

Single-Use Bioreactors and Microcarriers

Scalable Technology for Cell-Based Therapies

by Mark Szczypka, David Splan, Heather Woolls, and Harvey Brandwein

Cell-based therapies hold promise for treating many acute and chronic diseases (1). Optimism surrounding that therapeutic potential has driven the initiation of multiple clinical trials in pursuit of such treatments. Procedures for preparing these therapeutic agents begin with selective isolation of cells from desired tissues. That is followed by ex vivo expansion of cells of desired phenotype and functionality. Once expanded to acceptable levels, cells are stored to preserve their viability during transportation to treatment facilities. The final step in the process of cell therapy treatment involves infusion of cells into patients through a number of administration routes (2, 3).

Although significant progress has already been made within each step of that process, specific challenges still require addressing in further developmental studies if cell therapies are to become widely available. Advances in cell characterization techniques, media formulation, and identification of specific cell markers; development of potency assays; and improvements in processing technologies are rapidly moving this field forward as many groups focus their efforts to optimize each process step. It is clear, however, that development of scalable methods for expanding large numbers of cells represents a challenge that will require significant effort to overcome (4).

Photo 1: Pall's XRS-20 rocking incubator functions through biaxial agitation (rocking motion on two horizontal axes at 90 degrees separation), which improves agitation properties, providing for shorter mixing times and higher mass transfer rates. It is a fully enclosed platform with a safety interlock that improves operator safety, is suitable for production environments, and protects light-sensitive culture media.



ADHERENT STEM CELL CULTURES

Adherent stem cells (ASCs) are present in many organs and tissues, including bone marrow, peripheral blood, and adipose tissue (5–14). These cells represent <0.1% of the total tissue mass. Once harvested, they possess a limited doubling capacity. It is generally accepted that ASCs must remain in an undifferentiated state when expanded in culture to retain their therapeutic properties. Studies have shown that when ASC cultures reach high cell densities, they begin to differentiate into more mature cell types and lose their ability to replicate (15). All these attributes present a considerable challenge for expanding large numbers of such cells.

Common tissue sources for harvest of adherent ASCs include bone marrow, adipose tissue, placenta, and dental pulp. Harvested cells adopt a

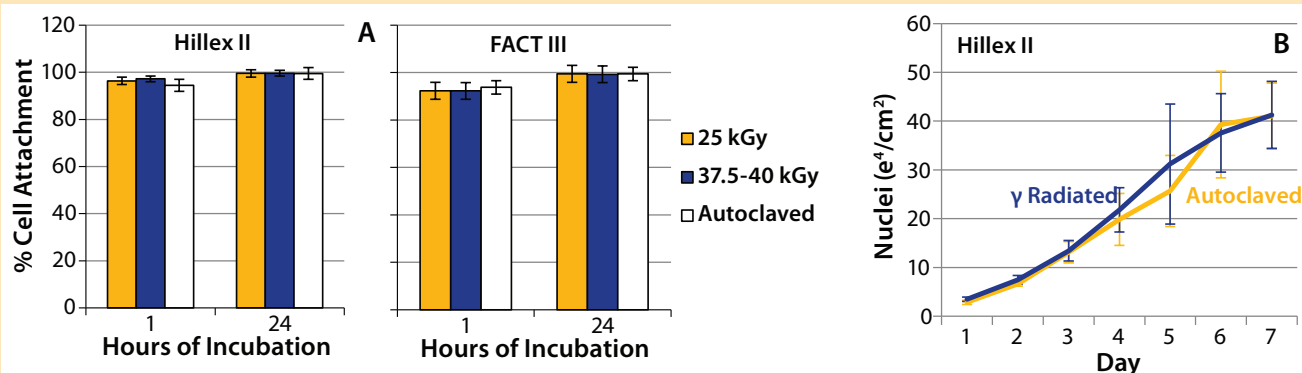
fibroblast-like morphology when cultured in vitro, and they occupy a relatively large footprint, thus requiring a significant amount of surface area (SA) for expansion. Current estimates predict that clinically relevant dosages will range from 10^5 to 10^9 cells per dose depending on the indication (16, 17). In ongoing clinical trials, companies are attempting to identify the lowest effective dose required for treatment of various diseases.

Considering that a single clinical indication could have a patient population >250,000 individuals per year (requiring the expansion of 10^{12} – 10^{14} cells to treat these individuals), there is a significant demand to develop robust, efficient, reproducible, and scalable processes for generating therapeutically active cells. For perspective, the SA required for expanding 10^{12} anchorage-dependent ASCs is about equivalent to the area of two American football fields. That highlights the need for exploring technologies and processes that can best meet such demand. And here is where the benefits of single-use bioreactors and microcarriers are realized.

TWO-DIMENSIONAL CELL CULTURE

Traditional stem cell culture involves two-dimensional (2D) surfaces under static conditions. Examples of such technology include tissue culture plates and T flasks, which provide SAs in the hundreds of square centimeters. Given the SA

Figure 1: (A) Cell-attachment kinetics are not adversely compromised when Pall SoloHill microcarriers are irradiated at 25 or 40 kGy. The number of cells attached to autoclaved and gamma-irradiated microcarriers were quantified, and no difference in attachment was observed (24). (B) Comparison of Vero cell growth on autoclaved and gamma-irradiated Hillex II microcarriers; no difference in growth was observed in spinner cultures (25).

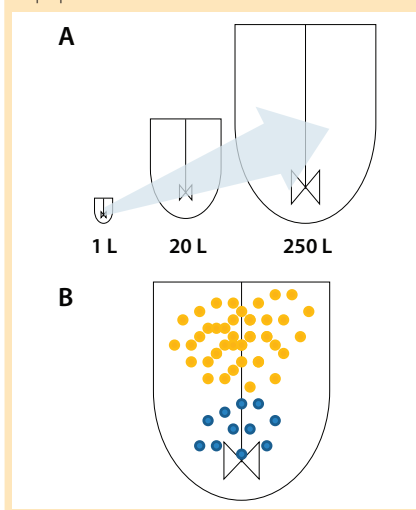


requirements for generating large numbers of cells and many open handling steps required when using those standard vessels, it is clear that such platforms quickly become impractical as solutions for large-scale cell expansion. It would require some 220,000 standard T150 flasks to generate 10^{12} human mesenchymal stem cells (hMSCs) derived from bone marrow harvested at a cell density of 3.0×10^4 cells/cm².

Roller bottles and multiplate culture flasks (e.g., Thermo Scientific's Nunc Cell Factory and Corning's Cell Cube and HyperFlask systems) have provided a next-generation solution for cell expansion by introducing vessels with increased SA. Roller bottles and the Nunc systems provide SAs that can reach thousands of square centimeters per vessel. Both platforms are attractive in that they are extensions of 2D cell culture practices, but they also can become impractical when larger numbers of cells are needed. Cell Factory systems have successfully generated >200 million (2×10^8) hMSCs per vessel for clinical use. But larger vessels can be difficult to physically handle, require specialized equipment, and occupy large amounts of incubator space when >5–10 vessels are needed (18, 19).

A lack of environmental control and difficulties in obtaining uniform cell growth have limited such platforms, making them problematic for reliable generation of functional cell products. To enhance the former, manufacturers have prospectively designed and integrated continuous automated feed

Figure 2: Simplified schematics of bioreactor and microcarrier-based cell-expansion processes; (A) cells dissociated and separated from microcarriers are sequentially transferred to larger vessels containing fresh microcarriers; (B) fresh microcarriers and media are added to a vessel after initial expansion period, and cells are encouraged to transfer from populated beads (blue spheres) to fresh microcarriers (orange spheres) by direct migration to unpopulated beads.



systems into the vessels or retrofitted systems to include such features. The Pall Xpansion bioreactor is one example of a multiplate reactor that includes an integrated environmental control system for cell expansion (20). That system offers SAs up to 11 m², so it provides a possible solution when intermediate numbers of cells are needed.

BIOREACTORS AND SINGLE-USE TECHNOLOGIES

Bioreactors have been used for decades to produce a range of therapeutic

biomolecules, affording operators the opportunity to monitor and control environmental conditions continuously throughout the culture period with the added benefit of maintaining an entirely closed system. Adding microcarriers is a mechanism for providing large surface areas for growth of anchorage-dependent cells. Stainless steel tanks are used at volumes ranging from tens to thousands of liters in vaccine and biologics production, and many manufacturers have incorporated microcarriers into their processes (21–23). Successful implementation of small- and large-scale bioreactor culture has contributed to the accumulation of a substantial library of data from which manufacturers can draw when developing new processes. Innovative methodologies for gas transfer, maintenance of pH, and optimal feeding and cell quantification have been developed. All innovations have contributed to the possibility of adopting this technology for cell expansion of adherent ASC on microcarriers (24, 25).

Introduction of single-use technologies (SUTs) including disposable bioreactors to manufacturing processes has created a cost-effective platform that can be conveniently coupled with microcarriers. A number of such bioreactors are commercially available, including Pall's PadReactor system.

Many SUTs are used by contract manufacturing organizations (CMOs) and pharmaceutical companies for manufacturing vaccines and therapeutic molecules using adherent

Table 1: Characteristics of Pall SoloHill microcarriers

Type	Core Material	Animal-Component Free?	Charge?	Relative Density	Bead Diameter (μm)	Surface Area (cm ² /g)
FACT III	Crosslinked polystyrene	No	Yes	1.02 and 1.04	90–150 and 125–212	480 and 360
Collagen	Crosslinked polystyrene	No	No	1.02 and 1.04	90–150 and 125–212	480 and 360
Hillex II	Modified polystyrene	Yes	Yes	1.11	160–180	515
Hillex-CT	Modified polystyrene	Yes	Yes	1.11	160–180	515
Plastic	Crosslinked polystyrene	Yes	No	1.02 and 1.04	90–150 and 125–212	480 and 360
Plastic Plus	Crosslinked polystyrene	Yes	Yes	1.02 and 1.04	90–150 and 125–212	480 and 360
ProNectin F	Crosslinked polystyrene	Yes	Yes	1.02 and 1.04	90–150 and 125–212	480 and 360
Glass	Crosslinked polystyrene	Yes	Yes	1.02 and 1.04	90–150 and 125–212	480 and 360

Table 2: Continuous passages on Pall SoloHill Plastic microcarriers in spinner culture; six consecutive passages on microcarriers demonstrate the ability to expand MSCs continuously without decreasing maximal confluent density or doubling time.

Passages	1	2	3	4	5	6
Seeding Density (×10 ⁴ cells/cm ²)	0.3	2.0	1.0	0.3	0.3	0.3
Harvest Density (×10 ⁴ cells/cm ²)	4.6	3.3	2.7	3.6	4.3	4.5
Days of Growth	8	5	5	7	8	8
Number of Doublings	4.0	<1.0	1.3	3.6	3.8	3.9

cell lines. The attractiveness of such platforms — along with their successful implementation at manufacturing scale — has prompted cell therapy companies to engage in developmental efforts to determine whether SUTs coupled with microcarrier technologies can provide a viable solution for stem cell expansion.

MICROCARRIERS IN STIRRED-TANK REACTORS

Microcarriers are tiny spheres or particles (typically 90–300 μm in diameter) used for culturing adherent cells in stirred-tank bioreactors. The geometry of microcarriers provides investigators with an ability to increase the amount of SA in a given volume (cm²/mL) of medium. Therefore, one driver for transitioning from 2D platforms to microcarriers is the benefit of increasing SA-to-volume ratio when scaling up to larger systems. Increasing that ratio improves product consistency and decreases costs through full use of culture medium for growing large numbers of cells (4).

However, that advantage is realized only when cells are attached to and grow on the surfaces of most of the microcarriers in a vessel, thus maximizing SA coverage. Conditions that promote efficient attachment and even distribution of cells across an

entire microcarrier population therefore must be optimized. Generating a single-cell suspension using favorable harvest conditions is absolutely essential to accomplishing that goal. Cell dissociation times, enzyme concentrations, and incubation temperatures are examples of parameters that should be optimized. Ideally, every microcarrier in suspension will have the same number of cells attached to its surface in one to four hours after cells are seeded into a reactor. Although that is the ultimate goal, in practice a statistical distribution of cells on the microcarriers is inevitable.

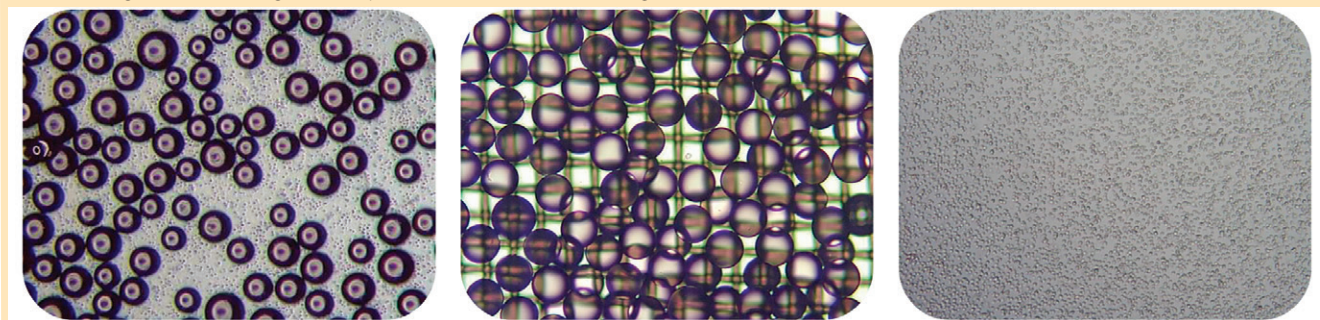
The same parameters that are important to successful static cell culture are also required for implementing microcarrier-based adherent cell culture. Some environmental conditions and cell handling techniques that may not affect static cultures can positively or negatively influence microcarrier systems. Such parameters must be optimized in developmental studies. Cell attachment, spreading, and growth are the three most important physical processes required for successful microcarrier culture, and media components influence those processes in different ways. For example, high protein concentrations in cell culture media formulations can block or impede cell attachment to

microcarriers (26). When that occurs, microcarrier attachment efficiency — the distribution of cells onto the microcarrier population — can be suboptimal, which leads to inefficient use of the available SA in the reactor.

Different microcarrier surface modifications also influence cell-attachment kinetics and efficiencies. Positive charges imparted onto microcarrier surfaces facilitate attachment of most cell types. Pall SoloHill Plastic Plus, FACT III, and Hillex II microcarriers have positively charged surfaces to which cells typically exhibit rapid attachment. Cell-adhesion molecules — such as collagen, fibronectin, laminin, and proteins or peptides that contain the Arg-Gly-Asp (RGD) attachment site — can enhance attachment efficiency and facilitate cell spreading. Pall SoloHill ProNectin F, Collagen, and FACT III microcarriers are all coated with cell-adhesive molecules. Such coatings facilitate attachment of fastidious cells, promoting an environment that is favorable for cell growth.

Pall SoloHill microcarriers are made of rigid, modified-polystyrene spherical cores, with each different type involving unique surface modifications. They come in a range of sizes and relative densities (1.02–1.10), which can be beneficial for distinct applications and cell types. The rigid properties of these microcarriers ease cell harvesting and serial passaging and facilitate subsequent three- or four-step scale-up through bead-to-bead transfer. Such properties are critical when cells are final products. Table 1 describes important characteristics of the eight different types of

Photos 2: Cell and microcarrier slurries are passed through a bag and microcarriers are retained on a screen within it so single-cell suspension can be used to seed next reactor; microscopic images of microcarrier/cell slurry before passing through through (LEFT), microcarriers devoid of cells retained within the bag (MIDDLE), and single-cell suspension obtained in flow-through (RIGHT).



commercially available microcarriers from Pall SoloHill. All can be gamma irradiated (Figure 1) without loss of function (27). That is an important property providing the opportunity to supply sterile microcarriers in bags for direct connection and easy transfer into single-use bioreactors.

Microcarrier-to-microcarrier cell expansion in closed-system, single-use bioreactors represents an attractive manufacturing platform (Figure 2). This can be accomplished either by using a bioreactor seed train of increasing size or by increasing SA within a single reactor through addition of fresh media and microcarriers. Implementing such systems requires multiple conditions to be met: First, cells that retain therapeutic properties must propagate on microcarriers for multiple passages. Second, the cells must either be harvestable and transferrable to a larger reactor containing new microcarriers, or they must be capable of directly migrating to freshly added beads in an existing reactor. Conditions optimized in small-scale vessels also must be scalable to larger production formats. Studies using small-scale spinners have demonstrated the feasibility of Pall SoloHill microcarriers for propagation of hMSCs over multiple passages (Table 2).

Large numbers of hMSCs can be readily harvested from Pall SoloHill microcarrier types while retaining high viability (Table 3). At small volumes, cells can be separated from microcarriers using cell strainers or by allowing microcarriers to settle and decanting the culture supernatant. Systems for separating viable cells from

Table 3: Efficient harvest of cells from Pall SoloHill microcarriers (see Photos 2, above)

Experiment	Total Nuclei Before Trypsin	Total Cells After Trypsin	Recovery (%)
MSC1201-Collagen	8.78×10^8	5.9×10^8	67
MSC1301-Collagen	1.25×10^9	8.98×10^8	72
MSC1303-Collagen	—	6.3×10^8	—
MSC-Hillex II	5.9×10^6	5.0×10^6	84
MSC-Plastic	5.2×10^6	4.6×10^6	88

microcarriers at larger volumes are under development, and at least one commercially available solution has been recently introduced. Pall SoloHill has demonstrated feasibility of using a cell-separation bag: a sterile bag containing a 70- μ m screen. In that system, a slurry of microcarriers and dissociated cells are unidirectionally pumped through a separation bag, in which cells are liberated to the medium and flushed into the next vessel through sterile connections and microcarriers are retained in the bag. Applications of this device have demonstrated its ability to efficiently harvest ASCs as a single-cell suspension from Pall SoloHill's rigid microcarriers (Photos 2).

MICROCARRIERS FOR STEM CELL EXPANSION

Pall SoloHill and other spherical microcarriers have been used as substrates to grow a range of adherent stem cell types (29, 30). Corning has released microcarriers that appear to support stem cell growth under specific conditions (31). Other commercially available nonspherical microcarrier types also may be used for cell growth. The ability to harvest healthy stem cells from microcarriers for therapeutic purposes is absolutely critical. Even though a number of microcarrier types

support cell growth, it can be difficult to efficiently liberate and separate viable cells from them (32). The process should be optimized before transitioning to larger-scale reactors.

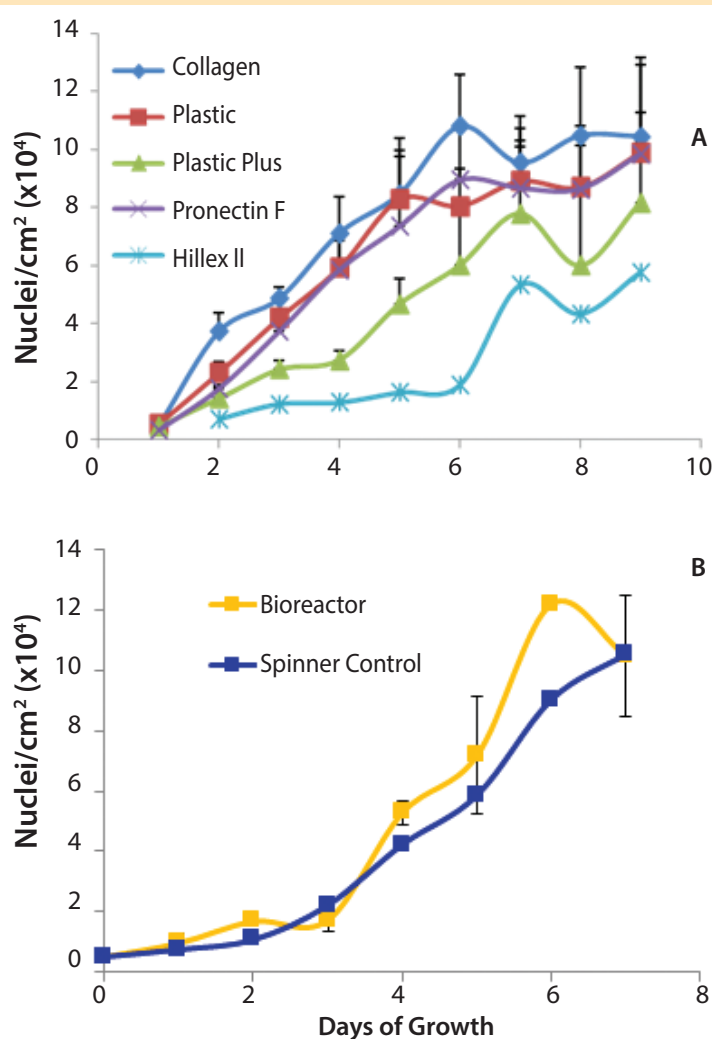
Results obtained in a number of studies have demonstrated that human MSCs grow extremely well on Pall SoloHill microcarriers (Figure 3) (32). After expansion with serum-containing medium in small-scale spinners at volumes of 125–200 mL, cells retain their identity and reach acceptable densities (33, 34). Similar results have been obtained using larger glass and disposable reactors, in which hMSCs have been expanded and harvested from Collagen microcarriers at 2-L volumes (Figure 3).

Distinct cell types will behave differently when environmental conditions are changed. A microcarrier that supports cell growth in serum-containing medium may not be optimal for use under chemically defined conditions. Therefore, it is important to determine the best cell–microcarrier media combination for growth at small scale before transitioning to large-scale production.

STREAMLINING PROCESS DEVELOPMENT

Minimizing costs and shortening timelines are goals for developing all

Figure 3: (A) Nuclei counts show growth of hMSCs on five different types of Pall SoloHill microcarriers in spinner cultures. Data are presented as means \pm SEM ($n = 3$). (B) Growth curves of hMSC grown on Pall SoloHill Collagen-coated microcarriers in bioreactor and spinner control cultures.



therapeutic manufacturing processes. Because cell therapies are living cells, they must overcome unique difficulties to achieve regulatory approval. Cellular identity and function must be defined by developing meaningful potency assays that predict the therapeutic properties of cells. Those attributes must be maintained as new processes are developed and adopted. Fortunately, many critical parameters needed for successful microcarrier culture can be identified and optimized through well-designed and highly targeted developmental efforts. Microcarrier concentration, cell-seeding density, enzymatic dissociation times and temperatures, optimal media conditions, and vessel stir speeds are some parameters that

can be examined using small-scale stirred tanks.

Small-scale studies in static cultures and stirred vessels allow investigators to save on material costs and expedite development timelines. Initial cell, media, and microcarrier compatibility studies can be performed in static cultures. Although such cultures are of somewhat limited utility, those studies provide useful information about cell-attachment kinetics and growth on different microcarrier types. Such studies are rapid and cost effective, and successful microcarrier-media combinations identified under static conditions then can be tested in small-scale stirred vessels.

Many groups use 100-mL to 500-mL reusable or disposable vessels to

perform initial screening studies.

Bellco Glass and Corning both sell reusable glass spinner vessels that are cost effective options for these studies. Corning has recently introduced a line of small-scale, plastic, disposable stirred reactors that range 100–300 mL in working volume. Those single-use reactors can be used to expedite developmental timelines if costs are not a consideration or if cleaning vessels and autoclaving becomes burdensome. Conditions such as microcarrier concentrations, seeding densities, media formulations, and minimal stir speeds can be identified using small-scale platforms.

Those parameters are typically representative of conditions that will be favorable to cell expansion in larger bioreactors. However, scaling to larger vessels is a critical component of the developmental process that should not be overlooked. Processes such as fluid transfer or cell harvesting are routinely performed at laboratory scale, and engineering expertise is required to identify suitable methods for effectively performing them at larger scales.

PROMISING ADVANCEMENTS

Several recently introduced small-scale multiplexing experimental systems could provide a suitable platform to accelerate microcarrier-based cultures and decrease production costs. Global Cell Solutions offers a programmable, eight-position stirrer called the Biowiggler. Each magnetically driven position on this device can be independently programmed for continuous or intermittent stirring in a working volume of 5–45 mL. Other potentially useful systems include the DASBox system from Eppendorf, Applikon's Micro-Matrix bioreactor, the ambr microbioreactor from TAP Biosystems, and Pall's Micro-24 MicroReactor system. The latter is a compact solution for efficient cell-line evaluation and process development (35). It runs up to 24 simultaneous bioreactor experiments with independent control and monitoring of each reactor's gas supply, temperature, and pH at working volumes of 3–7 mL. All these

systems can be operated at low volumes, making them potentially cost-effective options for initial development studies. Determining the compatibility of such systems with microcarrier processes — and whether they will generate results that are directly translatable to larger vessels — is becoming increasingly important.

Numerous examples of successful cell therapies are beginning to emerge, and more clinical trials using cells are being initiated. This signals an exciting time in the fields of cellular therapy and regenerative medicine. New tools and innovations in cell culture, molecular biology, manufacturing techniques, and SUTs are providing the necessary enabling technologies for expediting developmental efforts to meet the demand for the large cell numbers that will be required to treat expected patient populations. Microcarriers coupled with single-use bioreactors represent an attractive platform for these promising therapies.

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Corresponding author **Mark Szczypka** is senior director of applications and new product development, **David Splan** is a senior scientist, and **Heather Woolls** is a scientist, all at Pall Life Sciences in Ann Arbor, MI 48108; 1-734-973-2956; mark_szczypka@pall.com. And **Harvey Brandwein** is vice president of business development at Pall Life Sciences.

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