by the selection of the pore size or molecular weight cutoff (MWCO) of the fiber.

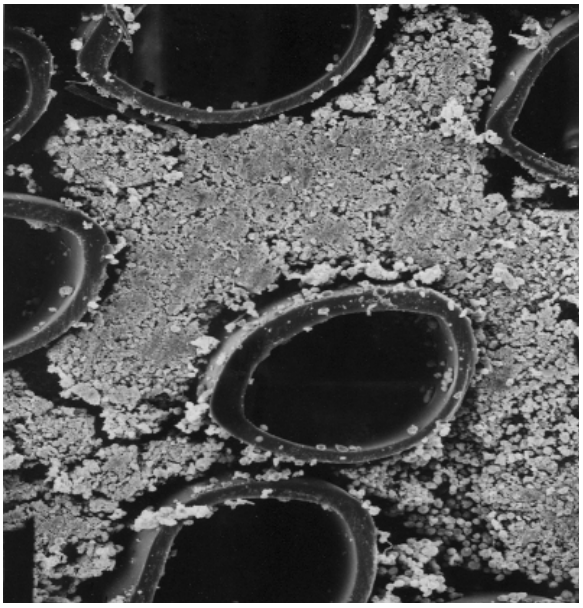

Figure 1 Hollow-fiber cartridge in cross-section showing the fibers and lymphocytes in culture.

The first description of hollow-fiber systems for cell culture was described by Knazek et al.,[1] who sought a method to grow cells at densities similar to those found in vivo, i.e., 10^8 cells/mL or higher, as opposed to the 10^6/mL or so achieved by standard cell culture techniques. Hollow-fiber modules can provide a tremendous amount of surface area in a small volume, as much as 200 cm^2 per mL. This allows a large number of cells to attach in a very small volume. Extremely efficient exchange of nutrients and waste products occurs across the fiber wall, and since the MWCO of the fiber can be controlled during manufacturing, the

filtration characteristics of the fiber can be adjusted to either retain fiber particular proteins or cytokines or allow them to pass through the fiber into the circulating medium.

Although hollow-fiber bioreactors are not new, improvements in materials and protocols have reinvented the technology. Two examples are described below. The first is a new type of polysulfone that offers: 1) An extremely high gross filtration rate—the gross filtration rate of a typical cellulosic fiber with a 30-kD MWCO is about 15 (mL/min/cm^2/mm Hg pressure/hr). The gross filtration rate of the FiberCell 20-kD MWCO polysulfone fiber (catalog nos. C2011 and C2018, **FiberCell Systems,** Frederick, MD) is in excess of 140. 2) Waves cast into the fiber—the fiber bundle is then evenly distributed within the housing. The waves push against one another to produce a uniform fiber bundle, eliminating channeling effects and areas of necrosis produced by the fiber spacing being too great.

The medium-sized hollow-fiber bioreactor cartridges (catalog nos. C2011 and C2008, **FiberCell Systems**) provide 2200 cm^2 of surface area in a volume of only 15 mL. The larger cartridges (C2018 and C2003) provide 1.2 m^2 of surface area in a volume of 60 mL.

One of the defining characteristics of hollow-fiber bioreactors is their ability to support cell growth at densities greater than 10^8 per mL. The normal spinner flask culture of mammalian cells occurs at densities around 10^6 mL. High cell densities result in a high concentration of secreted proteins, efficient virus infection, and the ability to reduce serum requirements or more easily adapt the cells to a serum-free medium.

Another fundamental difference between hollow-fiber bioreactors and other cell culture techniques is that hollow fibers form a porous support for cell attachment. This is the most in vivo-like manner in which to grow cells. Since nutrient delivery is from the bottom up, cells are free to pile on top of one another to form a layer many cells thick. It is not necessary to split cells in a hollow-fiber bioreactor. Depending on their growth characteristics, these cultures can be maintained for extended periods of time. Researchers have used a single FiberCell hollow-fiber bioreactor to produce a monoclonal antibody for more than one year of continuous production. Chinese hamster ovary (CHO) and HEK 293 human embryonic kidney cell lines will produce protein for three months or longer in the same cartridge.

Monoclonal antibody production

The third defining characteristic of hollow-fiber bioreactors is their ability to modulate the effects of cytokines on cell growth based on their molecular weight. The most common example of this occurs in monoclonal antibody production using hybridoma cell lines. This was the first larger-scale application for hollow-fiber bioreactors. Hybridoma cells secrete tissue growth factor-beta (TGF-β), a cytokine that can be inhibitory to hybridoma growth. The molecular weight of the active form of TGF-β is around 27 kD. A hollow-fiber filter with the correct MWCO will allow the TGF-β to diffuse away while the secreted antibody is accumulated to a high concentration in the ECS of the cartridge. The inhibitory effects of TGF-β are reduced or eliminated by dilution of the cytokine into the circulating medium. The same mechanism is in effect for lymphocytes; there it is tumor necrosis factor-alpha (TNF-α), which inhibits lymphocyte growth.

The advantages of hollow-fiber bioreactors for monoclonal antibody production are well documented:

- Antibody concentrations are typically 100× higher than flask culture in the range of 0.5–5 mg/mL
- Harvest volumes are 15–60 mL, collected every two days
- Adaptation to serum-free medium is facilitated by the high cell density (see *Figure 2*)
- Endotoxin burden can be reduced when compared to ascites fluid production
- Chimeric, humanized, and nonmurine antibodies can be easily produced
- Antibodies can be produced from a single cartridge for more than six months of continuous culture.

A hollow-fiber bioreactor system is well suited for the production of 100 mg to several grams of antibody.

Secreted protein production

Another reason hollow-fiber bioreactors are gaining favor is their use for the production of recombinant proteins. CHO and HEK 293 cell lines are the most popular, but other types of cells have been used as well, including some constitutively expressing insect cell lines such as Drosophila. In this case, there are no inhibitory cytokines to consider, and the MWCO of the fiber is selected based solely on the molecular weight of the protein to be produced. Expression levels are typically 100× higher

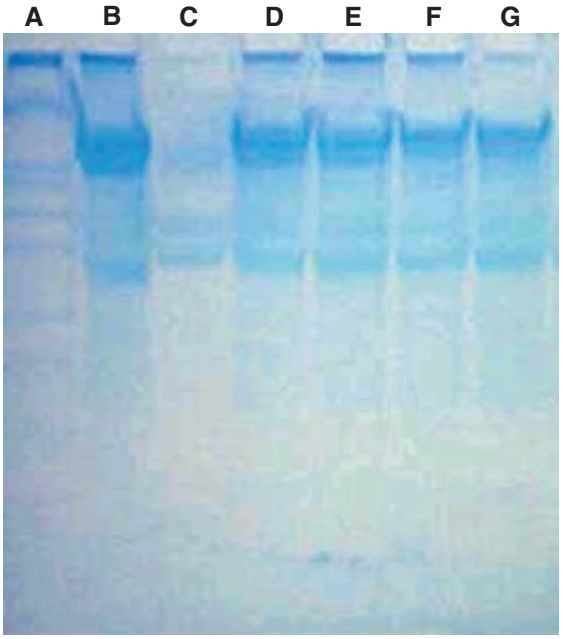

Figure 2 *Gel showing circulating serum-free medium and several harvests from the ECS of the cartridge. Lane A: MW standards. Lanes B, D, E, F, and G: Harvests from ECS. Lane C: Circulating serum-free medium. This gel demonstrates that IgG of 147 kD is retained by the fiber, while proteins in the range of 60 kD and smaller can cross the fiber into the circulating medium.*

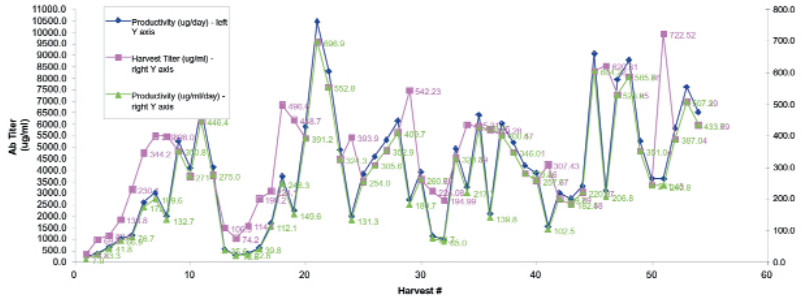

Figure 3 *HEK 293 production of a recombinant IgG1 in serum-free medium.*

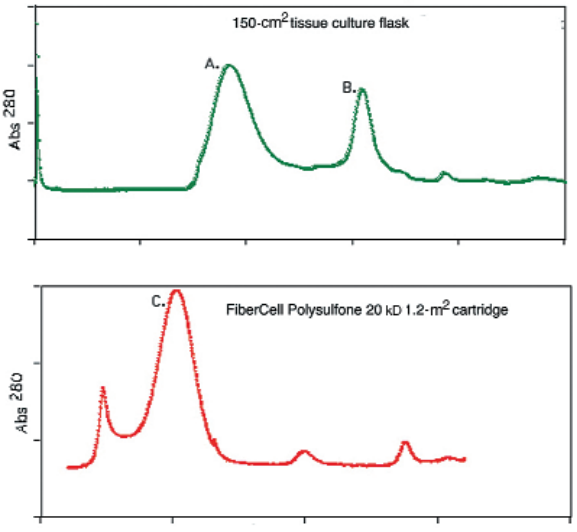

Figure 4 *Comparison of a hexerimized IgG1 produced in flask culture and the FiberCell hollow-fiber bioreactor.*

than those seen in flask culture, with levels in the range of 100–500 µg/mL of harvest volume.

Figure 3 shows the protein data and lactate data from an HEK 293 cell line expressing a recombinant IgG cultured in serum-free medium. The medium was changed when its glucose was 50% of the initial value, except on the weekends. On Friday afternoons, a vigorous harvest was performed to reduce cell mass, and a single liter of fresh medium was attached to the system to support the cartridge over the entire weekend. The system typically consumes 1 L of serum-free medium every two days. Two aspects are most interesting. The first is the productivity data. Although there is significant variability in the day-to-day production, on some days the harvested protein is nearly 800 µg/mL, with a harvest volume of 20 mL. In total, 276 mg of protein were produced in two months of culture in a volume of 900 mL, for an average concentration of 300 µg/mL and 4.5 mg/day.

Secondly, it is easy to see where Monday falls on the graph. These are the peak days of lactate concentration and protein levels. The medium has not been changed and no harvest has been performed for more than two days. The protein has had a chance to accumulate over the weekend and the medium is completely spent. Glucose is below 100 mg/mL; pH is well below 6; and the lactate levels are extremely high, reaching a maximum of 3.8 mg/mL. Although it can be seen that cell metabolism drops significantly for a day or two afterwards, it is impressive to note that the majority of cells survived this level of lactate and continued production. As the culture matures and cell density increases, the recovery time is even shorter. This high resistance to lactate is a result of the high-gross-filtration rate fiber and is observed with most cell types grown in the FiberCell cartridge.

Figure 4 compares protein production in a recombinant CHO cell line in T150 flasks to a FiberCell hollow-fiber bioreactor system. The cells were grown in the T150 flasks until nearly confluent, and were then harvested and seeded into the bioreactor. The protein expressed is a hexeramized IgG1 consisting of six IgG1 subunits held together with three IgA tails. When cultured in flasks, approximately 40% of the protein is expressed as an improperly folded monomeric subunit; when transferred to the FiberCell bioreactor, approximately

95% of the protein is expressed as a properly folded hexamer. Four hundred seventy-five milligrams of protein was produced in 10 weeks in a total volume of 4 L.

The advantages of hollow-fiber systems for the production of recombinant proteins are the same as for hybridomas, with the additional benefit that if the protein is extremely complex and/or heavily glycosylated, improved protein folding may result in comparison to flask culture. Hollow-fiber bioreactors are useful for the production of ten milligrams to several hundred milligrams of recombinant protein.

Virus production

Virus production has long been an attractive application for the use of hollow-fiber bioreactors. Cells grown in high density should provide rapid and uniform infection kinetics for virus propagation, and the resultant virus should be obtained at very high titers. Effective protocols for the production of different viruses have proven elusive. Many of the first attempts at virus production centered on retrovirus production in 3T3 or PA317 packaging cell lines. These cell lines are of fibroblastic origin and are extremely adherent. Cells quickly overgrow the hollow-fiber cartridge, reducing the harvest volume from 10–15 mL to as little as 0.5 mL. Although the virus harvested was of a high titer, the small total volume of virus recovered made this method ineffective. Additionally, if it were necessary to propagate the virus by infection, only the outer layer of cells would become infected and produce virus.

The advent of improved serum-free mediums that could adapt certain cell lines to suspension culture solved one of the shortcomings of virus production in hollow-fiber systems. If HEK 293 cells were adapted to suspension culture, then infection with adenovirus or other virus types requiring infection for propagation could proceed. The success of this method was first reported by Chung et al.,[2] and the method was expanded upon by Dr. Alex Kotov at the University of Alabama Gene Therapy Center (Birmingham).[3] The general principle is that if the packaging cells can be placed into suspension culture and the virus is transmitted by infection, then it should be possible to produce large quantities of virus in a hollow-fiber system.

Epstein-Barr Virus and HIV have also been successfully produced in this manner. In the case of HIV, titers were sometimes observed to be 1000× higher than those observed in flask culture. Virus production is a relatively new application for hollow-fiber bioreactor systems and is currently the subject of further research at **FiberCell Systems.**

Malaria culture

Plasmodium culture can be difficult and time consuming. Plasmodia require red blood cells to infect. The malaria parasite is a relatively anaerobic organism and utilizes glucose inefficiently. This means that the medium must be changed frequently. The red blood cells are usually placed in flasks at a concentration no higher than 6% hematocrit. Using a protocol developed at the National Institutes of Health (Bethesda, MD),[4] it is possible to inoculate up to 100% hematocrit into a FiberCell cartridge and infect with plasmodia directly in the cartridge. A single harvest from this cartridge is equal to more than 60 T25 flasks.

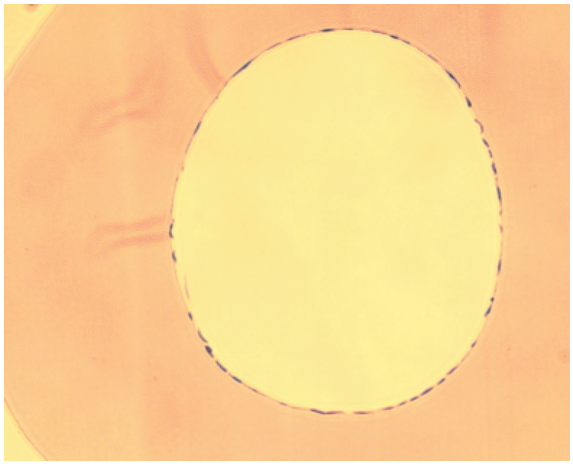

Figure 5 Cross-section of human umbilical vein endothelial cells (HUVECs) seeded on the inside of the fiber.

Polysulfone Plus (PS+)

Antibody production, protein expression, and the generation of conditioned medium are some of the classic applications of hollow-fiber bioreactors. Further examples of their use do not revolve around their capacity to support tremendous numbers of cells but on other abilities. The Polysulfone Plus (PS+) fiber (**FiberCell Systems**) permits the attachment of proteins, cytokines, antibodies, or other proteinaceous materials to the fiber surface. The fiber is activated by 70% ethanol, allowing proteins to adsorb to a concentration of 10–100 $\mu g/cm^2$. The fiber enables the study of the effects of surface biochemistry on long-term cultures of specific cell types.

Endothelial cell culture

Endothelial cells cultured in standard cell culture flasks grow in an active state and continuously divide without expressing tight junctions. Grown under conditions of constant shear stress, the effect of the medium flowing over the cells, endothelial cells would respond in a physiologic manner. Endothelial cells cultured in this manner form a monolayer and orient to the flow and tight junctions form. Gene expression patterns are affected, as are protein expression patterns.[5–7] Unpublished data provided by Dr. Barbara Ballerman and Dr. Eudora Eng, formerly with Johns Hopkins Univeristy (Baltimore, MD), showed that the binding of vascular endothelial growth factor (VEGF) caused an increase in the proliferation rate of the endothelial cells under low shear conditions.

Morphological changes can be induced in endothelial cells responding to different amounts of shear stress. *Figure 5* demonstrates the effect of low shear (5 dynes/cm^2) versus high shear (15 dynes/cm^2) on human pulmonary endothelial cells. The low shear photograph (*Figure 6*) depicts a monolayer of cells formed on the surface of the fiber. At high shear stress, the cells pile on top of one another in a plexiform lesion (*Figure 7*). This is also observed in vivo but not in flask culture.

It is also possible to culture a second cell type on the outside of the fiber while endothelial cells are cultured on the inside as shown in *Figure 8*. Endothelial cells on the inside of the fiber cocultured with vascular smooth muscle cells on the outside of the fiber showed that, as the flow rate was

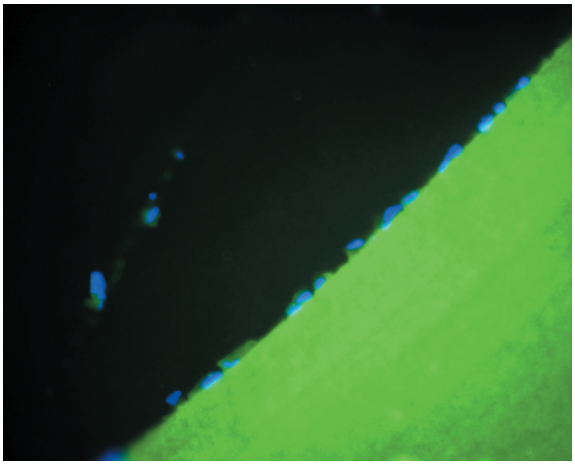

Figure 6 Pulmonary endothelial cells at physiologic shear stress (5 dynes/cm^2).

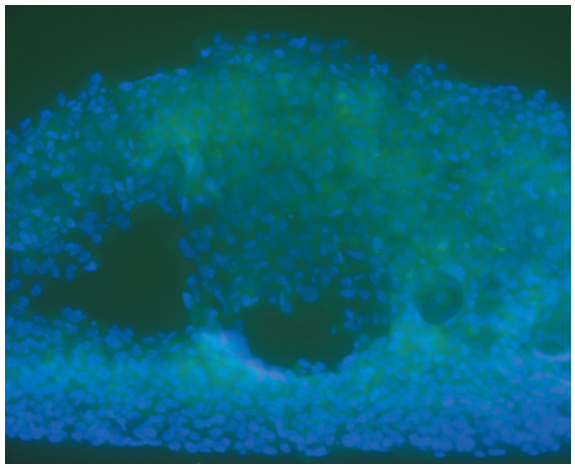

Figure 7 Pulmonary endothelial cells at pathologic shear stress (15 dynes/cm^2).

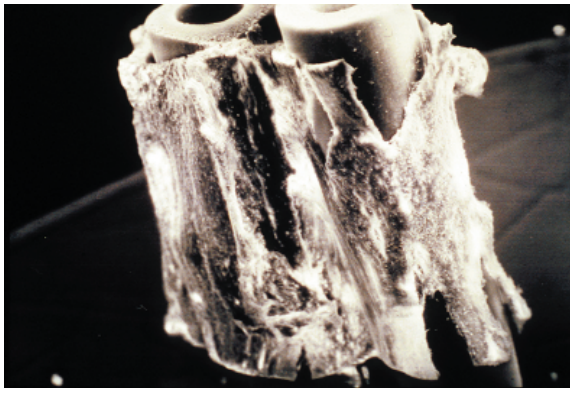

Figure 8 Bovine aortic endothelial cells cocultured with vascular smooth muscle.

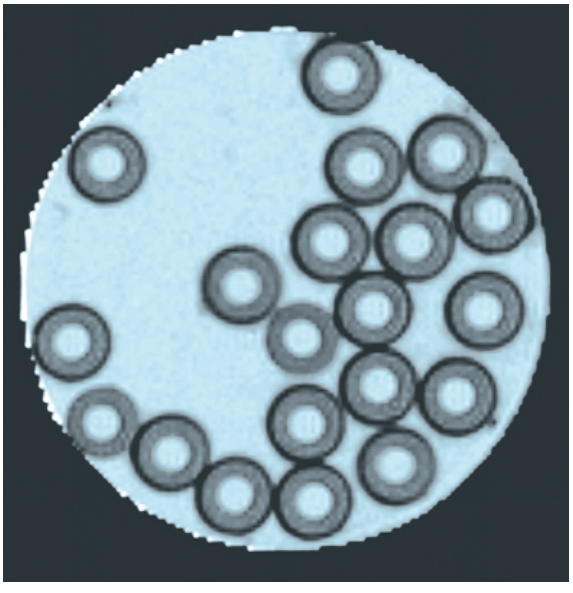

Figure 9 Osteoblasts seeded on the surface of fibers coated with fetal bovine serum.

changed, G-protein formation and endothelin receptor expression in the smooth muscle were directly affected. The endothelial cells secreted something that crossed the fiber and changed the smooth muscle cells.

The PS+ fiber permits the study of extracellular matrix effects on the long-term culture of different cell types. *Figure 9* shows osteoblasts grown on the surface of a fiber that has been coated with serum proteins. Because this is a relatively low-resolution nuclear magnetic resonance (NMR) image, the cells themselves cannot be resolved. However, the black rings around the fiber demonstrate the deposition of mineral, the first step in bone formation. The effects of other types of matrixes and bone morphogenic peptides can also be studied.

Other possible applications for the fiber include the attachment of specific antibodies for lymphocyte stimulation, attachment of specific ligands to promote growth of hepatocytes or pancreatic islets, and study of the effects of extracellular matrix on long-term cell growth and differentiation. The fiber permits studies on growth and differentiation that were not previously possible with conventional cell culture systems.

Although the technology is not new, significant advances in fiber materials have resulted in hollow-fiber bioreactor systems with improved productivity and ease of use. Hollow-fiber bioreactors remain the method of choice for the production of 100 mg to several grams of antibody or recombinant proteins or the culture of 10^9–5×10^{10} cells in any research laboratory. Protocols continue to be developed for the culture and production of viruses where the advantages of a more concentrated product are even more pronounced. Even after 30 years, hollow-fiber bioreactors remain the preferred method for large-scale laboratory cell culture.

References

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