Equipment design considerations for large scale cell culture

David M. Marks

DME Alliance Incorporated, 5012 Medical Center Circle, Allentown, PA 18106, USA (e-mail: david.marks@dmealliance.com)

Received 28 June 2002; accepted in revised form 3 February 2003

Key words: Bioreactor, Cell culture, Large scale, Mass transfer, Mixing, Scale up, Stirred tank

Abstract

Equipment design is frequently recognized as a key component in the success of GMP biologics manufacturing, but is not always implemented with full appreciation of the processing implications. In the case of mammalian cell culture, there are some recognized issues and risks that develop when transitioning to a large scale of operation. The developing demand for cell culture production capacity in the biopharmaceutical industry has led to a progressive increase in the scale of operation in the last decade. This review will provide a high level summary of the documented process difficulties unique to serum-free large scale (LS) cell culture, analyze the engineering constraints typical of these processes, and suggest some practical equipment design considerations to enhance the productivity, reliability and operability of such systems under GMP manufacturing conditions. A systems approach will be used to establish a good LS bioreactor design practice, providing a discussion on gas distribution, agitation, vessel design, SIP/CIP and control issues.

Introduction

The success of biopharmaceutical manufacturers in filling parenteral drug pipelines has fueled speculation that the world's cell culture production capacity will be insufficient for the growing demand for monoclonal antibody and recombinant protein production in coming years. This has stimulated an increased interest in large-scale (>10,000 liter) production techniques. The conventional approach to large scale biologics production is through a stirred tank bioreactor. Several biopharmaceutical manufacturers have invested in LS stirred-tank cell culture manufacturing capability by implementing production bioreactors at 10,000 to 20,000 liter scale (Glaser 2000).

Perfusion technology is available to support commercial production without a large equipment scale up. A variety of cell retention devices have been developed for use in stirred perfusion bioreactors (Woodside et al. 1998). These devices allow media replenishment without a loss of viable cells, supporting higher cell densities and extended production time. Perfusion is attractive because it enables a pilot

scale bioreactor to produce a production scale yield. However, the price for this productivity bonus is the acceptance of greater risk associated with a more complex process. As very few biopharmaceutical manufacturers have been able to justify the implementation of perfusion technology for large scale GMP production, the scope of this paper will be limited to LS stirred tank bioreactors that are operated in batch and fed-batch modes.

Several alternative technologies show promise, notably transgenic plants and animals, but a transgenic production facility has yet to be licensed by a regulatory agency for the production of human therapeutics. As these processes are still in their pioneering stage of development, the approach represents significant risk in terms of uncertainties regarding the time required for process development and regulatory hurdles. In addition, it remains to be proven that the capital savings in replacing the cell culture unit operation are not offset or exceeded by the increases in purification costs (Morrow 2002). Therefore the stirred tank bioreactor is expected to remain the biologics workhorse and most viable approach for

GMP production of complex proteins in the foreseeable future.

Early initiatives with LS cell culture production approached bioreactor design in much the same way as one would design a microbial fermentor. The microbial design conventions and rules of thumb for agitation, vessel geometry and aeration were often applied, if for no other reason than the fact that there were no established conventions for cell culture and there was no evidence that these methods would not work. Subsequent studies have demonstrated that there are unique challenges presented by a LS cell culture process, which necessitate a different approach to equipment design and scale up.

Agitation and aeration

At large scale, the agitation and aeration characteristics of the bioreactor are critical and clearly the most difficult aspect of scale up. The LS cell culture issues can be differentiated into three categories for this discussion:

1. Factors that contribute to physical cell damage or lysis.
2. Concentration gradients resulting from poor mixing.
3. Problems associated with inadequate gas-liquid phase mass transfer.

Of the factors that can lead to physical cell damage, the two principal mechanisms are agitation induced hydrodynamic shear and bubble damage caused by gas sparging. Animal cells, unlike microbial organisms, lack a protective cell wall and are therefore relatively sensitive to disruptive physical forces in the fluid. A culture's 'shear sensitivity' is influenced by choice of cell line, presence of key nutrients, concentration of inhibitory cell metabolites and batch age (cells are more susceptible to shear damage in lag and stationary phases). Though the focus of this discussion is on equipment design, it is recognized that the problem of shear damage in cell culture is multifaceted, requiring consideration of process methods and medium composition as well.

Bioreactor mixing is always an issue at large scale, as the time required to achieve bulk homogeneity is generally longer as fluid volume is increased (Enfors et al. 2001). For cell culture process, mixing is an even greater concern because of the gentle agitation systems that are typically implemented to minimize

the hydrodynamic shear effects on animal cells (Leist et al. 1990). Problems associated with nutrient, pH and dissolved oxygen gradients due to poor mixing can develop in systems with inadequate agitation. As we shall see in the discussion that follows, many of the mixing problems experienced in LS cell culture result from an erroneous view of the sensitivity of mammalian cell lines to mechanical agitation.

Oxygen, a key nutrient for all aerobic organisms, requires constant replenishment for cell growth to continue. At first consideration, the oxygen transfer requirement for LS cell culture would seem relatively easy to accomplish because animal cell respiration rates are substantially lower than that of microbial organisms. However, unlike microbial fermentations, animal cells cannot tolerate voluminous sparging in conjunction with mechanical gas dispersion to maximize aeration. The shear sensitivity of mammalian cells places practical constraints upon the mass transfer technologies that can be used. Oxygen transfer, then, becomes progressively more difficult as one scales up the process, as the mass transfer efficiency of stirred tank bioreactors will generally degrade as scale is increased. Likewise, the ability to remove carbon dioxide produced by cell respiration is also affected, and problems associated with high CO₂ accumulation have been documented for LS cell culture systems as well (Garnier et al. 1996; Kimura and Miller 1996; DeZengotita et al. 1998; Taticek et al. 1998).

Mass transfer

Mass transfer in stirred tank bioreactors can be accomplished via a gas permeable membrane, surface aeration, and subsurface sparging. Aeration by means of a permeable membrane has been demonstrated in bioreactors up to 150 liters (Lehmann et al. 1988; Vorlop et al. 1989) but has not proven scalable for commercial GMP applications. Surface aeration occurs in all stirred tank bioreactors, and is often sufficient alone for mass transfer in small-scale systems. However, the contribution of surface aeration to the overall mass transfer requirements of the culture is much less significant at large scale. The mass transfer contribution from surface aeration diminishes rapidly as one scales up the process because there is an approximate log-log relationship between production scale and the surface area available for mass transfer in a bioreactor vessel (Figure 1). Even when used in

conjunction with a surface agitator, surface aeration is usually only practical at small scale. Therefore, the only viable method to meet the mass transfer requirements of LS cell culture is through a subsurface introduction of sparge bubbles through the culture medium.

One cannot adequately address the mass transfer issues within LS cell culture without also considering the damaging effects of sparge bubbles. Bubble effects are widely regarded as the principle cause of cell damage in mammalian cell culture using serum-free medium. It is postulated that cells collect along the gas-liquid interface of the sparge bubbles as they rise within the vessel, and then are exposed to damaging effects when the bubble bursts at the liquid surface (Handa et al. 1987; Chalmers 1994). Some studies have also suggested that cells are damaged when they are captured by sparge bubbles and sequestered in the foam layer at the surface. Detrimental effects have been noted at sparge rates as low as 0.05 VVM. Studies on the effects of sparge bubbles have demonstrated a linear relationship between the specific gas flow rate and the cell death rate (Jöbses et al. 1990). The degree of the cellular damage is dictated by bioreactor design and operation, as well as the cell line characteristics, media formulation, and nutritional state of the cells. The conventional approach to addressing bubble damage is to add 0.5 to 3 g/liter Pluronic F68, a non-ionic surfactant copolymer of polyoxyethylene and polyoxypropylene. It coats the

surface of objects immersed in the fluid, creating a protective layer that mitigates the adverse effects of sparge bubbles in serum-free medium. The use of Pluronic surfactants should be restrained, as they are also known to create problems with foaming, downstream processing and loss of mass transfer efficiency.

Another problem associated with mass transfer in LS cell culture is a progressive increase in the partial pressure of carbon dioxide ($p\text{CO}_2$) as the batch progresses. A high concentration of dissolved carbon dioxide can have an inhibitory effect on cell growth and will drive the culture pH more acetic because it will increase the concentration of H_2CO_3 . This problem occurs when the rate of CO_2 stripping is less than the rate of oxygen transfer. It has been noted in cell culture systems that deliver oxygen by membrane diffusion or very small bubbles that dissolve before reaching the surface of the medium. As the only remaining method of eliminating metabolically produced CO_2 is through surface mass transfer (Mitchell-Logean and Murhammer 1997) it is not surprising that larger bioreactors are more susceptible to this phenomenon.

Production bioreactors should be designed to achieve an oxygen transfer capacity that will at least match the oxygen uptake rate of the culture at the harvest cell density. The rate of oxygen transfer in a bioreactor is directly proportional to the system mass transfer coefficient, $k_L a$, which is a function of medium constituents, vessel geometry, gas distribution design, operating pressure and vessel agitation. It is normal to experience some loss in mass transfer efficiency when stirred tank bioreactors are scaled up. The $k_L a$ for production-sized bioreactors can diminish by as much as 1/2 that of similar pilot scale reactors. To compensate, mass transfer performance can be boosted through oxygen enrichment, increased sparge flow, and improved mixing. Oxygen enrichment will increase the mass transfer driving force (C^*-C) by increasing the O_2 concentration in the gas phase. The oxygen transfer rate is directly proportional to the mass transfer driving force in accordance with the equation $\text{OTR} = k_L a (C^*-C)$, where C is the oxygen concentration of the gas phase and C^* is the oxygen concentration that would be in equilibrium with the liquid phase. Sparge flow, typically scaled by Vessel Volumes per Minute (VVM), dictates the rate of oxygen delivery to the system and the number of bubbles available for aeration. An increase in the O_2 mass flow rate per unit volume will improve $k_L a$ due to greater surface area available for mass transfer,

Figure 1. Surface area per unit volume diminishes exponentially with increase in batch size.

though the degree of freedom in this area is limited by the aforementioned problems with bubble damage and foaming. The mass transfer efficiency of a culture can also be improved by better agitation. Fluid mixing will affect a remarkable improvement on $k_L a$ because a well-agitated vessel prevents bubble coalescing and maintains bubbles in suspension for a longer period of time. Both sparge flow and agitation parameters exhibit a linear relationship with $k_L a$, a relationship that can be characterized for the specific operating and design configuration of each bioreactor. In most LS cell culture systems the disruptive energy released by bubble bursting is at least an order of magnitude greater than that of mixing induced hydrodynamic shear. This suggests a strategy of keeping the sparge rate at a minimum and focusing on oxygen enrichment and mixing improvements to achieve the necessary oxygen transfer rate.

Mixing

Early concerns about the fluid shear produced by agitation led to the predominant use of marine impellers, as well as the development of a variety of 'low shear' pitched blade impellers in the 1980s. These mixers provide gentle bulk mixing without the vigorous fluid turbulence typical of microbial fermentors (Figure 2). Subsequent studies have indicated that shear stress in the bulk fluid is not as significant a factor as it was once thought to be (Chisti 1993). Indeed, it is the bubble entrapment resulting from high agitation rates and impeller placement (too close to the surface) that often represents the greatest threat to the culture (Kioukia et al. 1996). Nevertheless, bubble damage can be mitigated by Pluronic F68 as previously discussed, and so it is still prudent to use low-shear mixing technology to avoid unnecessary localized fluid turbulence. Mechanical mixing becomes a process constraint where localized fluid shear is produced by eddies in the wake of impeller blades. The potential for cell damage resulting from localized fluid shear can be quantified by examining the smallest of the turbulent fluid eddies, commonly called Kolmogorov eddies, which can be calculated for a bioreactor vessel as a function of kinematic viscosity and local power per unit mass in accordance with the equation, $\lambda^4 = \nu^3 / \varepsilon$, where λ = Kolmogorov eddy size, ν = kinematic viscosity, and ε = localized power dissipated per unit mass.

The risk of cell damage resulting from these micro

Figure 2. The 'Elephant Ear' Low Shear impeller is designed to distribute fluid shear equally along the trailing edge of each blade (from ABEC Incorporated with permission).

eddies becomes significant as they approach the size of the cell (McQueen et al. 1987). Detrimental effects have been shown to occur when the Kolmogorov eddy length drops below $1/2$ – $2/3$ times the diameter of the cellular unit (Croughan et al. 1989). This is usually not problematic for suspension cell culture, where there are free floating cells on the order of 10 – $20\ \mu\text{m}$. Anchorage-dependant cells, however, frequently require 100 – $200\ \mu\text{m}$ diameter microcarrier beads to provide a growth substrate. These are much more sensitive to the shear effects of microeddies due to their large size (Croughan et al. 1987). When scaling up, it can be difficult to provide sufficient agitation to keep the microcarriers in suspension while maintaining the Kolmogorov size above the threshold that strips cells from the substrate, which may partially explain the prevalence of suspension cell culture at large scale.

Poor mixing is a common problem with LS cell culture, leading to undesirable concentration gradients and diminished mass transfer efficiency within the bioreactor (Nienow et al. 1996). In addition, concentration gradients can be exacerbated in high-density cultures by the formation of cell aggregates or agglomerates within the turbulent eddies of the fluid. In poorly mixed systems, these aggregates can become large enough to create segregated cellular clumps that establish concentration gradients within

the culture (Ozturk 1996). The impact on the heterogeneity of the culture can adversely affect cellular growth and productivity (Sen et al. 2001).

Mixing can be improved by (1) increasing the agitation speed, (2) increasing the diameter of the impeller, and (3) adding impellers to the system. Interestingly, some studies suggest that mixing time is independent of impeller type in systems with equivalent power input and tank geometry (baffled tank; 1:1 liquid aspect ratio) (Nienow 1997). In systems with good fluid circulation patterns, the medium mixing is related to the power per unit volume imparted by the agitation system, which is defined by the equation $P/V = N_p N^3 D_i^5 \rho / V$, where N_p = impeller power number, N = agitation speed, D_i = impeller diameter, ρ = medium density and V = medium volume. As indicated by this equation, the mixing energy imparted to the medium is very sensitive to both agitation speed and impeller diameter. However, the Kolmogorov eddy size is also dictated by the power input, which defines a limitation where the hydrodynamic shear becomes damaging to the cells. The equation for Kolmogorov eddy length can be employed in conjunction with the power equation to predict this limit for design purposes, using the cube of the impeller diameter as an estimate of volume for the calculation of localized power dissipated per unit mass (Nelson 1988). Moreover, these formulas predict that the Kolmogorov eddy size in the medium is inversely proportional to fluid density, which exacerbates the problem approaching the end of the batch when both cell density and oxygen demand are at their highest level.

Scale up

Cell culture bioreactors are usually scaled up with similar vessel geometries to duplicate pilot scale mixing patterns, but beyond that there are no reliable scaling parameters that correlate well with production performance. For lack of a better method, aeration and agitation systems are often scaled by equivalent VVM and impeller tip speed, but these criteria are not reliable indicators that can be used to predict the performance characteristics and productivity of a LS cell culture system (Aunins and Henzler 1993). Processing parameters are ultimately chosen by empirical observations of the sparge rates and agitation speeds required to scale the $k_L a$ of the system. For that reason, aeration and agitation systems are usually

specified with a generous safety factor with regard to capacity and turndown control.

The Oxygen Uptake Rate for a given cell line cultured at the desired harvest cell density can be used to establish the minimum required bioreactor $k_L a$, or oxygen transfer threshold, below which respiration becomes growth limiting (Figure 3). For each equipment design and operating configuration, this oxygen transfer threshold can be characterized as an exponential function of sparge flow rate and mixing speed (Figure 4). If we consider all of the design constraints discussed thus far, we can construct a process operating window within which agitation and aeration parameters are sufficient to achieve the desired cell density (Figure 5). The specific boundaries of this window ultimately must be determined experimentally for each cell line and equipment configuration. As cell density increases, the area of the operating window diminishes until one or more of the process constraints becomes growth limiting. The scale up objective is to make this operating window as large as possible, maximizing the degrees of freedom for each process parameter.

Figure 3. At constant $k_L a$, the bioreactor mass transfer performance can be characterized in terms of an exponential relationship between aeration and agitation (modeled data).

Figure 4. In any given set of equipment and operating conditions, the relationship between aeration and agitation can be characterized for several oxygen transfer thresholds (modeled data).

When designing equipment for LS cell culture, one must take into account all of the common scale up problems previously discussed, as well as environmental and nutritional requirements of the cells.

Figure 5. The objective of equipment design for LS cell culture is to mitigate process constraints such that the process operating window is as large as possible.

These constraints clearly have the greatest impact on the design of the agitator and gas distribution systems. Both of these critical systems have a significant impact on the mass transfer characteristics of the equipment.

Gas distribution design

Equipment gas distribution systems typically utilize thermal mass flow controllers to meter aeration to the bioreactor. Oxygen is frequently used to supplement air for a reduced sparge flow rate, thereby mitigating the problems associated with sparge bubbles. Bio-reactor aeration systems may also include carbon dioxide gas for pH control, and nitrogen for dissolved oxygen probe calibration and control. These gases are combined in a 0.2 μm sterile filter, which provides a dual function of mixing the gases and removing biologic contaminants.

Gas spargers used in GMP equipment must be designed such that they are easily cleanable, steam sterilizable, and free draining. In practice, it is difficult to achieve ideal GMP construction in conjunction with optimal mass transfer performance. The designs in common use are the point sparger, ring sparger, and frit sparger. Each represents a compromise in the performance or operability of the system. The sparger selection for a given application should be one that will provide the best GMP operation and meet the minimum mass transfer requirements at the same time. Ideally, spargers should be designed to disperse bubbles sufficiently to avoid coalescence into larger bubbles as they rise within the vessel. The pressure drop across the sparger should be sufficient to ensure uniform flow distribution, but not so great as to create a turbulent jet that could potentially damage cells. At least one study has suggested that an excessive pressure drop across the sparger can affect cell growth (Murhammer and Goochee 1990). A good rule of thumb is to size the sparger such that the pressure drop is 0.5–2.5 psi throughout the anticipated operating range.

Point spargers are the simplest of designs, providing a single bullet-shaped nozzle through which gas is introduced to the vessel. The simplicity of this design makes it preferable from a GMP perspective because it is the easiest to clean and sterilize. The orifice on the tip of the point sparger is designed to provide flow restriction such that a continuous stream of bubbles is provided throughout the entire opera-

tional gas flow range. The principle disadvantage of point spargers is that there is little control of bubble size, and large bubbles provide relatively little bubble surface area for mass transfer.

Ring spargers are often employed to increase the surface area available for mass transfer by creating multiple smaller bubbles. The air nozzles on this sparger are drilled on the bottom of a tubular ring such that it can freely drain to meet GMP requirements. Therefore, ring spargers need to be inserted in a specific orientation and should be indexed to the vessel nozzle. If used in conjunction with a bottom-mounted agitator, ring spargers are usually designed in the shape of a shepherd's crook instead of a full ring, to allow for installation clearance relative to the agitator shaft. In practical comparison with the point sparge design, ring spargers often do not provide a significant enough improvement in the mass transfer performance to justify the added expense and cleaning difficulties introduced.

Frit spargers utilize a porous tube to create thousands of very small bubbles within the bioreactor vessel. The miniature bubbles produced by a frit are ideal for mass transfer because they provide ample gas-liquid surface area and allow additional contact time for gasses to reach equilibrium (Figure 6). The frit itself, usually constructed of sintered stainless steel or porous Teflon, can be sized to create a uniform curtain of bubbles with a fairly narrow size distribution. Bubble size is further affected by sparge rate and Pluronic F68 concentration. As smaller bubbles tend to create higher shear gradients that are

damaging to cells, cultures with micro-sparging systems are more susceptible to bubble damage at low flow rates. In high density CHO and BHK cultures, bubble damage has been documented at sparge rates above 0.054 VVM using a 0.15 μm sparger, and above 0.025 VVM using a 0.5 μm sparger (Qi et al. 2001).

The frit porosity is chosen to optimize the balance between oxygen transfer and CO_2 stripping within the medium. If the bubbles are too small, they will dissolve before reaching the surface – effectively removing the CO_2 stripping capability of the sparge. If the bubbles are too large, the sparge rate will need to be increased to compensate for the loss of mass transfer area. An experimental study of high density CHO culture at 500 L scale has suggested an optimum pure oxygen bubble size of approximately 2–3 mm diameter, which is sufficiently large to affect CO_2 stripping while minimizing the total sparge rate (Gray et al. 1996). Larger bubbles may be necessary to produce the same effect in LS cell culture systems. Manufacturers generally recommend frit porosities of 2–5 μm . The length of the frit element is then sized to create a reasonable pressure drop across the sparger.

The principle problem with frit-type spargers is the difficulty cleaning the porous element. In non-GMP applications they are typically cleaned by a caustic soak followed by thermal oxidation of any organic residuals in a dry heat oven. However, it is extremely difficult if not impossible to validate that all process residuals imbedded in the frit have been removed. Therefore the frit elements used in GMP cell culture

Figure 6. In comparison with ring spargers (left), frit spargers (right) produce thousands of miniature bubbles, which optimize the bubble-liquid interface area available for mass transfer (from Mott Corporation with permission).

are generally considered disposable. Replacement sparge elements are either attached via an aseptic connection or rewelded to the sparge insert tube.

Over-foaming can become problematic if the surfactant level and sparge rates are too high, especially when the bubble size is very small (as produced by a frit-type sparger). The surface foam layer threatens the cell culture process when it is high enough to entrain foam in the gas exhaust line and foul the vent filter. Several devices have been developed for foam separation from the bioreactor off-gas stream, but their application is costly and not recommended for long-term aseptic applications such as GMP cell culture. A variety of antifoam and defoaming agents are commercially available to address the foam layer, but these chemicals can degrade mass transfer performance and create difficulties in downstream processing. If possible, it's best to avoid conditions that are conducive to excessive foam build up in the first place.

Agitator design

Cell culture impellers are typically sized at 1/3 to 1/2 of the vessel diameter. If space allows, additional impellers can be added to improve mixing. Since the Kolmogorov eddies occur in the local turbulent zone adjacent to each impeller, the addition of a second or third impeller is an effective strategy to improve mixing without significantly contributing to the problem of hydrodynamic shear from micro eddies. It is best to use axial flow impellers with good pumping characteristics, such as marine, hydrofoil or low shear pitched blade designs, to provide good vertical mixing. Axial flow impellers improve mass transfer efficiency by increasing the average residence time of the bubbles. These impellers should be spaced at least one impeller diameter from each other and from the surface of the medium. If an impeller operates too close to the liquid surface, it can create a vortex that entrains air bubbles from the headspace, mimicking the damaging effects of the sparge bubbles (Kunas and Papoutsakis 1990).

Agitation systems present some unique operational challenges in LS cell culture applications. These systems are required to maintain a long-term aseptic environment, and the financial loss associated with a contamination at this stage is substantial. Therefore, the sanitary design detail of the agitator internals - including impeller construction, couplings and me-

chanical seals - is critical to the success of LS cell culture. Magnetic coupling systems are available as a replacement for mechanical seals, but have not gained widespread acceptance for LS manufacturing (perhaps because of torque limitations and perceived cleaning and maintenance difficulties). A good reference for GMP agitation design in this application is the ASME BPE standard (American Society of Mechanical Engineers 2002) section DS-4.8, which illustrates specific preferred and recommended design practices to ensure the sterility and cleanability of bioprocessing equipment. Both top and bottom mounted drives have been used for cell culture, though large systems frequently do not have the ceiling clearance required for maintenance and removal of top-mounted agitators. Impeller installation and removal also presents a problem at large scale, as cell culture impellers are frequently too large to fit through a standard vessel manway. This may require the installation of an oversized manway or full opening head, as well as the overhead lifting mechanisms required for handling.

The agitator positioning in stirred tank vessels may be specified with either angular off-center positioning or centerline orientation. Angular off-center drives are installed with an agitator shaft angle that is positioned approximately 15 degrees off the vertical centerline of the vessel so as to collapse the fluid vortex on the surface. Used in conjunction with axial flow impellers, these drive orientations are commonly employed in pilot scale cell culture bioreactors because they can be implemented in an unbaffled tank - thereby removing the need for baffles that complicate the cleaning of the vessel and are perceived as a contributor to hydrodynamic shear stress on the cells. Angular off-center agitators have been used in LS cell culture systems as well, but require a structurally robust design because the unbalanced fluid forces in the bioreactor can become severe when the power delivered by the agitator exceeds 3 HP (Perry and Green 1997).

Centerline agitators create relatively balanced fluid forces in comparison with angular systems, but require the addition of 2–4 baffles to ensure uniform mixing. Vessel baffles, vertical metal strips that are equally spaced in radial positions along the tank wall, are typically designed with a width 1/10 to 1/12 of the tank diameter and extend the full tangent height of the vessel. In GMP applications, they should be seal welded to the tank wall and constructed so as to avoid sharp edges, corners, cracks and crevices. Despite the

undesirable baffle requirement, there are several factors that favor the implementation of centerline drives in large scale systems. As centerline agitators create a balanced mixing pattern throughout, it is less likely that sequestered regions with poor mixing will develop within the bioreactor vessel. The mechanical forces acting upon a centerline drive are better balanced as well, supporting an expectation that the maintenance frequency of bearings and mechanical seals would be less frequent than that which would be required for angular systems. In addition, the maintenance handling of centerline-mounted agitators is easier relative to an angled drive because the weight of the components, which can become quite heavy at this scale, is better balanced for lifting.

Vessel design: CIP and SIP

The chosen agitation and gas distribution approach will ultimately impact bioreactor vessel design, control and operation. For example, in the early 1990s it was commonly believed that very gentle agitation in conjunction with a hemispherical bottom head on the vessel was necessary to keep cells in suspension while avoiding shear damage. Further experience revealed that animal cells are more resistant to hydrodynamic shear than initially believed, particularly in the case of hybridomas, and can usually tolerate more thorough mixing (Varley and Birch 1999). Consequently, contemporary cell culture vessels are typically designed with less costly ASME Flanged and Dished heads.

Bioreactor vessel design should take into account processing and recovery requirements as well. If the production method will be fed batch, the system needs to be designed to allow adequate mixing and temperature control at the starting volume, as well as a means for the sterile addition of media during the batch. Minimum volume processing can be facilitated by low impeller placement and the implementation of a bottom jacket on the vessel, something that is not always practical on smaller systems. Cell culture vessels are typically designed for a 1:1 to 2:1 aspect ratio, but higher aspect ratios are sometimes employed to allow for control of a smaller starting volume. Upon recovery, the vessel bottom drain is usually used to harvest the cells. If tangential flow filtration will be used in conjunction with the bioreactor vessel for the recovery operation, a second large steam sterilizable port may be required for medium recirculation.

The principles of GMP vessel design do not change upon scale up, but the consequences of poor aseptic construction do become economically more significant. Vessel internals are typically constructed of 316 L stainless steel with 15–20 μ in Ra electropolished finish and free-draining surfaces free of pits, cracks and crevices that are difficult to clean and sterilize. In contrast with small and pilot scale bioreactor systems, LS cell culture vessels are typically 100% cleaned in place because most components are too large and difficult to access for out of place cleaning. Reliability is a key economic requirement at this scale, and the need for consistent and effective aseptic operation frequently drives a decision to invest in full automation of CIP and SIP operations. Particular attention should be given to the design of subsurface vessel penetrations and internal objects that may be shadowed during CIP. The ASME BPE standard, section SD 4.7, provides a good guideline for the design of vessel internals and nozzles in this service.

Vessel cleaning at large scale typically requires multiple CIP spray devices to ensure complete coverage of all internals. The need for complete and reproducible CIP coverage favors static sprayballs that are custom-drilled to direct cleaning chemicals to nozzles and other areas that would not normally encounter the falling film of CIP fluid as it drains from the vessel. As these sprayballs can appear superficially similar to each other and are usually removed during intermediate processing, they should be match-marked to the specific vessel nozzle they are mated to. A radial index to the nozzle also needs to be provided to ensure that the specific orientation of the spray device is duplicated for every CIP operation (Figure 7).

Vessel sterilization presents other unique problems at large scale. As many of the constituents of serum-free cell culture media are heat labile, the customary approach to sterilization in place is to inject clean steam directly into an empty bioreactor vessel. Following equipment SIP, the culture medium is then transferred into the vessel via sterile filtration. The clean steam condensate produced by SIP is drained through the vessel bottom drain valve and diverted to a steam trap or flow orifice to regulate condensate flow. In large systems, the condensate flow rate through the bottom drain can be substantial, and the downstream piping and steam trap must be sized correctly to prevent condensate from backing up into the bioreactor vessel during SIP. Following the sterilization dwell period, vessel cooling can produce addi-

tional difficulties. The specific volume of saturated steam at sterilization temperature is nearly 800 times larger than that of an equivalent mass of steam condensate. Therefore, a large vessel filled with 100% saturated steam will lose positive pressurization rapidly as steam collapses upon cooling. For GMP operation, it is important that the vessel stay pressurized after sterilization. Consequently, the SIP operation needs to be controlled such that a vacuum is not produced within the vessel during cool-down. This can be accomplished in two ways: (1) by restricting the rate of cooling and (2) by allowing for the rapid introduction of sterile air back into the vessel after sterilization. The bioreactor gas distribution system, normally designed for a maximum flow capacity in the neighborhood of 0.10 VVM, is usually insufficient to replace the volume of collapsing steam during cooling. To address this limitation, the bioreactor can be designed with a bypass around the mass flow controllers to route sterile compressed air directly to the vessel during SIP cool down. This strategy, in combination with a controlled rate of cooling until the vessel temperature drops below 100 °C, is an effective way to maintain positive pressure in the vessel. Alternately, the vessel can be partially filled with WFI

during sterilization, and then drained prior to the media transfer. The WFI acts as a heat sink to slow the rate of cooling until enough air can be reintroduced to the vessel through the gas distribution system to pressurize the vessel. The disadvantage to this approach is that it extends the sterilization time and wastes a substantial amount of WFI, requiring enough water volume to ensure that it can be adequately heated and mixed during SIP.

Instrumentation and control

The economics of large scale production can often justify instrumentation and control redundancy that is not feasible for smaller systems. As an example, redundant pH and dissolved oxygen sensors are frequently implemented at large scale. These probes can be easily damaged and have been known to drift after sterilization and long-term use. It creates a serious problem when one of these critical sensors fail in the middle of a run that can last several weeks. *In situ* sterilizable probe housings are available that will allow one to replace the probe during a run, but with a single probe it is still difficult to detect a process

Figure 7. Many vessel vendors weld a tab onto the sprayball nozzle so that it can be indexed to a mating pin on the spray device (from Lee Industries with permission).

signal error in time to prevent an erroneous response from the control system. For this reason it is advisable to consider backup probes and transmitters for dissolved oxygen and pH data acquisition. This redundancy can be coupled with a discrepancy alarm that will alert the operator in time to determine which sensor is failing and switch control if necessary to use the accurate signal. Redundant probes also provide an opportunity to easily gauge the effectiveness of mixing by noting signal divergence after a tracer chemical is added, particularly if the probes are placed so as to measure opposite ends of the medium.

When translating biologics production to large scale, the effect of hydrostatic pressure on polarographic dissolved oxygen (DO) sensors is frequently overlooked. The unit of measure for DO, percent saturation, indicates the ratio of oxygen to other gasses in the medium. This reading is a relative measure of oxygen saturation at the pressure conditions of the DO sensor, not an absolute measure of oxygen partial pressure and activity. An absolute measurement would need to take into account the cumulative effects of the vessel head pressure and medium hydrostatic pressure on the reading. As vessel overpressure sensors are typically mounted in the vessel head space, the added hydrostatic pressure contributed by the higher liquid level in LS cell culture is easy to miss. In practice, a pressure increase of 1 atmosphere (or 10 m of liquid height) will increase the DO signal by 100%, so each 0.3 m of liquid height will effectively increase the DO reading by 2.94%. When scaling up a bioreactor the equivalent DO reading for a larger vessel can be calculated as

follows: $DO_2 = DO_1 (P_2/P_1)$, where pressure (P) is equal to the sum of the atmospheric pressure, head-space pressure, and hydrostatic pressure for the probe position in each vessel (R. Garrahy, unpublished presentation by Broadley-James Corporation, RAFT-IV Conference on Recent Advances in Fermentation Technology). This equation can be employed to predict the minimum operating DO level for operation in a larger vessel, or to calculate a virtual DO reading for any measurement position in a bioreactor vessel.

As many LS cell cultures are particularly susceptible to deleterious effects of high carbon dioxide concentrations, the subject of dissolved carbon dioxide (dCO_2) measurement and control merits further discussion. YSI, Inc. offers a model YSI 8500 instrument that is capable of providing reliable *in situ* dCO_2 measurement in a bioreactor environment (Figure 8). This instrument utilizes an *in situ* fiber optic chemical sensor and expresses dissolved carbon dioxide levels in units of % gas-phase concentration. An online dCO_2 signal can be used to implement closed loop control of dissolved carbon dioxide via N_2 sparging. When used in conjunction with closed loop control of dissolved oxygen via O_2 and pH control via CO_2 sparging, this strategy can be employed to achieve all of the mass transfer requirements of the process while minimizing the overall sparge rate (Pattison et al. 2000) (thereby mitigating the cellular damage resulting from sparge bubbles).

In general, a wider variety of technologies for online measurement of culture nutritional environment and cell growth become economically feasible at large scale. The use of automated chemistry analyzers for

Figure 8. YSI 8500 dissolved carbon dioxide measurement (—) in comparison with measurements from a blood gas analyzer (*) during fermentation of W3100 E. coli in Super Broth with glucose (Spokane et al. 1999) (from YSI, Inc. with permission).

media monitoring and *in situ* optical density probes for cell density measurements is gaining wider industry acceptance. Several emerging technologies, such as radio frequency impedance measurements of viable cell concentration, also show promise. Our purpose is not to evaluate these methods in detail, but to point out that the value of additional *in situ* monitoring is proportionally greater at large scale. By closely monitoring the cell physiology and growth rates, one can optimize resource utilization, minimize transfer times between scales and maintain the cell population in exponential growth (Schoengerr et al. 2000). The implementation cost of these systems is essentially scale independent, while the benefits, in terms of nutritional control improvements and harvest timing, are appreciable at large scale. Therefore process scale up should include a reevaluation of the process instrumentation scope and its design implications for bioreactor probe ports and controls.

Equipment implementation

The high cost of clean room space in biopharmaceutical production facilities has led to some interesting innovations that allow for all or part of the bioreactor systems to be segregated in an unclassified space. At large scale, the financial driver for such segregations is significant. As the aseptic requirements of a cell culture process require a closed system, the principle reason for clean room access is to facilitate manual operations. To the extent that these operations can be automated, an opportunity exists to consider locating much of the equipment outside of the classified space. Systems that do not require clean room access can be mounted on a separate skid, to interface via a piping panel with the rest of the equipment in classified space. Alternately, piping modules can be constructed with a built-in clean room barrier to segregate systems that do not require clean room access. These modules are essentially built into the wall of the clean room, and simplify the facility interface by including all of the piping penetrations in a single structure. In some installations, the entire bioreactor vessel is located in unclassified space, with the only clean room interface in the form of a barrier alcove to access the sample assembly. An added advantage of such arrangements is that much of the routine preventative maintenance can be performed without clean room gowning or environmental contamination concerns.

Unlike pilot scale equipment, which can be easily

packaged on a single skid, LS cell culture equipment is likely to require at least partial assembly on site. Process assemblies can be prefabricated and mounted in one or more piping modules, but connections to the vessel often require a field fit. In addition, the large size of the bioreactor vessel dictates a tighter integration with the facility. They frequently require high bay clearance, mezzanine or platform access, and special hoist apparatus for maintenance. Overall, the implementation of equipment at this scale is complicated and requires detailed project execution planning.

Conclusion

Mammalian cell culture offers the best method for large-scale production of complex proteins with correct folding and posttranslational modifications. While the cell culture unit operation is essentially identical to that of industrial fermentation with microbial organisms, there are some distinct differences with regard to processing and economics that advise the approach to bioreactor scale up and design. Likewise there are unique process risks and constraints to consider when designing for large scale, as opposed lab and pilot scale cell culture. Successful LS cell culture involves a thorough understanding of the cell line growth characteristics, a bioreactor design approach that recognizes and mitigates process constraints, and a pragmatic evaluation of the equipment investment in the light of process economics.

References

- American Society of Mechanical Engineers 2002. ASME BPE-2002. <http://www.asmeny.org/catalog/html/catgcs.htm>
- Aunins J.G. and Henzler H.-J. 1993. Aeration in cell culture bioreactors. In: Stephanopoulos G., Rehm H.-J., Reed G., Pühler A. and Stadler P. (eds), *Biotechnology Vol. 3*. 2nd edn. VCH Verlag GmbH, Weinheim/D, pp. 219–281.
- Chalmers J.L. 1994. Cells and bubbles in sparged bioreactors. *Cytotechnology* 15: 311–320.
- Chisti Y. 1993. Animal cell culture in stirred bioreactors: Observations on scale-up. *Bioproc Eng* 9: 191–196.
- Croughan M.S., Hammel J.F. and Wang D.I.C. 1987. Hydrodynamic effects on animal cells grown in microcarrier cultures. *Biotechnol Bioeng* 29: 130–141.
- Croughan M.S., Sayre E.S. and Wang D.I.C. 1989. Viscous reduction of turbulent damage in animal cell culture. *Biotechnol Bioeng* 33: 862–872.
- DeZengotita V., Kimura R. and Miller W. 1998. Effects of CO₂ and

- osmolality on hybridoma cells: growth, metabolism and monoclonal antibody production. *Cytotechnology* 28: 213–227.
- Enfors S.O., Jahic M., Rozkov A., Xu B., Hecker M., Jürgen B. et al. 2001. Physiological responses to mixing in large scale bioreactors. *J Biotechnol* 85: 175–185.
- Garnier A., Voyer R., Tom R., Perret S., Jardin B. and Kamen A. 1996. Dissolved carbon dioxide accumulation in a large scale and high density production of TGF β receptor with baculovirus infected Sf-9 cells. *Cytotechnology* 22: 53–63.
- Glaser V. 2000. Fermenters and bioreactors for bioprocessing. *Genetic Engineering News*. Vol. 20, No. 14, pp. 13, 15, 81.
- Gray D.R., Chen S., Howarth W., Inlow D. and Majorella B.L. 1996. CO₂ in large-scale and high-density CHO cell perfusion culture. *Cytotechnology* 22: 65–78.
- Handa A., Emery A.N. and Spier R.E. 1987. On the evaluation of gas-liquid interfacial effects on hybridoma viability in bubble column bioreactors. *Dev Biol Standard* 66: 241–253.
- Jöbses I., Martens D. and Tramper J. 1990. Lethal events during gas sparging in animal cell culture. *Biotechnol Bioeng* 37: 484–490.
- Kimura R. and Miller W. 1996. Effects of elevated pCO₂ and/or osmolality on the growth and recombinant tPA production of CHO cells. *Biotechnol Bioeng* 52: 152–160.
- Kioukia N., Nienow A.W., Al-Rubeai M. and Emery A.N. 1996. Influence of agitation and sparging on the growth rate and infection of insect cells in bioreactors and a comparison with hybridoma culture. *Biotechnol Prog* 12: 779–785.
- Kunas K.T. and Papoutsakis E.T. 1990. Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment. *Biotechnol Bioeng* 36: 476–483.
- Lehmann J., Vorlop J. and Büntemayer H. 1988. Bubble-free reactors and their development for continuous culture with cell recycle. In: Spier R.E. and Griffiths J.B. (eds), *Animal Cell Biotechnology* Vol. 3. Academic Press Ltd., London, UK, pp. 221–237.
- Leist C.H., Meyer H.P. and Fiechter A. 1990. Potential and problems of animal cells in suspension culture. *J Biotechnol* 15: 1–46.
- McQueen A., Meilhoc E. and Bailey J.E. 1987. Flow effects on the viability and lysis of suspended mammalian cells. *Biotechnol Bioeng* 32: 1001–1014.
- Mitchell-Logean C. and Murhammer D.W. 1997. Bioreactor headspace purging reduces dissolved carbon dioxide accumulation in insect cell cultures and enhances cell growth. *Biotechnol Prog* 13: 875–877.
- Morrow J.K. 2002. Economics of antibody production. *Genetic Engineering News*. Vol. 22, No. 7.
- Murhammer D.W. and Gooch C.F. 1990. Sparged animal cell bioreactors: mechanism of cell damage and Pluronic F-68 protection. *Biotechnol Prog* 6: 391–397.
- Nelson K.L. 1988. Industrial scale mammalian cell culture, part II: design and scale-up. *Biopharm*. Vol. 1, No. 3, pp. 34–41, 50.
- Nienow A.W. 1997. On impeller circulation and mixing effectiveness in the turbulent flow regime. *Chem Eng Sci* 52: 2557–2565.
- Nienow A.W., Langheinrich C., Stevenson N.C., Emery A.N., Clayton T.M. and Slater N.K.H. 1996. Homogenization and oxygen transfer rates in large agitated and sparged animal cell bioreactors: Some implications for growth and production. *Cytotechnology* 22: 87–94.
- Ozturk S.S. 1996. Engineering challenges in high density cell culture systems. *Cytotechnology* 22: 9–13.
- Pattison R.N., Swamy J., Mendenhall B., Hwang C. and Frohlich B.T. 2000. Measurement and control of dissolved carbon dioxide in mammalian cell culture processes using an *in situ* fiber optic chemical sensor. *Biotechnol Prog* 16: 769–774.
- Perry R.H. and Green D.W. 1997. *Perry's Chemical Engineers' Handbook*. 7th edn. McGraw-Hill, Sec. 18, pp 10.
- Qi H., Jovanoic G., Michaels J. and Konstantinov K. 2001. The art and science of micro-sparging in high-density perfusion cultures of animal cells. In: Lindner-Olssen E., Chatzissavidou N. and Lüllau E. (eds), *Animal Cell Technology: From Target to Market*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 412–415.
- Schoengerr I., Stapp T. and Ryll T. 2000. A comparison of different methods to determine the end of exponential growth in CHO cell cultures for optimization of scale-up. *Biotechnol Prog* 16: 815–821.
- Sen A., Kallos M.S. and Behie L.A. 2001. Effects of hydrodynamics on cultures of mammalian neural stem cell aggregates in suspension bioreactors. *Ind Eng Chem Res* 40: 5350–5357.
- Spokane R.B., Pitts G.E., Wu P., Cordonnier M.J. and Ordaz D. 1999. An optical sensor for the *in situ* monitoring of dissolved carbon dioxide in Microbial Fermentations (Abstract). American Chemical Society National Meeting, March 21–25. *Biochemical Technology*.
- Taticek R., Petersen S., Konstantinov K. and Naveh D. 1998. Effect of dissolved carbon dioxide and bicarbonate on mammalian cell metabolism and recombinant protein productivity in high-density perfusion culture. Presented at Cell Culture Engineering Conference VI, San Diego, CA, USA.
- Varley J. and Birch J. 1999. Reactor design for large scale suspension animal cell culture. *Cytotechnology* 29: 177–205.
- Vorlop J. and Lehmann J. 1989. Oxygen transfer and carrier mixing in large scale membrane stirred cell culture reactors. In: Spier R.E., Griffiths J.B., Stephenne J. and Crooy P.J., *Advances in Animal Cell Biology and Technology for Bioprocesses*. Butterworths, Stevenage, UK, pp. 366–369.
- Woodside S.M., Bowen B.D. and Piret J.M. 1998. Mammalian cell retention devices for stirred perfusion bioreactors. *Cytotechnology* 28: 163–175.