Meeting Lot-Size Challenges of Manufacturing Adherent Cells for Therapy

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Adherent cells such as adult primary cell lines and human multipotent (MSCs) and pluripotent stem cells (hPSCs) present a manufacturing challenge as lot sizes increase from 10^9 (billions) to 10^{12} (trillions) cells (**1**). Typically, manufacturing platforms are good for one log of expansion. So new methods will be required to achieve commercially relevant lot sizes. Traditional two-dimensional culture methods have been used to grow anchorage-dependent cell types. Although such methods are reliable and well defined, they are very labor intensive and limited in scale-up production potential by the available growth surface area (Table 1). Allogeneic "off-the-shelf" therapies based on adherent-cell platforms may require manufactured lot sizes from 100 billion to a trillion cells depending on a given indication's market size (**2**).

Here, we examine the three platforms available for producing adherent cells — planar technologies, packed-bed systems, and suspension platforms such as microcarriers and aggregate cultures — for their potential of meeting lot requirements at different scales. As new production methods are introduced, we propose addressing downstream processing bottlenecks before they occur and introduce some large-volume downstream process technologies.

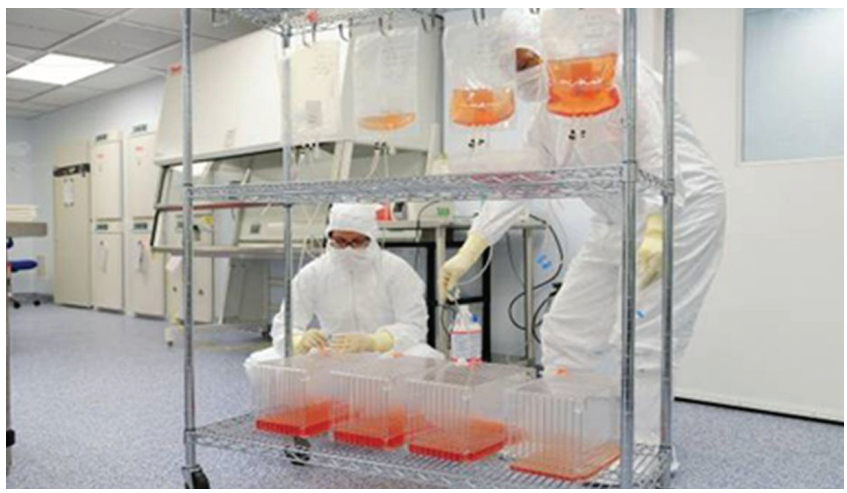

Figure 1: Closed system manufacturing of therapeutic adherent cells in 10-layer vessels using bagged media

Scaling of Planar Flask Cultures

Adherent therapeutic cells (e.g., dermal fibroblasts, chondrocytes, and MSCs) are typically produced using planar technologies (flasks). Ten-layer vessels (Figure 1) have been used to progress several allogeneic cell therapy products into mid- to late-stage clinical development. By some estimations, planar technologies will reach lot-size limitations of 3–5 million cm^2 per lot (Table 1) — capping lot sizes in the 100–400 billion cell range for most adult primary cells.

Scaling up traditional flask-based culture processes from laboratory scale usually involves commercially available stacked-plate systems such as Nunc Cell Factories and Corning CellStacks. These multilayer vessels have been used for over 30 years for large-scale cell culture (**3**). They were first published for therapeutic dendritic cells (DCs) (**4**) and large-scale culture of MSCs (**5**) in the early 2000s. More recently, Millipore and BD have brought small-scale parallel-plate vessels to market. Traditional 10-layer vessels have been adopted for closed-system processing and are being used as a platform in the good manufacturing practice (GMP) production of allogeneic therapeutic adherent cells (**6**).

The main strategy for maximizing lot size in planar vessels is to scale up the total surface area manipulated per unit operation and then scale out multiple units. Scale-up can be achieved by increasing the size and number of layers per vessel and then scaling out that unit operation to a manageable size for manufacturing. Traditional 10-layer vessels with ~6,300 cm^2 of surface area have successfully been scaled out to lot sizes of 50–70 vessels for a

Table 1: Projected lot sizes by production platform in number or unit operations, total surface area per harvest, and total cell numbers, with robotic manipulation of four large vessels in a single unit operation; cell density at harvest used in the calculations represent harvest densities at confluence for multipotent stem cells, fibroblasts, and pluripotent stem cells.

		Types of Planar Methods							
		Manual 10/12 Layers		Manual 36/40 Layers		Robotic 36/40 Layers		Robotic 120 Layers	
Vessels/Unit Operations		Low 60/60	High 100/100	Low 40/40	High 60/60	Low 64/16	High 80/20	Low 64/16	High 80/20
Total Surface Areas (cm^2)		372,000	620,000	1 million	1.5 million	1.6 million	2 million	3.84 million	4.8 million
Cell Types	**Harvest Density ($cells/cm^2$)**	**Estimated Billions of Cells Produced**							
MSCs	25,000	9	16	25	38	40	50	120	150
HDFs	80,000	30	50	80	120	128	160	384	480
hPSCs	160,000	60	99	160	240	256	320	768	960

total of ~400,000 cm^2 (**6**). These vessels were designed to be scaled-up to 40 layers (**3**).

Robotic instrumentation has been used to manipulate four 40-layer vessels in a single manipulation — or 16-fold greater surface area per unit operation than a single 10-layer vessel. Recent innovations in Hyper technology (Corning) has tripled the surface area per unit volume of traditional multilayer vessels (**7**). Applying robotics to that technology could allow the manipulation of four 120-layers to achieve >240,000 cm^2 per unit operation, with the potential to scale out to several million square centimeters per harvest (Table 1). Table 1 shows approximate surface area per harvest that is achievable for various flask-based vessels, with total harvest yields for typical cell types at estimated harvest densities. Harvest densities of GMP processes can vary greatly depending on media composition, cell type, and confluency at harvest.

The two main variables dictating harvest size in planar culture methods are total surface area harvested per lot and cell density at harvest. Increases in lot size require maximizing both variables. Because different cell types can achieve different cell densities at harvest, we estimated lot sizes in billions of cells per harvest over various culture systems (Table 1). For adult primary cells such as MSCs, lot sizes >100 billion cells are not readily achievable with planar technologies outside of massive automation and parallel processing. Suspension technologies are required to achieve scales of 1 trillion cells per lot. Human dermal fibroblasts (HDFs) can achieve much higher densities at harvest, and lot sizes may approach 500 billion using planar technologies. Human pluripotent stem cells (hPSCs) are very small and grow as tight clusters. So if more robust cell lines are developed, they may achieve 1 trillion cells per lot more readily than adult primary cells.

Researchers have made several attempts to apply bioreactor control to planar culture systems, including the RepliCell (Aastrom) system (**8**), CellCube (Corning) cell culture unit (**9**), and Xpansion (Artelis) bioreactor (**10**). Those systems automate many operations and monitor and control many traditional culture variables such as dO_2, dCO_2, and pH.

Packed-Bed Cultures

Cultures based on packed-bed bioreactors (PBRs) are systems in which adherent or nonadherent cells are immobilized to a substrate that is enclosed in a packed bed within a bioreactor system. Two PBR configurations exist: one in which a packed bed is placed within a medium reservoir, and one in which a packed bed is external to a medium reservoir. A wide array of substrate materials and configurations can be used in a packed bed. The most widely used are beads, porous structures, fibers, and hollow fibers (**11–13**).

Packed-bed systems are primarily used to produce proteins and antibodies. Cells secrete their product into a medium that can be continuously collected without disturbing them. However, PBRs are increasingly being used for culture of therapeutic cells. Because of the advantages they provide, packed-bed configurations are emerging as favorable candidates for scaling up adherent-cell production. The amount of published research is very limited, however, regarding culture of MSCs and hESCs in commercial-scale packed-bed systems. Much of the knowledge regarding large-scale adherent-cell packed-bed culture is derived from hands-on experience of a few companies. For example, the iCELLis bioreactor (ATMI) is based on polyester microfibers, Pluristem Therapeutics uses a nonwoven-fiber–based bioreactor (**14**), and Beckman-Coulter offers its Quantum hollow fiber reactor.

Primary advantages of PBR perfusion-based systems for culture of therapeutic cells are the

- ability to reach high densities of cells per milliliter of packed-bed volume
- real-time ability to control cell culture parameters (pH, dO_2, temp, perfusion, agitation)
- low shear-stress forces on cells during culture compared with microcarrier and aggregate stirred-culture systems
- beneficial biological attributes of cell cultures in 3D scaffolds.

With porous carriers or woven or nonwoven fiber carriers, cell densities can reach 1×10^8 cells/mL with a carrier surface-area to volume (SA:V) ratio of up to 119×10^3/m (**13**). By comparison, the SA:V ratio of a two-dimensional multilayered flask is ~0.5×10^3/m. A wide range of carriers and materials are used in packed-bed systems, including glass beads and ceramic, polyester, and polyurethane materials. They can be used in a smooth, porous, woven, or nonwoven configuration (**13, 15, 16**). Table 2 compares packed-bed systems with suspension methods. Current packed-bed volumes fall between 10 mL and 40 L. Yields of 1×10^8 cells/mL are the best to date, but a wide range of cell densities can be achieved

Table 2: Achievable cell densities projected to commercial-scale lot sizes (total volumes and cell harvests) for bioreactors

Methods for Producing One Lot of Stem Cells		Cell Density (cells/mL)	Volumes (L)	Total Cell Harvest
Packed-bed reactors	Low	100 million	1 (packed-bed volume)	100 billion
	High	100 million	40	4 trillion
Suspension (microcarriers/aggregates)	Low	0.5 million	1,000	500 billion
	High	5 million	1,000	5 trillion

Table 3: Estimated harvest volumes and number of product doses per lot produced by planar and bioreactor technologies

Bioreactor Types	Scale	Harvested Volume (L)	Number of Doses per Lot	
			50 million/dose	250 million/dose
10-layer trays	60 vessels	30	200	40
40 layers per rack	20 racks, 80 vessels	200	1,000	200
120 layers per rack	20 racks, 80 vessels	600	3,000	600
Bioreactor*	250 L	250	5,000	1,000
Bioreactor*	1,000 L	1,000	20,000	4,000

*Assuming a cell density of 1 million cells/mL

depending on cell type, carrier type, and culture conditions.

Similar to other cell culture methods, culturing adherent cells in a PBR includes three main stages: cell seeding, cell culture, and cell harvest. Each step must be optimized based on cell type, carrier properties, and packed-bed volume to achieve an effective process and optimal cell number and quality.

Several factors affect cell seeding dynamics and efficiency, including

- adherence properties of the cells
- physical properties of the carrier (smooth, porous, or fibrous)
- chemistry and electrical charge of the carrier
- rate and pattern of media flow during seeding (**17**)
- dimensions of the packed bed.

During the culture phase, online control of culture conditions is a central advantage of PBR systems. Those parameters must be optimized to ensure efficient cell proliferation and maintainance of cell health at high cell densities (**16, 17**). One challenge is to maintain a homogeneous axial and radial gradient of nutrients (e.g., oxygen and glucose). Doing so maintains a homogeneous cell density throughout the packed-bed volume, which is especially crucial when scaling up the volume. Media agitation can be achieved using impellers and must be optimized to ensure flow throughout a packed bed while providing appropriate shear stress to cells.

The final process step is removal of cells from their carriers. This may represent the most challenging aspect of an adherent-cell PBR culture process. Generally speaking, the better the scaffold in terms of efficiency of cell seeding and endpoint cell numbers (maximal surface area), the more challenging the cell removal process. A balance between those conflicting goals must be reached.

Cell removal should be optimized to attain high yield while maintaining cell health and properties. PBR systems must be tailored based on cell type, carrier type, and properties of a final product. Although scaling up PBR systems is a very attractive and promising prospect, the feasible volume has limitations depending on specific properties of cultured cells and carriers. All three phases of the culture process must be examined and optimized when increasing packed-bed size.

To date, only relatively small packed bed bioreactors are available for cell production. Three examples are the Celligen 2.5–40 L PBR system (New Brunswick), which uses Fibra-Cel nonwoven polyester base carriers (**11**); the Quantum (Beckman-Coulter) hollow-fiber–based PBR (**18**); and the iCELLis bioreactor, which uses polyester microfibers. Given their capacity for high cell density and control over culture parameters, PBR systems are promising in terms of scalability potential. They face limitations in that nutrient gradients in culture limit the height of the bed, so that must be addressed. (**13**, **19**). Thus, PBR systems present many advantages and are certainly attractive as a platform technology for highly controlled and efficient commercial scale culture of therapeutic cells.

Suspension Microcarrier and Aggregate Cultures

Achieving lot sizes of several hundred billion to trillions cells efficiently and cost effectively will be imperative for commercial success. Suspension culture of therapeutic cells in existing single-use bioreactor manufacturing platforms is likely to be the only way to accomplish that.

Nonetheless, other strategies for adherent therapeutic cell scale-up have been investigated. One such method uses microcarriers in a bioreactor-based system. A potential benefit of using microcarriers for large-scale production is that the surface-area-to-volume ratio is greatly increased over traditional static culture processes. So cell density may be increased and the required footprint reduced.

Many different types of microcarriers are commercially available. Microcarriers can be made from polystyrene such as HyQSphere (HyClone) and Hillex (SoloHill Engineering) brands or made from cross-linked dextran such as the Cytodex brand (GE Healthcare). Although most microcarriers are spherical and smooth, others have macroporous surfaces such as Cytopore brand (GE) and alternatives such as rod-shaped carriers (**20**). Additional technological advances include infusion of magnetic particles that may help in cell separation from beads (GEM particles from Global Cell Solutions) and chip-based microcarriers such as the μHex product (Nunc) that provide a traditional flat surface for cell growth while maintaining the high SA:V ratio of traditional microcarriers. Selecting a microcarrier for cell expansion is not a trivial task. Different properties of microcarriers may significantly affect expansion rates and cell multi- or pluripotency (**21**). Some surface chemistry modifications can improve cell adhesion. Such methods include applying positive or negative charges and coating with extracellular matrix proteins such as laminin or vitronectin (**22**).

One advantage of using microcarriers is increased control of a culture's environment in a bioreactor-based system. Technology borrowed from the development of single-use bioreactors for biopharmaceutical processes can be applied to growing therapeutic adherent cells (**23**). MSCs typically achieve $<1 \times 10^6$ cells/mL, whereas ESCs can achieve 1×10^6 to 3×10^6 cells/mL. Using similar bioreactor-based systems of >1,000-L volume for scaling up stem cells may result in lot sizes approaching or surpassing 1 trillion cells (Table 2). Bioreactor technology offers the ability to precisely control process parameters such as gas exchange, nutrient feeding, and pH. In smaller-scale systems, however, factors such as shear stress must be controlled closely because stem cells are susceptible to spontaneous differentiation in an unoptimized system (**24**, **25**). An alternative to a traditional impeller-driven bioreactor system is the Wave bioreactor (GE Healthcare) that uses a single-use bag and a rocking motion to agitate cell suspension. As a result, shear stress is potentially reduced. The system chosen, microcarrier, and cell culture medium will influence cell proliferation. Maintaining cell phenotype and differentiation potential is critical.

Harvesting: For the biotherapeutic and vaccine markets, in which a supernatant contains the product, there is no need to separate cells from a microcarrier. In cell therapy processes, cells are the product and must be harvested. Cell harvesting and yield of microcarrier-based methods depends on efficiency of cell dissociation and separation from beads.

Enzymatic treatment using commercially available recombinant animal-origin–free proteases is commonly used to remove cells from microcarriers. Using new surface chemistries that allow nonenzymatic removal of cells may increase a system's effective yield. Tangential flow filtration (TFF) and sequential differential centrifugation techniques are options for cell harvesting but require extensive optimization and validation for processing large lot sizes (>1,000 L) to ensure that all microcarriers or particulates are removed from a cell suspension.

Because many cell therapies will be administered intravenously, carryover of particulates or intact microcarriers into final products poses a serious safety risk. Using magnetic-particle infused beads can facilitate cell separation, and incorporating biodegradable and thermosensitive materials may help reduce that concern (**26**).

An alternative approach is culturing stem cell as aggregates. The advantage is that it does not need a carrier or extracellular matrix. The key to ensuring success of this technique is using single-cell seeding and maintaining high viability (e.g., using a ROCK inhibitor) (**27**). Aggregate size, however, is more difficult to control, and large aggregates will suffer transport limitations. To overcome that problem, aggregates will have to be

broken up or passaged every few days. But high cell-density limitations will pose a greater challenge.

Studies have demonstrated suspension aggregate cultures for both pluripotent hESCs and iPSCs. Other studies have shown the same for MSCs — albeit at relatively low cell densities — and sometimes slower doubling times result (**28–30**). Similar to microcarrier cultures, agitation can cause cell differentiation and unstable cell growth in aggregate cultures (**27**). Regardless of the method used for harvest and cell concentration, robust quality control (QC) assays will be necessary to demonstrate product consistency and efficacy. So suspension systems can achieve homogenous cultures of high densities, but that will require fine control to maintain stem cell function as the volumes increase.

Downstream Processing

As therapeutic-cell lot size is scaled from several billions to hundreds of billions (Table 1), manufacturing bottlenecks will shift to the downstream processing (DSP) areas. The cell therapy industry will need to proactively address DSP requirements so that technology is in place to accept larger lot sizes as new culture technology is implemented.

Process bottlenecks will shift to two DSP process steps: volume reduction and wash, and final product filling. Volume reduction and washing process requirements will be driven by harvest volume, which is dictated by the culture platform and volumes used during harvest (Table 3). Volumes >5–10 L cannot be easily reduced using laboratory centrifugation or blood processing equipment, and scale-out is cumbersome. Scalable single-use technologies have been adapted from bioprocessing to enable presterilized, closed systems. Other technologies include process automation such as therapeutic cell TFF (**31**) and continuous centrifugation (e.g., from kSep Systems). Both TFF and continuous centrifugation processes are scalable from tens of liters to hundreds of liters. The kSep technology has the potential to scale up to 1,000 L processing volume.

Larger lot sizes may shift bottlenecks to the final-product dose-filling step, which is driven by lot size and the number of cells per dose. Lot sizes will range from several hundred to several thousand doses per lot, requiring a shift from traditional blood bags to pharmaceutical vials and compatible filling automation. New plastic vials from West (**32**) and Aseptic Technologies (**33**) coupled with traditional pharmaceutical fill line automation will enable lot sizes in the several hundred to several thousand doses per lot.

Holding times for cells in dimethyl sulfoxide will dictate process timing, so the ability to fill several thousand vials per hour should enable lot sizes of least three to five thousand vials per lot. The DSP manufacturing bottleneck is likely to shift depending on manufacturing platform, harvest process volumes, and product dose size. Final fill will be the manufacturing bottleneck of greatest concern at the highest culture volumes and lowest product doses where fill time may become unmanageable.

Challenges Ahead

As lot sizes increase from tens of billions to trillions of cells, alternatives to planar technologies will have to be considered. The two main variables dictating harvest size in planar culture methods are total surface area harvested per lot and the density of the cells at harvest. Increases in lot size will require maximizing both. Manipulation of multiple units, however, will limit the scale of this technology to one trillion cells.

Packed-bed reactors can achieve very high densities (10^8/mL), up to 200× more than planar surface densities. The challenge is to increase volumes beyond 40 L with good process control to achieve a trillion cells. Suspension technologies such as microcarriers and aggregate cultures can already achieve densities >10^6/mL and potentially can scale to thousands of liters. So they are the most promising approaches for meeting lot sizes of trillions of cells. As those commercial-scale lot sizes are reached in the upstream portion of a process, bottlenecks downstream should be addressed proactively so that technology is in place to accomodate large product doses.

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