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REVIEW

The potential of hydrodynamic damage to animal cells of industrial relevance: current understanding

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Abstract Suspension animal cell culture is now routinely scaled up to bioreactors on the order of 10,000 L, and greater, to meet commercial demand. However, the concern of the 'shear sensitivity' of animal cells still remains, not only within the bioreactor, but also in the downstream processing. As the productivities continue to increase, titer of $\sim$10 g/L are now reported with cell densities greater than 2×10^7 cells/mL. Such high, and potentially higher cell densities will inevitably translate to increased demand in mass transfer and mixing. In addition, achieving productivity gains in both the upstream stage and downstream processes can subject the cells to aggressive environments such as those involving hydrodynamic stresses. The perception of 'shear sensitivity' has historically put an arbitrary upper limit on agitation and aeration in bioreactor operation; however, as cell densities and productivities continue to increase, mass transfer requirements can exceed those imposed by these arbitrary low limits. Therefore, a better understanding of how animal cells, used to produce therapeutic products, respond to hydrodynamic forces in both qualitative and quantitative ways will allow an experimentally based, higher, "upper limit" to be created to guide the design and operation of future commercial, large scale bioreactors. With respect to downstream hydrodynamic conditions, situations have already been achieved in which practical limits with respect to hydrodynamic forces have been experienced. This review mainly focuses on publications from both the academy and industry regarding the effect of hydrodynamic forces on industrially relevant animal cells, and not on the actual scale-up of bioreactors. A summary of implications and remaining challenges will also be presented.

Keywords Hydrodynamic forces · Animal cell culture · Agitation · Sparging · Scale up · Shear sensitivity

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Introduction

It has been more than 40 years since Telling and Elsworth (1965) made one of the first reports of the growth of a suspension culture of animal cells. Specifically they reported on the use of baby hamster kidney (BHK) cells, suspended in a 30 L stainless

steel bioreactor, for vaccine production. One of the early, large scale, commercial suspension animal cell culture was first reported over 25 years ago. With the subsequent need to produce recombinant human therapeutics, significant commercial motivation has existed, and continues to this day, to increase the productivity of suspended animal cells. As was recently summarized in a review by Wurm (2004), between 1986 and 2004, the final product concentrations have increased approximately 100 fold, the final cell density approximately 10 fold, and the specific productivities increased from about 10 pg/cell/day to 90 pg/cell/day. These significant increases are the results of a number of advances in: cellular engineering, culture media development, and bioprocess engineering. This review will focus on our current understanding of one aspect of bioprocess engineering: the effect of hydrodynamic forces upon suspended animal cells.

From the early studies of animal cell culture, the concern of "shear sensitivity" has been frequently mentioned. Given the native state of animal cells with all of the mechanical and nutrient support from vascularized solid tissue, this concern is logical, and is further underscored by the relative large size and lack of cell wall in comparison to the typical microorganisms used in fermentation. The only separation of the animal cell from the surrounding fluid is the thermodynamically-controlled and self-assembled bilayer (plasma membrane) consisting of phospholipids, triglycerides, cholesterol, and transmembrane proteins, all of which is supported by an internal protein matrix.

This concern was further, experimentally, supported by the early studies of the negative impact on cell concentration by increasing the rpm of the stir bar in a spinner vessel. It is important to note, however, that this inhibitory effect of rpm was typically observed on VERO or CHO cell attached to microcarriers (Hirtenstein and Clark, 1980; Croughan et al. 1987, 1989; Cherry and Papoutsakis 1988, 1989; Venkat et al. 1996). These results led to a perception by some people that suspension animal cell culture in a large scale, typically agitated and sparged bioreactor was considered to be unpractical. A number of bioreactor designs were consequently proposed, such as air-lift and membrane bioreactors, to address this concern (Varley and Birth 1999; Katinger et al. (1979))

However, this concern of "shear sensitivity" obviously contrasts to historical and current industrial practice; in fact, the first commercial human therapeutic protein from recombinant animal cells was in 1986 (tPA, Genentech), which used (uses) suspended, CHO cells in stirred tank bioreactors with sparger aeration. This misunderstanding is the result of a number of factors, not the least of which are (1) the complex nature of turbulent flow and our current lack of being able to characterize the flow and corresponding hydrodynamic forces on a micron scale, and (2) the lack of a model (relationships) which relates hydrodynamic forces to lethal and non-lethal cell effects. This lack of first principle knowledge leads to inhibitory "shear forces" usually being considered as a top-ranking contributor to unpredicted cell damage, or sub-optimal productivities in bioprocesses.

There is no doubt that commercial pressure will continue to push the upper limits of animal cell productivity. For example, Luan et al. (2006) reported that they achieved a product titer of 9.8 g/L in a 21-day fed-batch CHO cell culture and Huang et al. (2010) reported two CHO fed-batch cell culture processes with a final titer of 10–13 g/L. These high productivities obviously require higher mass transfer rates than the commercial animal cell cultures of 20 years ago, which, correspondingly, require higher agitation and/or aeration rates. If these productivities continue to increase at a similar rate, future mass transfer demands could become quite significant.

This review mainly focuses on the recent research regarding the characterization of hydrodynamic forces in bioprocessing equipment with respect to the lethal and non-lethal effects of hydrodynamic forces, including damage in foam layers, on cells. This knowledge, combined with our current understanding of a cells tolerance of hydrodynamic forces, will guide concluding statements with respect to the implications for improved operation strategies and suggestions for future research. For a more complete discussion of the actual engineering of animal cell bioreactors, we suggest Nienow (2006).

Hydrodynamic forces

Strictly speaking, "shear stress" is only one of two types of fundamental hydrodynamic forces, the

second of which is referred to as normal stresses. Both stresses are commonly defined as a viscosity multiplied by a velocity gradient, with the flow direction being perpendicular for shear stress (i.e., du_x/dy) and parallel for normal stresses (i.e., du_x/dx). In addition, in complex flows, these stresses vary with time, further adding to the complexity. Beyond the biotechnology community, the larger field of fluid mixing is also interested in understanding and optimizing these forces. Recognizing the current inability to solve for the fluid flow and corresponding forces from first principles, a number of different parameters have been, and are currently used, to characterize and estimate the local fluid conditions.

One of these parameters from the mixing community is the energy dissipation rate, EDR, which was first proposed by Bluestein and Mockros (1969) to characterize cell damage. A broader review of the use of EDR in the mixing community can be found in a review by Kresta (1998). Fundamentally, EDR, can be expressed for a incompressible Newtonian fluid as:

$$\begin{aligned}\varepsilon &= \tau : \nabla U = \mu\left[\nabla U + (\nabla U)^T\right] : \nabla U \\ &= \mu \sum_i \sum_j \left[\nabla U + (\nabla U)^T\right]_{ij} \nabla U_{ji}\end{aligned} \tag{1}$$

where ε is EDR (W/m^3), τ is the stress tensor (N/m^2), μ is the viscosity (Pa·s), U is the velocity vector, ∇U is velocity gradient vector, and ∇U^T is the transpose of ∇U. ∇U can be further defined as:

$$\nabla U = \begin{bmatrix} \frac{\partial U_x}{\partial x} & \frac{\partial U_y}{\partial x} & \frac{\partial U_z}{\partial x} \\ \frac{\partial U_x}{\partial y} & \frac{\partial U_y}{\partial y} & \frac{\partial U_z}{\partial y} \\ \frac{\partial U_x}{\partial z} & \frac{\partial U_y}{\partial z} & \frac{\partial U_z}{\partial z} \end{bmatrix} \tag{2}$$

For flow systems/geometries in which analytical solutions exits, or direct numerical solutions can be performed, EDR can be directly calculated Mollet et al. (2004, 2007). For highly complex flows (and turbulent conditions) average EDR values are typically obtained. These values can range from bulk averages, such as the rate of total input energy to the dissipated volume, often power per volume, (i.e., the number of watts put into a bioreactor through the agitator, divided by the fluid volume), to local EDR values obtained through a variety of experimental techniques that measure local fluid velocities (Kresta 1998).

Another well known parameter to describe hydrodynamic forces is the Kolmogoroff microscale of turbulence, λ, which is defined in Eq. 3, assuming the turbulence is isotropic:

$$\lambda = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4} \tag{3}$$

where ν is kinematic viscosity of the liquid (m^2/s). It was suggested (Cherry and Papoutsakis 1986; Croughan et al. 1987) that when the value of λ is the same size as a microcarrier with cells attached, or single suspended cells, significant cell death occurs. However, challenges exist in the determination of λ; not the least of which is the need to accurately know the local value (relative to the cell) of the EDR, ε.

A summary of parameters reported in the literature to correlate the effect of hydrodynamic forces on cells is presented in Godoy-Silva et al. (2010).

Cell damage from agitation in stirred tanks

The experimental determination of local EDR in a cell culture bioreactor is a very difficult task since it is a three-phase system (gas–liquid–solid) typically in the turbulent flow regime. The flows around the impeller region have been qualitatively and quantitatively visualized and studied through stereoscopic visualization technology by a number of researchers for many decades, (van't Riet and Smith 1975; Tatterson et al. 1980; Mollet et al. 2004). These studies indicated the presence of recirculation flows, jet flows, vortices in the impeller region, and strong trailing vortex originating from the impeller tips. All of these types of flow contribute significantly to the energy transfer and gas dispersion.

As can be imagined, the local EDR is significantly higher in the impeller discharge zone compared to the bulk liquid zone. Based on one-dimensional laser Doppler anemometer (LDA) data, and appropriate assumptions, Zhou and Kresta (1996a) reported that 43.5 and 70.5% of total mechanical power input is dissipated in the impeller discharge region for Rushton turbine (BT) and pitched blade turbine (PBT), respectively. They also showed that the impeller diameter and off-bottom position as well as the number of baffles have great impact on EDR and its distribution (Zhou and Kresta 1996b). Mollet et al. (2004) used a three-

dimensional particle tracking velocimetry (PTV) system to investigate the EDR distribution in a 2 L Applikon bioreactor equipped with a 6-bladed Rushton turbine and no baffles. They reported 27.8% of the total energy is dissipated in just 5.74% of the total liquid volume; this high amount in such a small volume was similar to what has been reported by Zhou and Kresta (1996a) in bigger vessels with similar geometric ratios.

Zhou and Kresta (1996b) further suggest that the maximum EDR value can be estimated from:

$$\varepsilon_{\max} = E \cdot N^3 \cdot D^2 \cdot \rho \quad (4)$$

where N is the agitation speed (1/s); D is the impeller diameter (m); ρ is density (kg/m^3), E is a non-dimensional constant depending on impeller type, impeller diameter/vessel diameter, and off-bottom distance/vessel diameter. Table 1 summarizes a number of representative, literature values of estimates of normalized values of local EDR (local EDR divided by the mean EDR for the whole vessel) in regions away from the impeller (bulk) and in the impeller volume. As can be observed, local values of EDR can vary by several orders of magnitude.

From an operational parameter perspective in actual animal cell culture processes, the effect of agitation speed on animal cells has been studied and reported since the first published studies of animal cell culture. When one considers any of these past, or more recent reports, two important observations are in order before these studies are interpreted: (1) are the cells in suspension or attached to microcarriers?, and (2) does a central vortex form from the headspace and lead to bubble entrainment?

When cells are attached to microcarriers, the removal of cells attached to microcarriers is clearly a function of the agitation speed of the system, and it was this observation that lead to the original linking of the Kolmogoroff microscale to cell damage (Cherry and Papoutsakis 1986; Croughan et al. 1987). It is generally accepted that once a cell is removed from the microcarrier, due to intense mixing, the cell is effectively, dead. It is recognized, however, that gentile particle to particle transfer of cells is possible; in fact, a common method to transfer cells to new microcarriers is to take a well mixed culture of microcarriers, introduce new microcarriers, stop the mixing and let all microcarriers settle. This allows any free cells, or cells on a microcarrier to potentially migrate to a new microcarrier. Early reports of microcarrier culture also suggest that if the culture is not excessively mixed, it is possible that cells can migrate from microcarrier to another while the microcarriers remain suspended (Crespi and Thilly 1981)

Therefore, the hydrodynamic sensitivity in this case is really a question of what hydrodynamic forces are needed to remove a cell attached to a surface. It is interesting to note that the experimentally estimated EDR that started to remove CHO cells from microcarriers in a spinner vessel (Venkat et al. 1996), is the same order of magnitude level of EDR that removes

Table 1 Representative, literature values of estimates of normalized values of local EDR divided by mean, vessel EDR in regions away from the impeller (bulk) and in the volume sweep out by the impeller

Study	Type of impeller[a]	Baffled	Local EDR/vessel mean EDR	
			Bulk	Impeller region
Cutter et al. (1966)	RT	Y	0.25	280
Okamoto et al. (1981)	RT	N	0.21	50
Okamoto et al. (1981)		Y	0.16	40
Costes and Couderc (1988)	RT		0.06	100
Wu and Patterson (1989)	RT	Y	–	22
Zhou and Kresta (1996a, b)	A310	Y	0.652	46
Zhou and Kresta (1996a, b)	PBT	Y	0.652	120
Zhou and Kresta (1996a, b)	HE3	Y	0.484	180
Zhou and Kresta (1996a, 1996b)	RT	Y	0.652	140
Mollet et al. (2004)	RT	N	~0.1	200

RT: Rushton turbine, *A310: Fluidfoil turbine*, *PBT*: pitched blade turbine, *HE3: high-efficiency axial flow impeller*

cells from microcarriers when the suspended microcarriers are passed through a flow contraction device (Ma et al. 2002). In addition, they reported that the levels of laminar shear stress (N/m^2) that begins to metabolically effect animal cells corresponds to similar levels of EDR as reported to remove CHO cells from microcarriers.

In contrast to anchorage dependent animal cells, a large number of studies indicate that suspended animal cells can withstand, and in fact, thrive, and produce functional products at significantly higher agitation than hydrodynamic conditions that remove cells from microcarriers. For example, Oh et al. (1989, 1992) reported cell growth, viability, and antibody production was similar under different agitation speeds from 100 to 450 rpm on three hybridoma cell lines (anchorage independent cell lines) as long as surface aeration was used to avoid bubble entrainment and subsequent rupture of bubbles at the gas-medium interface. In another study, the apparent cell growth rate did not change with the increase of agitation speed up to 600 rpm in a fully-filled bioreactor (Kunas and Papoutsakis 1990). In this system, no bubble ruptures were observed since there was no head space and the small bubbles present just flowed with the flow at this high agitation speed. Consistent with these previous observations, Zhang and Thomas (1993) reported the viability of hybridoma cells did not drop even at 1,500 rpm in a baffled 2 L bioreactor with a Rushton turbine and without an air/liquid interface present. Kioukia et al. (1996) studied the effect of agitation on the insect cell line, Sf9 noting only a small difference in cell growth at elevated agitation rates, and attributed this change due to bubble entrainment when agitation speed increased from 100 to 400 rpm; however, no change in viral infections was observed as agitation was increased. With respect to the primary industrial animal cell type, CHOs, numerous industrial practioners have noted that CHO cells can also withstand elevated agitation rates as long as bubbles are not entrained. Li et al. (2006) published a study on the cell growth and viability of an industrial CHO cell line at different agitation speeds, with the presence of sparging aeration, in order to develop a valid scale-down model for a 2,000 L commercial process. They reported that 350 rpm in a 2 L bioreactor, which has the equivalent "power per volume" ratio as a specific 2,000 L vessel at Li's organization, had a similar performance. Upon further increase in agitation speeds in the 2 L system, in the range of 450–550 rpm, negative effect on cell growth and viability after day 5 began to emerge.

However, cell damage can be observed as low as 200 rpm in an open system when the entrainment of bubbles resulting from the instability of the center vortex occurs (Kioukia et al. 1992). The formation of this central vortex is the result of the rotating impeller, and occurs at low rpms depending on the system geometry and volume of the cell suspension. To prevent this low rpm induction of the central vortex, the installation of baffles is recommended. However, many bioreactors typically used are not equipped with baffles. Consequently, Markopoulos and Kontogeorgaki (1995) correlated the vortex depth to several parameters such as impeller type, diameter, speed, and tank diameter. Assirelli et al. (2008) confirmed the correlation and suggested a broad application with respect to mixing in unbaffled vessels.

With respect to the effect that baffles have on EDR, Vakili and Esfahanny (2009) conducted a CFD simulation with a three-compartment model to study turbulence at the impeller, baffle, and bulk zone. Their results showed that EDR in the baffle zone is lower than that in impeller zone in terms of mean and maximum values. An increase in baffle width causes a decrease of EDR in the baffle zone due to the low tangential velocity at baffle edge. While, historically, many animal cell bioreactors have not had baffles, one reason being the concern for increased potential to damage cells from shear stress, the above results, taken together with the general knowledge in the mixing community for the positive attributes of the presence of baffles, indicates that if possible, they should always be used.

Cell damage from sparging

Bubble-associated cell damage has been extensively studied in animal cell cultures. It is generally accepted that most of the cell damage from gas sparging occurs in the bubble disengagement region at the liquid surface. This damage results from either the actual rupture of an individual bubble, with cells attached, or cells in the foam layer being damaged. With respect to bubble rupture, the process can briefly be described: a bubble rises beyond the

horizontal level of a static liquid surface, the upper liquid film thins until a hole forms in the dome and the bubble ruptures. From this breaking point, the fluid in the film rolls up into a toroidal ring and rapidly retreats toward the bulk liquid. This accelerating toroidal ring flows to the bottom of the bubble; the resulting collision creates an upward and downward jet of fluid (Fig. 1a). This process has been mathematically modeled (Boulton-Stone and Blake 1993; Garcia-Briones et al. 1994), and from these models, the maximum EDR present in this process was calculated (Fig. 1b). The maximum EDR significantly increases with the decreasing of bubble size, reaching 10^7–10^9 W/m^3 when bubbles of 1–2 mm in diameter (Fig. 2) rupture. This maximum EDR is orders of magnitude higher than that generated from normal agitation in bioreactors. Also, a corresponding Kolmogoroff microscale can be calculated, and the value of this microscale falls into the range of the diameter of freely suspended animal cells (Chisti 2000). Consistent with these high values of EDR, Oh et al. (1992) reported negative impact of sparging on hybridoma cells at 0.02 vvm. For Sf9 cells, sparge

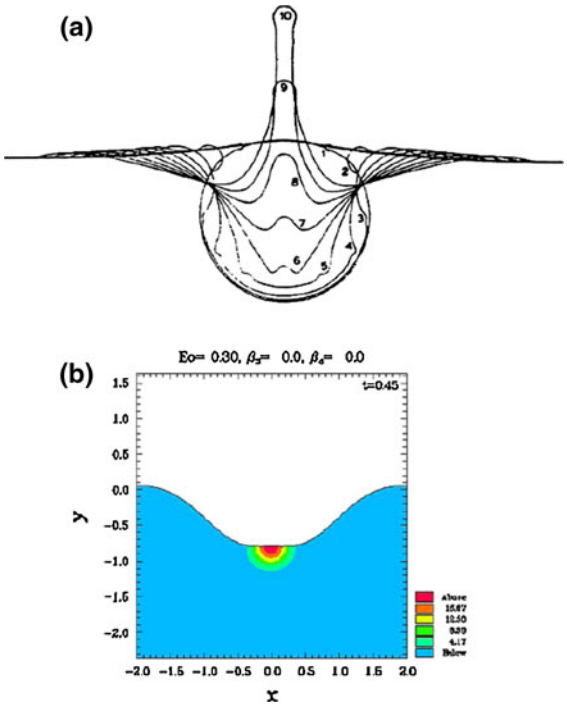

Fig. 1 Bubble rupture (**a**) a diagram of whole process (Macintyre 1972) (**b**) hydrodynamic force distribution before the start of upward jet from a mathematical model (Boulton-Stone and Blake 1993)

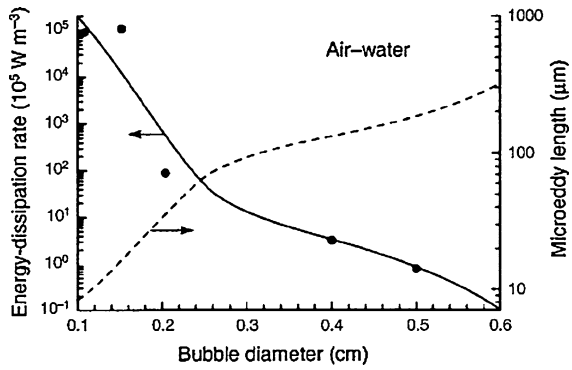

Fig. 2 The effect of bubble diameter on maximum EDR during bubble rupture and corresponding micro-eddy length (Chisti 2000, adapted from Boulton-Stone and Blake 1993)

rates as low as 0.007 vvm already showed detrimental effect on cell growth as well as viral titer (Kioukia et al. 1996).

To further quantify the bubble rupture process, Trinh et al. (1994) conducted a well controlled experiment in which the number of cells killed per bubble rupture could be determined, in a statistically significant manner. Using an insect culture with approximately 1×10^6 cells/mL, grown in medium containing FBS, but not Pluronic F-68, on average 1,050 cells were killed per 3.5 mm bubble rupture. Further, for some of the ruptured bubbles, the upward drop ejected was recovered and microscope investigation indicated that the majority of cells in this jet were dead, based on viability stains. The cells damaged during bubble rupture consist of three parts: (1) cells in suspension near the bubble; (2) cells trapped in (on) the bubble lamella, and (3) cells attached to bubbles themselves, which was microscopically observed by Bavarian et al. (1991).

A variety of medium additives, such as Pluronic F-68 (PF-68), polyvinyl alcohol (PVA), polyethylene glycol (PEG), dextran, and methylcellulose, have been tested to alleviate the bubble-associated cell damage (Murhammer and Goochee 1990; Michaels and Papoutsakis 1991; Michaels et al. 1992; van der Pol et al. 1995). PF-68, an amphiphilic triblock copolymer consisting of poly(propylene oxide) center and two poly(ethylene oxide) tails, has been the primary additive used industrially to protect cells from damage. It should be added that the original selection of PF-68 was an empirical process (Swim and Parker 1960).

A number of mechanisms for the protective effects of PF-68 have been proposed which can be categorized into two groups: biological and physical. Biological mechanism includes the interaction between PF-68 and cell membrane. Early reports suggested that PF-68 reduced the plasma-membrane fluidity and increased the shear-resistant capability of the cell, in a manner similar to cholesterol (Ramirez and Mutharasan 1990). In a related report, PF-68 was reported to increase membrane strength, as measured by micromanipulation (Zhang et al. 1992). Palomares et al. (2000) reported that PF-68 can change the physiology of Sf9 cells in terms of recombinant baculovirus and protein production, indicating the physical interaction between PF-68 and cells. In contrast, Niagam (2006) suggested that PF-68 was completely excluded from model lipid monolayers, based on surface pressures measurements. These model lipid monolayers consisted of two common phosphatidylcholine membrane lipids, DPPC and POPC, in a device used to carefully measure interfacial surface pressures. While PF-68 did not penetrate a two lipid system, it easy penetrated a pure DPPC monolayer. The effect of cholesterol on PF-68 penetration behavior was complicated and depends on surface pressure, the ratio of cholesterol to phospholipids, and most probably other variables. Considering the complexity of actual cell membranes and typical cell culture media, these results with pure systems indicate that no simple assumptions on the role of PF-68 actually becoming part of a cell membrane can be made.

Adding further complexity to the question of the actual interaction of PF-68 with cells, Gigout et al. (2008) utilized a fluorescent derivative to show that PF-68 is taken up by CHO cells up to 12 μg/10^6 cells. This amount of uptake does not vary in static versus agitated culture, and did not vary as a function of PF-68 concentration in the culture. Further, it was demonstrated that intracellular fluorescent PF-68 can be cleared by CHO cells in 7 days. Based on this uptake and a concentration of 10×10^6 cells/mL, a total of 0.12 g/L PF-68 will enter cells. This might explain why the protective effect of Pluronic F-68 can be limited under high cell concentration and or extended runs (Ma et al. 2004). In addition, the PF-68 uptake rate is cell line dependant, and can increase 4–5 fold in chondrocytes compared to CHO cells.

From a purely physical point of view, PF-68 clearly lowers the interfacial tension of cell culture media (Chattopadhyay et al. 1995a), which has at least two effects with respect to cell damage. According to the Young–Laplace equation, the pressure difference across the bubble boundary is proportional to the surface tension. Given that the driving force of the bubble rupture process is a pressure difference, lowering the surface tension lowers the driving force which lowers the overall EDR associated with the bubble rupture. Lowering the surface tension of the gas–liquid interface also makes the attachment of cells to gas-medium interfaces thermodynamically unfavorable (Chattopadhyay et al. 1995b). Michaels et al. (1995) reported that PF-68 can significantly increase the induction time required before a cell-to-bubble attachment can occur to 5–20 s. This led Meier et al. (1999) to create a mathematical model simulating the process of cells following streamline flows around a sphere to investigate the role of cell-to-bubble attachment in the total bubble-associated cell damage and reconcile some contradictory observations in the literature. It was found that the cell attachment to rising bubbles plays an important role in cell death with the increase of reactor height, especially without the presence of protective additives. Furthermore, the impact of cell-to-bubble attachment is less important when large bubbles are present than in the microbubble case (<100 μm) due to the significant difference in terminal rise velocities of large and small bubbles (Fan and Tsuchiya 1990). A lower surface to volume ratio thus reducing area for cell attachment relative to the total amount of air introduced most probably also contributes to the lesser impact of large bubbles.

Even though PF-68 provides significant protection in most cases, there is still a concern about its effectiveness at high cell concentration. In a quantitative study, Ma et al. (2004) demonstrated that approximately 10^3 cells per bubble were still entrapped in the foam layer when the cell concentration was 10^7 cells/mL and the PF-68 concentration was as high as 1 g/L (Fig. 3). Given the typical rate of sparging, especially at high cell concentrations, such cell loss per bubble can be significant. It is suggested that the mechanism of cell damage in this situation would result predominately from cells trapped in the foam layer, not necessarily cells specifically attached to rupturing bubbles. An

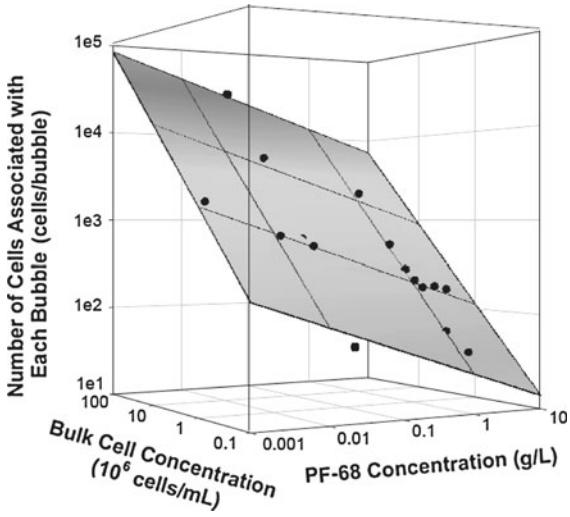

Fig. 3 Effect of bulk cell and PF-68 concentration on the number of cells associated with each bubble in the foam (Ma et al. 2004)

increase in PF-68 concentration may be needed to further lower the number of cells associated with a bubble.

In an attempt to find other protective additives, Hu et al. (2008) conducted a screening of small-molecule surfactants. Compared to PF-68 which consists of a block copolymer of relatively complex structure, small-molecule surfactants can rapidly lower the dynamic surface tension. Using the results of a number of sugar surfactants that show promise in drug delivery formulations, several small molecule, sugar based surfactants, were screened for the potential to prevent cell-bubble attachment. Of the ones tested, n-Nonyl-β-D-Maltopyranoside was demonstrated to be the most effective.

Beside the bubble disengagement and/or foam layer, cell damage at bubble formation site has been suggested (Murhammer and Goochee 1990). Recently, Zhu et al. (2008) reported that NS0 cell damage at the sparger site followed first-order kinetics when the gas-entrance velocity was higher than 30 m/s. This observation was achieved in an 8 L spinner by changing the number of nozzles, nozzle diameter, and total aeration rate. A similar phenomenon was also reported in microalgae cases with critical gas-entrance velocity around 30–50 m/s (Barbosa et al. 2004). None of the additives was reported to be able to alleviate this damage. It is still not clear how the cells get damaged at the sparger site; the turbulence caused by air jet or other flow pattern out of nozzle at high gas entrance velocity are potential cause(s).

Cell damage in downstream equipment and other bioprocess equipment

While lethal cell damage to typically used animal cells in commercial scale bioreactors has been demonstrated to be preventable, hydrodynamic forces in other bioprocess equipment can cause inhibitory effects. In particular, in downstream process units, sufficient cell damage can occur such that undesired material released from the cells can affect product quality and overall productivity (Kao et al. 2010). In addition, this material can further complicate purification steps.

Different model systems have been used to emulate shear stress conditions in bioprocess equipment. Computer Fluid Dynamics (CFD) was used by Boychyn et al. (2001) to estimate values of energy dissipation rate in the feed zone of an industrial multichamber-bowl centrifuge. Simulation results indicated that the greatest energy dissipation rates are concentrated around the disc region of the feed distributor and along the base and exit of the inner chamber. In addition, the same authors developed a scale down model to study centrifuge performance that includes a rotating disc shear device to mimic critical zones with maximum EDR and subsequent clarification with a laboratory centrifuge. Complementing this simulation approach, a rotating device was used by Levy et al. (1999a, b) and later applied by others for the evaluation of harvesting and clarification stages of mammalian cell culture broths (Boychyn et al. 2000, 2001, 2004; Hutchinson et al. 2006). Some of these studies used the rotating device to reproduce the feed zone of the industrial scale centrifuges as well clarification of the broth at industrial scale in a disc stack centrifuge with different feed configurations. In terms of efficiency the authors claim that the level of fine solids is increased in the supernatant as a result of exposing broth to high shear stress in the rotating device. This increased level of fine solids reduce the filterability of the process and could cause fouling in the chromatographic steps. While their results indicate that there is no change in the level of product, they made clear

that the cell concentration is lower than industrial cell concentrations at the harvest stage. At low cell concentration cell disruption and additional released material might be not significant, but could become a problem at higher concentrations. In addition, protein analysis of the product indicated that no structural changes occurred that could alter the charge profile during the large-scale, industrial clarification. In contrast, Kao et al. (2010) did demonstrate, at least for one therapeutic antibody produced in a CHO line, that centrifugation and filtration steps in the harvesting process resulted in significant cell lysis sufficient to release intracellular enzymes that resulted in an extensive reduction of inter-chain disulfide bonds in the antibody product. This reduction was sufficient to make the product unusable.

Continuing a quantitative approach to understanding the effect of hydrodynamic forces in downstream processing equipment on animal cells, Westoby et al. (2011) used EDR measurements in a capillary shear device to model industrial scale continuous-flow centrifugation. They reported that exposing cells to a EDR value of 3×10^4 W/kg (3×10^7 W/m^3) was a critical EDR value at which they could mimic the compromised performance of the large scale centrifuges.

Shear damage to suspended CHO cells during filtration was modeled (Vickroy et al. 2007). This model was subsequently validated with experiments using capillary as well as filtration units. Results indicated that there are critical shear stress values that lead to cell death when the exposure time is sufficiently long.

While not a routine downstream unit, fluorescent activated cell sorting (FACS) systems are a commonly used technology for selection of specific cell types (subsets) and, in some organizations, is used for clone selection. Mollet et al. (2008) experimentally, and through CFD simulations, demonstrated that the EDR in the nozzle of FACS is sufficient to damage a number of different cell lines, including CHO cells.

Table 2 and Fig. 4 summaries these, and other studies, of downstream equipment with respect to the estimates of the shear stress or EDR created. If no EDR was given, for comparison purposes, the reported values of shear stress were converted to energy dissipation rate using Eqs. 5, 6 and 7:

$$\tau = \mu * \gamma \tag{5}$$

$$\varepsilon = \tau^2/\mu \tag{6}$$

$$\varepsilon = \eta * \gamma^2 \tag{7}$$

Table 2 Devices and equipment used in shear stress studies for downstream processing

Device	Shear rate, s^{-1}	EDR, W/Kg	EDR, W/m^3	References
Rotating disc shear device, 2,000 rpm	3.00E + 04	1.50E + 03	1.50E + 06	McCoy and Hoare (2009)
Rotating disc shear device, 3,000 rpm	5.70E + 04	5.50E + 03	5.49E + 06	McCoy and Hoare (2009)
Rotating disc shear device, 4,000 rpm	5.70E + 04	5.50E + 03	5.49E + 06	McCoy and Hoare (2009)
Pilot multichamber-bowl centrifuge, 167 rps		6.00E + 05	5.99E + 08	Boychyn et al. 2001
Pilot multichamber-bowl centrifuge, 255 rps		1.40E + 06		Boychyn et al. 2001
Disc stack centrifuge		5.00E + 04	4.99E + 07	Neal et al. (2003)
Batch laboratory-scale centrifuge		1.95E + 04	1.95E + 07	Hutchinson et al. (2006)
Capillary rheometer	1.40E + 05			Levy et al. (1999a, b)
	3.50E + 05			
	4.80E + 05			
Rotating disc shear device, 27,000 rpm	1.00E + 07	4.00E + 05		Levy et al. (1999a, b)
Capillary	1.00E + 04		1.00E + 05	Vickroy et al. (2007)
	5.20E + 05		2.70E + 05	
	1.04E + 05		1.08E + 05	
	2.07E + 05		4.28E + 07	
	3.11E + 05		9.67E + 07	
Channels Filtration system (wall)	1.10E + 04		1.21E + 05	Vickroy et al. (2007)

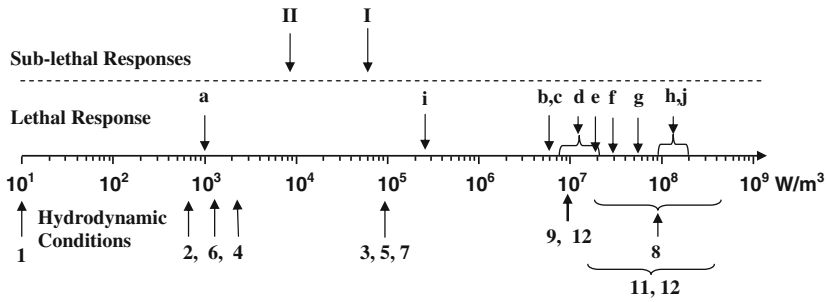

Fig. 4 Summary of the reported energy dissipation rate at which cells are damaged as well as the reported levels of energy dissipation rate in various bioprocess environments

Sub-lethal Physiological Responses Critical to Bioprocesses

Symbol	Cell	Mode of growth	Mode of test	Response	Reference
I	CHO	Suspended	10 days repetitive exposure	Recombinant protein Glycosylation profile change	Godoy-Silva et al. 2009b
II	LnCap	Attached	Time exposure to well defined shear stress	Membrane integrity failure, change in receptor number	McCoy et al. 2010

Lethal Responses (necrosis including LDH release)

Symbol	Cell	Mode of growth	Reference
a	CHO-K1	Anchorage for growth and test	Gregoriades (2000)
b	PER.C6	Suspended (naïve or adenovirus infected)	Ma (2002)
c	CHO (GS)	Suspended	Godoy et al. 2009a
d	Hybridoma	Suspended	Thomas et al. (1994); Zhang et al. (1993)
e	MCF-7	Suspended	Ma et al. (2002)
f	Mouse myeloma	Suspended	McQueen and Bailey (1989)
g	Hela S3, mouse L929	Suspended	Augenstein et al. (1971)
h	CHO-K1, Hybridoma HB-24	Suspended	Ma et al. (2002)
i	CHO-K1, apoptosis	Anchoraged for growth, suspended during test	Mollet et al. (2007)
j	CHO-K1	Suspended (wild type and bcl2 transfected)	Mollet et al. (2007)

Hydrodynamic Conditions

Symbol	Process	Description	Reference
1	Agitation	Volume average energy dissipation rate in typical animal cell culture bioreactors	Varley and Birch (1999)
2	Agitation	Volume average energy dissipation rate in a 10L mixing vessel (Rushton Turbine impeller, 700 RPM)	Zhou and Kresta (1996a)
3	Agitation	Maximum local energy dissipation rate in a 10L mixing vessel (Rushton Turbine impeller, 700 RPM)	Zhou and Kresta (1996a)
4	Agitation	Volume average energy dissipation rate in a 22,000L fermentor (Rushton Turbine impeller, 140 RPM)	Wernersson and Tragardh (1999)
5	Agitation	Maximum local energy dissipation rate in a 22,000L fermentor (Rushton Turbine impeller, 140 RPM)	Wernersson and Tragardh (1999)
6	Agitation	Maximum local energy dissipation rate in a spinner vessel	Venkat et al. (1996)
7	Bubble rupture	Pure water (bubble diameter: 6.32 mm)	Garcia-Briones et al. (1994)
8	Bubble rupture	Pure water (bubble diameter: 1.7 mm)	Boulton-Stone and Blake (1993) Garcia-Briones et al. (1994)
9	Membrane filtration	CHO suspension pumped through Millipore Membrane and capillary tubes	Vickroy et al. (2007)
10	FACS	CHO cell damage sorted through a FACS	Mollet et al. (2008)
11	Centrifugation	Bowl and disk centrifuge	Boychyn et al. 2001; Neal et al. 2002; Hutchinson et al. 2005
12	Capillary	Scale-down of industrial continuous centrifuge	Westoby et al. 2011

where: ε: energy dissipation rate (W/m^3), τ: shear stress (N/m^2), μ: dynamic viscosity (N*s/m^2), γ: shear rate (s^{-1}), η: kinematic viscosity (m^2/s)

Sub-lethal effect

Compared to lethal cell damage by hydrodynamic forces, sub-lethal effects of hydrodynamic forces in suspended culture are more difficult to identify, and conclusively prove. In contrast, endothelial cells (EC), which in their native state line the walls of arteries and veins, have been extensively studied, and a large number of non-lethal effects from hydrodynamic forces have been well documented. While a summary of all of the EC response to these forces is beyond the scope of this review, the responses of EC to shear stress include rearrangement of cytoskeleton, activation of molecules at the luminal cell surface such as kinases, integrins, and G proteins, and up and

down regulation of a number of genes and signaling cascades (Li et al. 2005). It is hypothesized that a number of these mechanisms might also be applicable to cells cultured in suspension. A number of reported sub-lethal effects potentially relevant to animal cells are:

(1) *Protein production* Keane et al. (2003) found that the production of human growth hormone (hGH) by anchorage-dependent CHO cells at the bottom of a well-defined flow chamber ceased when the shear stress was above 0.1 N/m^2.

(2) *Signal pathway* A significant increase of intracellular calcium concentration was found when Sf9 cells experienced force from an elevated agitation speed of a six bladed Rushton turbine (Aloi and Cherry 1996). Calcium is an important intracellular messenger, which has an effect on signal transduction, mitochondrial activity, and cytoskeletal alteration. The time dependency of calcium response was also observed due to the slow breakdown of cellular functions.

(3) *Cell membrane* The relationship between agitation intensity and the permeability of plasma membrane was demonstrated by the increased leakage of fluoresein diacetate (a positively charged dye) out of hybridoma cells at high agitation speed (Al-Rubeai et al. 1993). The fluidity of membrane lipid bilayer was also altered. It might have an impact on the substance exchange between cells and outside environments.

(4) *Glycosylation* The production of type II r-tPA, a partially glycosylated form compared to type I, increased at high shear condition (Senger and Karim 2003). Joosten and Shuler (2003) reported agitation in spinner flasks had a negative impact on glycosylation, specifically sialylation, of SEAP by *Trichoplusia ni* insect cell line. Godoy-Silva et al. (2009b) reported that the glycosylation pattern of a recombinant monoclonal antibody, produced in a CHO line, changed (i.e., decrease of G0 and increase of G1/G2 percentage) after being exposed to a EDR of 6×10^4 W/m^3 or higher, in a flow contraction device set up in a recycle loop with a traditional bioreactor.

(5) *Gene and protein expression* McDowell and Papoutsakis (1998) reported that a step increase of agitation from 80 rpm to 300–400 rpm at the middle of a 5-day cultivation of HL60 cells increased the CD13 receptor surface content and CD13 mRNA levels in a dose-dependant manner. C-fos protein, a transcriptional activator, can be induced at 25 dyn/cm^2 (2.5 N/m^2) in CHO cells (Ranjan et al. 1995).

(6) *Cell cycle* Motobu et al. (1998) found that more than 60% of CHO cells, which grew at the bottom of a flow chamber, were arrested in the G0/G1 phase after exposure to shear stress of 0.02 or 0.082 N/m^2 for 24 h.

Quantitative relationship of EDR to animal cells

The complicated turbulent flow and wide range of hydrodynamic forces (magnitude and elapsed time) inheritably present in a sparged and agitated bioreactor prevents using a simple, straightforward relationship relating cell response to constant, laminar shear stress and a specific condition(s) in a bioreactor. Nevertheless, a variety of tools and methods have been used to begin to quantify the sensitivity of animal cells, such as several types of rheometers (Joshi et al. 1996), capillary tube (Thomas et al. 1994), flow chamber (Keane et al. 2003), micromanipulation (Zhang et al. 1992), and atomic force microscopy (Hoh and Schoenenberger 1994).

More recently, a flow contraction, microfluidic device has been developed and applied to suspended animal cells. The concept of the device originated from a study of polymer degradation in a rapid, transient, extensional and shear flow device (Clay and Koelling 1997). The elongation forces in an extensional flow has been suggested to be more damaging to cells than a pure shear flow at the same level of energy dissipation rate (Croughan et al. 1989; Garcia-Briones and Chalmers 1994). Gregoriades et al. (2000) directly applied the flow contraction apparatus (two opposed pistons separated by an orifice) in evaluating the damage of CHO cells attached to Cytodex 3 microcarriers (175 μm). In this specific system, an abrupt contraction existed that created the high—extensional flow region followed by a zone of high shear stress.

An improved device was developed by Ma et al. (2002) in which a more gradual contraction–expansion was created through photolithographic technology. Mollet et al. (2007) further improved the system by creating the channel in a stainless steel sheet cut by wire electrical discharge machining technology. The specific design, facilitated by ultra-high resolution computation fluid dynamics, CFD, simulations (node locations separated by only a few microns in the contraction region) allowed highly accurate estimates of EDR to be made using Eq. 1. These CFD simulations indicate that comparable magnitudes of EDR are created by extensional flow in the entrance zone as well as by shear flow in the constricted flow zone. Comparisons of computer simulation and experimental, flow visualizations studies indicate the accuracy of these simulations, Mollet et al. (2007).

Using these microfluidic, flow contraction devices, 9 different types of cells, including industrially relevant CHO and insect cell lines, have been characterized with respect to what level of a rapid, single, high level exposure of EDR (as a result of shear and elongation forces) is needed to significantly damage/lysis the cells. The results of these studies, along with the levels of EDR reported in a variety of bioprocess equipment under different operating conditions are presented in Fig. 4. It should be noted that, within an order of magnitude, the level of EDR needed to damage suspended cells using the capillary tube and the microfluidic, flow contraction device, is the same as the level of EDR associated with bubble ruptures. In addition, the level of EDR needed to remove a CHO cell from a microcarrier as determined by the flow contraction device is comparable to the EDR in a spinner vessel operated at a rpm shown to remove CHO cells from microcarriers.

A criticism of these microfluidic, flow contraction device studies cited above is that the cells only experience a single pass through the microfluidic device, while in a typical bioprocess a cell will be subjected to multiple exposures of a range of EDR. To begin to address this, Godoy-Silva et al. (2009a) connected the microfluidic, flow contraction device to a bioreactor through a recycle loop and pump. This allows the evaluation of long-term effects of repeated exposure to transient values of high EDR, while still maintaining the cells under controlled culture conditions, such as DO, pH, and temperature. As might be expected, cell growth of a CHO K1 cell line (obtained from ATCC) was significantly inhibited at EDR values from approximately 1 to 2 orders of magnitude less than the values of EDR that cause cell rupture after a single pass. However, specific cell metabolism, as measured by glucose uptake and lactate production, did not change. More recently, Godoy-Silva et al. (2009b) used this recycle system on two industrial CHO GS cell lines producing two different recombinant antibodies. Unlike the CHO K1 cell line, the CHO GS cell line showed no growth or metabolic inhibition in response to multiple exposures to EDR in the microfludic flow contraction device until levels of EDR exposure approached the levels demonstrated to lysis cells in a single pass. However, while most measures of antibody product quality did not change with elevated, repeated exposure of EDR, a level of repeated exposures of EDR of 6.0×10^4 W/m^3 resulted in a change in glycosylation pattern of antibodies produced in two different CHO GS clones (Godoy-Silva et al. 2009b) (presented in Fig. 4). It should be noted that this level of EDR is still significantly higher than typical operating conditions in large scale, commercial bioreactors.

Beyond animal cells typically used in commercial applications, (i.e., CHO, insect cells, BHK, etc.), experimental data are beginning to emerge that other animal cells, typically coming out of medical research labs, can be significantly more sensitive to hydrodynamic forces than the typically used cell lines. For example, in 1998 McDowell and Papoutsakis reported that HL-60 cells (a human leukemic cell line) can respond metabolically and with respect to the number of cell surface receptor markers in response to changes in typical agitation rates in bench top bioreactors. More recently, Mollet et al. (2008) demonstrated that a different human leukemic cell line (THP1) is approximately two order of magnitude more sensitive to EDR than CHO cells, as measured in the flow contraction device described previously. McCoy et al. (2010) has most recently demonstrated that other human cancer cell lines, in this case a prostate derived line, LnCap, was also quantitatively more sensitive to cell lysis and non-lethal, cell surface marker receptor changes were observed in response to hydrodynamic forces.

Implications and challenges

Historically, suspended animal cell culture has been considered a process with relatively low requirements of mass transfer, and correspondingly, mixing. However, this situation is methodically changing as both the productivities and the general efficiencies of animal cell culture continue to significantly improve. *If the past rates of improvements in the productivity of commercial processes continues, the required mass transfer and mixing requirements will correspondingly increase, potentially substaintially.* For example, Huang et al. (2010) recently reported achieving product titers of 13 g/L in a 18 day culture that reached greater than 2×10^7 cells/mL. Even more striking, they reported that peak oxygen uptake rate tripled from baseline, and continued to increase after achieving maximum cell densities; a maximum oxygen uptake rate of 3.7 mmol/L/h was reported. These types of improvements will require increased mixing and aeration in bioreactors. Fortunately, with respect to mechanical agitation, both practical experience and the data summarized in Fig. 4 indicate that there is still room for improvement in mixing by an increase in agitation, from a hydrodynamic damage viewpoint. Increase in mixing also helps to eliminate heterogeneous distribution of pH (Langheinrich and Nienow 1999) and DO (Xing et al. 2009). See also: Nienow (2006) for a more complete discussion of current thinking with respect to large scale animal cell bioreactors.

In terms of aeration, and the removal of CO_2, the detrimental effect of bubble rupture and foam formation will potentially increase as culture densities increase. One potential solution is to microsparge pure oxygen in large scale systems. The bubbles will disappear before they even reach the top surface, minimizing foaming and bubble rupture. However, dissolved CO_2 removal becomes a serious problem due to lack of stripping in this case. A number of examples exist indicating the direct or indirect inhibition of productivity due to an accumulation of dissolved CO_2 (Mostafa and Gu 2003; Zhu et al. 2005). Somewhat paradoxically, as one continues to scale-up, CO_2 removal becomes more of a concern than oxygen addition, and larger bubbles become more advantageous for there capability in stripping, and transporting, the CO_2 out of the vessel. However, large bubble has less capability to supply oxygen. With the increase of cell density, the task to balance oxygen supplement and dissolved CO_2 removal while minimizing bubble-associated cell damage most probably will become more challenging. A potential operating scheme to address this would be to decouple O_2 addition and CO_2 removal. Such a decoupling could be achieved through independent microsparging of pure O_2, and large bubble air addition for CO_2 removal. This operating scheme has been informally discussed and presented at professional societies and allegedly used in an industrial setting. However, no official report exists with respect to the current authors' knowledge, nor have optimization studies been conducted. Therefore, it remains to be seen if such a scenario is viable and worth the added complexity.

Beyond the cultivation of animal cells in bioreactors, challenges of cell damage as a result of hydrodynamic forces in downstream processing have already been encountered. For example, Trexler-Schmidt et al. (2010) reported on a product failing to meet drug substance specification. Subsequent studies indicated that the product was damaged by cell enzymes released as a result of cell lysis during harvesting operations. While they were able to solve the problem, this process had previously worked without any detectable detrimental problems with other cell clones. This experience underscores the cell line variability as well as the potential upper limit in hydrodynamic forces to which suspended animal cells can be successfully subjected. Obviously, further research is needed to not only further quantify the hydrodynamic conditions within downstream processing equipment, but improved designs are needed that can reduce the zone in which high levels are achieved.

Finally, there is still a lack of understanding of the response of cells to hydrodynamic forces at the sub-cellular and/or molecular level. While the great commercial success of animal cell culture indicates that, in general, CHO cells are robust and not significantly inhibited by current operating conditions, as these systems continued to be pushed for greater performance, non-lethal, negative effects from hydrodynamic forces could potentially emerge. In addition, while it is generally accepted that CHO, insect cells, and hybridoma cells are robust, as is beginning to be quantitatively demonstrated, other cell lines are not as robust. It is highly likely that

the commercial success of large scale animal cell culture is the result of the use of a highly robust CHO line. As the field progresses, other cell lines could potentially emerge as viable candidates as hosts and the ability to rapidly and effectively screen them for large scale, highly productive hosts is needed. Therefore, the further, systematic characterization of animal cells response to hydrodynamic forces, and more fundamental understanding and characterization of the hydrodynamic in bioprocesses is needed.

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