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Mixing, aeration and cell damage, 30+ years later: what we learned, how it affected the cell culture industry and what we would like to know more about

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Greater than $100 billion in sales of human biopharmaceuticals are produced in large scale, animal cell culture in stirred tank bioreactors. Despite initial and continuing concerns of the ''shear sensitivity'' of animal cells, over the last 30 years, incredible advances have been made in the productivity of suspended animal cells to produce biopharmaceuticals, from mg/L of product to over 5 g/L. In this contribution, a summary of the current state of this ''shear sensitivity'' concerns will be discussed, demonstrating that it is not in general a problem with current bioprocesses. Examples of what is considered the current limits above which effects of hydrodynamic and interfacial phenomena become a concern and begin to negatively impact the cells and the bioprocess will be presented.

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The great success of commercial animal cell culture

While an early example of the industrial exploitation of animal cell cultures started over six decades ago with the production of the Salk polio virus vaccine in primary monkey kidney cells [1], it has been during the last 30 year period that the commercial use of animal cells to produce human biopharmaceuticals has dramatically increased. The primary reason for this growth was the realization that animal cells were needed to produce properly folded and post-translationally processed proteins, One recent report estimates that over 140 human biopharmaceutical made in animal cell culture were approved by the EU and US regulatory agencies between 1989 and April of 2014. This report estimates that the global biopharmaceutical market to be approximately 200 billion dollars in 2013, with a projected growth to 500 billion dollars by 2020. In 2012, 58% of the US and EU approved human biopharmaceutical were produced in animal cells, and in 2013, 8 of the ten top selling biopharmaceuticals were made in animal cells. A more complete discussion and a list of these biopharmaceuticals can be found in [2•].

This incredible success can be attributed to a number of factors, not the least of which is the productivity achieved in large scale, animal cell cultures. One of the first therapeutic monoclonal antibodies approved for human use in 1986, OrthoClone OKT3, only obtained a concentration during production in the range of 1–10 mg/mL [3] in the ascites of mice, while suspended animal culture only obtained concentrations on the order of 50 mg/L [4]. In contrast, it is routinely reported that concentrations on the order of 2–5 g/L in fed batch culture can now be obtained, and when a longer term perfusion culture is used, production of concentrations up to 25 g/L has been reported [5,6].

Ironically, this success has been achieved while a number of the fundamental areas of knowledge related to scaling up of these processes (i.e. mixing, aeration and cell damage) lacks theoretical, 'first principle' relationships. While successful, the actual scale-up and execution of these industrial processes have been based heavily on historical, empirical evidence, empirical correlations and 'rules of thumb.' This lack of first principle understanding is underscored by a quote of the Nobel Laureate, Richard Feynman who described turbulence as 'the most important unsolved problem of classical physics.' A similar statement can be made with respect to understanding the complex interactions of all of the hydrophilic and hydrophobic compounds that make up the animal cell culture suspension.

Despite reports showing that animal cell culture has been conducted for over a hundred years [7], the traditional, tried and true, stirred tank, or stirred tank bioreactor, *STB*, is, and continues to be, the primary method of large scale culture of animal cells. While the concern of the 'shear sensitivity' of animal cells still exists to this day, the *STB* is the preferred method of culture for a number of reasons, not the least of which is the vast empirical knowledge accumulated for the design, scale-up and operation of

STBs [8,9]. In addition to this empirical experience of STBs, another key to the current success of industrial animal cell culture was the practical development, and industrial demonstration, that several commonly used animal cell lines, most notably Chinese Hamster Ovary cells, CHO, can not only be manipulated to grow in suspension, but actually grow as well, if not better, than when attached to a surface. We will return to this attachment issue when discussing future needs.

In the early 1980s when it was realized that bacterial culture could not produce properly folded, glycosylated proteins (required for a majority of biopharmaceuticals) the only alternative was the genetic engineering of the few animal cell lines for which transfection techniques existed. Since at the time it was generally assumed that only 'transformed' cell lines can grow in suspension (not attached to a surface), and the use of 'transformed cell lines' to produce a human biopharmaceutical was considered a health risk, the only alternative for large scale culture was the use of animal cells in roller bottles or cells attached to microcarriers. However, at the time, and to this day, it was well known that too vigorous mixing can result in the removal of animal cells attached to microcarriers [10•]. To begin to quantify this removal of attached cells to microcarriers by hydrodynamic forces, Cherry and Papoutsakis [11], and Croughan *et al.* [12], correlated this removal to a Kolmogorov length scale [13,14]; this length scale was calculated from estimates of energy dissipation as a result of the mechanical stirrer. This correlation of cell damage to Kolmogorov length scale is still used to this day. During this same period, a number of institutions (both industrial and academic) developed/demonstrated that large scale, suspended (anchorage free) cell cultures of CHO cells can be achieved. Since the size of a single suspended CHO cell is more than an order of magnitude smaller than a microcarrier, this correlation of cell damage to Kolmogorov length scale implied that a significant increase in hydrodynamic (turbulent) energy release is needed before single, suspended cells are damaged. This combination of industrial demonstration of the capability of large scale suspended culture of CHO to produce a recombinant product, along with this semi-theoretical explanation using the Kolmogorov theory of turbulence, significantly improved the perception (provided the motivation/verification) for the large scale adaption of *STB* for commercial production of biopharmaceuticals produced in suspended CHO cultures.

Lack of relationships linking hydrodynamic conditions to cell damage

While the previously discussed results began to put a structure around considering the type of culturing vessels and conditions that can be used for suspended and anchorage-dependent cell lines, it left significant questions. Further, at its core, the Kolmogorov length scale calculation is based on correlations to experimental data and not derived from first principles. Independent of physical mixing with a mechanical agitator, it was also experimentally observed that suspended cell damage can still occur as a result of the addition of gas sparging to the culture used to introduce oxygen and remove carbon dioxide [15–22]. This cell damage associated with sparging was further confirmed when it was demonstrated that the omission of surface active compounds, such as the well-known surfactant, Pluronic F-68 could make the cell damage significant enough to prevent overall cell growth of suspended cultures [20,23–26].

Sparging of gas and cell damage

To further elucidate the role of sparging on suspended cell culture, Handa *et al.* [17] and Bavarian *et al.* [21] used high speed microscopic imaging to attempt to understand the interactions of cells, bubbles and surfactants, such as Pluronic F-68. Figure 1 presents images which pictorially represent the conclusion of these studies. Specifically, Figure 1a–c shows that suspended cells can adhere to rising bubbles and can be trapped in the foam layer at the top of the vessel (note these images were taken when no Pluronic F-68 was present) or on the bubble film of a bubble on the top air-medium interface, Figure 1d. Contrary to Figure 1d, when Pluronic F-68 is present, Figure 1e, no cells are attached to the air-medium interface. To determine if this qualitative observation of cells interacting with bubbles translates to actual cell death in significant numbers to account for observed cell death in cultures without Pluronic F-68, Trinh *et al.* [26] performed quantitative studies that demonstrated the detrimental effect of bubble rupture on insect cells using a specially designed bubble column which allowed a large number of 3.5 mm bubbles to be generated and ruptured, with and without the presence of the commonly used protective additive, Pluronic F-68. On average, 1050 cells were killed by each bubble rupture; conversely, when 0.1% Pluronic F-68 was present, no statistically significant cell death could be detected. Trinh *et al.* [26] further suggested that this rate of cell death per bubble could account for much of the cell damage observed in larger scale cultures.

Quantification of hydrodynamic conditions that damage cells

The results and observations presented above, as well as other published studies, led several laboratories to further investigate the hydrodynamic forces associated with bubbles, and especially bubbles breaking at the air-medium interface. Two studies, Boulton-Stone and Blake and Garcia and Chalmers [27,28], used advanced computing methods to approximate, quantitatively, the hydrodynamic forces associated with bubbles rupturing. These studies both indicated that using the scalar parameter, energy dissipation rate, *EDR* or ε (typically reported in units of W/m^3 or W/kg) for a rupturing bubble was orders