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Reactor engineering in large scale animal cell culture

Alvin W. Nienow

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Abstract This article mainly addresses the issues associated with the engineering of large-scale free suspension culture in agitated bioreactors >10,000 L because they have become the system of choice industrially. It is particularly concerned with problems that become increasingly important as the scale increases. However, very few papers have been written that are actually based on such large-scale studies and the few that do rarely address any of the issues quantitatively. Hence, it is necessary very often to extrapolate from small-scale work and this review tries to pull the two types of study together. It is shown that 'shear sensitivity' due to agitation and bursting bubbles is no longer considered a major problem. Homogeneity becomes increasingly important with respect to pH and nutrients at the largest scale and sub-surface feeding is recommended despite 'cleaning in place' concerns. There are still major questions with cell retention/recycle systems at these scales, either because of fouling, of capacity or of potential and different 'shear sensitivity' questions. Fed-batch operation gives rise to cell densities that have led to the use of oxygen and enriched air to meet oxygen demands. This strategy, in turn, gives rise to a CO_2 evolution rate that impacts on pH control, pCO_2 and osmolality. These interactions are difficult to resolve but if higher sparge and agitation intensities could be used to achieve the necessary oxygen transfer, the problem would largely disappear. Thus, the perception of 'shear sensitivity' is still impacting on the development of animal cell culture at the commercial scale. Microcarrier culture is also briefly addressed. Finally, some recommendations for bioreactor configuration and operating strategy are given.

Keywords Large-scale bioreactors · 'Shear sensitivity' · Homogeneity · pH control · Dissolved CO_2 · Osmolality

A. W. Nienow (✉)
Centre for Bioprocess Engineering, The University of Birmingham, Birmingham B15 2TT, UK
e-mail: A.W.Nienow@bham.ac.uk

Introduction

Probably the first description of an agitated, stainless steel animal cell culture bioreactor was one of 30 L for the production of inactivated foot and mouth disease vaccine from freely suspended BHK cells (Telling and Elsworth 1965); and from the late 1960s to the mid-1980s, the largest reported was one of 8 m^3 for the production of interferon from Namalwa cells (Pullen et al. 1985). However, recently, it has been reported that commercial bioreactors of 20,000 L or so have been commissioned at Lonza (Hoeks et al. 2004) and Genentech (Meier 2005). These bioreactors

and others are generally operated in the fed batch or draw and fill mode (Langheinrich et al. 1998) in order to reach high cell densities. Another approach to achieving high cell densities is to use cell retention techniques, e.g., spin filters, and the use of external recycling and cell separation to achieve retention at the industrial pilot scale has been reported by Wyeth (Moran 2004). Insect cell technology is relatively new when compared to mammalian cell technology. Therefore, there has been less demand to go to such large scales but no doubt it will come. However, this article is relevant to both cases because of the distinctly similar behaviour of these different types of cell at the small bioreactor scale (Kioukia et al. 1996).

In the main, this article assumes that a successful bioreactor protocol has been developed at the bench scale, e.g., at the 2–5 L scale and addresses the issue of what additional problems are likely to be of concern when scale up to the 10,000 + L scale is under consideration. It also addresses the issue colloquially called 'shear sensitivity', which has plagued the development of animal cell culture (the cells particularly being perceived as fragile because of the lack of a cell wall), both at the bench and the largest commercial scale, since the technology was first introduced and continues to do so. As a result, even though as early as 1965, the use of a 30 L stainless steel stirred bioreactor for successfully cultivating BHK cells was reported (Telling and Elsworth 1965), during the 1980's, many other systems were introduced such as air-lift (up to 2000 L at Lonza in the UK (Varley and Birch 1999)), hollow-fibre and circulating fluidised beds (for anchorage-dependent cells). It is now recognised that this concern for 'shear' sensitivity was excessive and the majority of industrial processes use free suspension stirred tank bioreactors, especially on the largest scales. If anchorage-dependent cells are to be utilised, then agitated bioreactors containing micro-carriers are employed. Hence, this article mainly relates to bioreactors containing one or more impellers. In addition, since much has been made, especially by manufacturers, of the use of specially designed, 'low shear' impellers, impeller selection will be considered too.

Agitator tasks in the bioreactor

The agitation system in the bioreactor provides the liquid motion that enables many different tasks to be fulfilled. It is important to understand the interaction between the fluid motion, the agitator speed and the power input into the bioreactor and these tasks. It is also necessary to know how a change of scale affects these relationships. Many of these aspects can be studied without carrying out a specific bioprocess and these physical aspects most relevant to animal cell culture are listed in Table 1. Table 2 sets out those aspects that are specific to the organism being grown and will usually be different for each case, even for organisms as similar as mammalian and insect cells.

The physical aspects in Table 1 have been discussed extensively for conditions relevant to a wide range of organisms elsewhere (Nienow 1996, 1998; Nienow and Bujalski 2004). Here, their relevance to animal cell culture will be emphasised, based on the aspects in Table 2 that are important in that case.

Special aspects of animal cell culture

General

Currently, cell densities at the commercial scale of up to about 5×10^6 ml^{-1} are being achieved

Table 1 Physical aspects of the agitation/agitator requiring consideration (Nienow 1998)

1. Mass transfer performance
2. Flow close to the agitator-single phase and air–liquid
3. Bulk fluid- and air-phase mixing
4. Air dispersion capability
5. Power draw (or mean specific energy dissipation rate, $\bar{\varepsilon}_T$ W kg^{-1})
6. Variation in local specific energy dissipation rates, ε_T W kg^{-1}
7. Heat transfer
8. Microcarrier suspension (for attached cells)

Table 2 Biological aspects that are system specific (Nienow 1998)

1. Growth and productivity
2. Nutrient and other additive requirements including oxygen
3. CO_2 evolution and RQ
4. Sensitivity to O_2 and CO_2 concentration
5. pH range and sensitivity
6. Operating temperature range
7. 'Shear sensitivity'

routinely and higher values have been indicated. This cell density produces some issues for achieving sufficient oxygen transfer (discussed in detail below) with specific oxygen demands reported for mammalian (Nienow et al. 1996) and insect cells from ~ 1×10^{-17} to 1×10^{-16} mol s^{-1} - $cell^{-1}$. However, even with this cell density, the viscosity of the cell/media suspension is close to that of water and from a 5 L bench bioreactor to the largest scale, the flow is essentially turbulent (Oh et al. 1989), i.e. Reynolds number, $Re = \rho_L ND^2/\mu > \sim 10^4$ where ρ_L is the media density (kg m^{-3}), μ is the viscosity (Pa s), D is the impeller diameter (m) and N, its speed (rev s^{-1}). Thus, turbulent flow theories can be used to analyse the fluid mechanics in the bioreactors. For example, though impeller power numbers, Po (dimensionless), depend on the agitator type, for any one type, they are essentially constant, regardless of their diameter relative to the bioreactor diameter, T (m) or their speed, N and of the bioreactor size (scale) provided geometric similarity is maintained across the scales (Nienow 1998). The power input, P (W), into the bioreactor imparted by the impeller is given by

$$P = Po\rho_L N^3 D^5 \tag{1}$$

where the mean specific energy dissipation rate, $(\bar{\varepsilon}_T)_I$ (W kg^{-1} or m^2 s^{-3}) from the impeller is the given by

$$(\bar{\varepsilon}_T)_I = P/\rho_L V \tag{2}$$

where V is the volume of medium in the reactor (m^3). The maximum local specific energy dissipation rate, $(\varepsilon_T)_{I\max}$, is close to the impeller, is very high relative to the average $(\bar{\varepsilon}_T)_I$ and depends on the agitator type. Also, the Kolmogoroff or micro-scale of turbulence, λ_K, which is often considered as an indicator of the potential for cell damage (see below for more details) is given by

$$\lambda_K = ((\varepsilon_T)_I/\nu)^{-1/4} \tag{3}$$

where $(\varepsilon_T)_I$ is the local specific energy dissipation rate and ν is the kinematic viscosity, $\approx 10^{-6}$ m^2 s^{-1} since the cell/medium suspension is water-like.

Heat transfer

The actual oxygen uptake rate (OUR) is still very low compared to bacterial and yeast fermentations. As a result, since the metabolic heat release Q (W m^{-3}) is proportional to the OUR (mol O_2 m^{-3} s^{-1}) (Van't Riet and Tramper 1991), i.e.,

$$Q \approx 4.6 \times 10^5 OUR \tag{4}$$

Thus, Q for animal cell culture is also relatively low compared to bacterial and yeast fermentations. With the latter organisms, this cooling load at the commercial scale is often a problem as Q scales with the volume of the reactor, i.e., T^3 whilst cooling surface area scales with T^2. Hence, for such systems, cooling coils are often required. However, with animal cell culture bioreactors, simple heating/cooling jackets are sufficient for good temperature control. Typical operating temperatures are cell line specific but for mammalian cells varies between 36°C and 38°C whilst for insect cells it lies between 25°C and 30°C.

Mass transfer performance and oxygen demand

Early work on growing BHK cells suggested that the optimum level of dissolved oxygen was < 50% saturation (Radlett et al. 1972) but that was probably because the reactor was unbaffled and sparged without the use of Pluronic F68 (see discussion below). More resent work indicates that the cultivation of mammalian cells generally

can be undertaken satisfactorily from 5% to 100% of air saturation (Oh et al. 1989). Insect cells have been reported to have a narrower operating range of 40–60% (Klopinger et al. 1990), though there is also some work indicating that they are similar to mammalian cells (Kioukia et al. 1996).

The overall oxygen demand of the cells throughout the cultivation (including post-infection in the case of insect cells) must be met by the oxygen transfer rate and the demand increases as long as the number of viable cells is increasing. Thus, a maximum oxygen transfer rate must be achievable and this depends on the mass transfer coefficient, k_La (s^{-1}), and the driving force for mass transfer. It has been shown many times (Nienow 2003) that k_La is only dependent on two parameters. These are, first, the total mean specific energy dissipation rate imposed on the system, $(\bar{\varepsilon}_T)_g$, made up of that (a) from air sparging, $(\bar{\varepsilon}_T)_S$, ($\approx v_s\, g$ where g is the acceleration due to gravity (9.81 $m^2\ s^{-1}$) and v_s ($m\ s^{-1}$) is the superficial air velocity (=(vvm/60)(volume of broth)/(X-sectional area of the bioreactor) and which can often be neglected) and (b) from the impeller under sparged conditions, $(\bar{\varepsilon}_T)_{Ig}$, where

$$(\bar{\varepsilon}_T)_{Ig} = Po_g N^3 D^5 / V \tag{5}$$

and Po_g is the power number under aerated conditions. Po_g is generally less than Po, and often significantly so for the aeration rates used in bacterial and mycelial fermentations (Nienow 1998). However, at the relatively very low air flow rates used in animal cell culture to date, $Po_g \approx Po$ (Langheinrich et al. 1998). Nevertheless, the relationship between Po_g and Po impacts on impeller selection (Nienow 1998; Nienow and Bujalski 2004) as discussed later. Secondly, k_La also depends directly on v_s. Thus,

$$(\bar{\varepsilon}_T)_g = (\bar{\varepsilon}_T)_S + (\bar{\varepsilon}_T)_{Ig} \tag{6}$$

$(\bar{\varepsilon}_T)_g$ and v_s must together (Langheinrich et al. 2002) be sufficient to produce the necessary k_La where

$$k_La = A(\bar{\varepsilon}_T)_g^{\alpha}(v_s)^{\beta} \tag{7}$$

This equation applies independent of the impeller type and scale and α and β are usually about 0.5 ± 0.1 whatever the liquid. On the other hand, A is extremely sensitive to composition (Nienow 2003) and the addition of antifoam which lowers k_La or salts which increase it, may lead to a 20 fold difference in k_La for the same values of $(\bar{\varepsilon}_T)_g$ and v_s. For example, Mostafa and Gu (2003) used antifoam at the 1000 L scale as part of a programme to tackle problems associated with high dissolved CO_2 levels (as discussed below) and reported at 25% fall in k_La. The addition of Pluronic F68, which is added to the medium to protect cells from damage especially due to bursting bubbles (as discussed below), also lowers k_La significantly (Nienow and Lavery 1987). However, up to the cell densities obtainable so far at the commercial scale, the oxygen demand and hence the k_La are very low compared to bacterial fermentations. Thus typical values of $(\bar{\varepsilon}_T)_g$ and v_s are of the order of 0.01 to 0.15 W kg^{-1} and 1×10^{-4} to 20×10^{-4} m s^{-1} respectively. These values cover results from 8 m^3 (Langheinrich et al. 2002) to 20 m^3 (Hoeks et al. 2004) and the latter study showed the applicability of Eq. (7) across the scales in 12 L, 500 L and 20,000 L bioreactors using animal cell culture media. Typical k_La values (Nienow et al. 1996; Langheinrich et al. 2002; Hoeks et al. 2004; Meier 2005) in animal cell culture media are in the range 1–15 h^{-1}.

During the earlier stages of a culture, lower $(\bar{\varepsilon}_T)_g$ values will suffice, so a variable speed drive motor for the impeller gives additional flexibility. Clearly, this level of agitation and aeration intensity must not significantly alter the cells ability to grow and generate the desired product (especially considering their perceived 'shear sensitivity') and in the cases referenced here, that was so. However, as discussed later, higher agitation intensities have been used on the small scale without ill-effects due to 'shear'. Thus, a variable speed motor allowing higher $(\bar{\varepsilon}_T)_{Ig}$ would enable higher oxygen demands to be met as higher cell densities are reached. Another way to do so is to increase the driving force by blending oxygen into the air or using a separate oxygen bleed into the bioreactor (which is a more

efficient way of using the oxygen because it maximises the effectiveness of the enhanced driving force). However, since the respiratory quotient for animal cells is about 1, the latter approach enhances the potential problems associated with increased levels of dissolved CO_2 and pH control which in turn may give rise to high osmolality (Meier 2005). These aspects will also be discussed below.

'Shear sensitivity'

Due to agitation alone

Because the specific oxygen demand per cell combined with the cell density so far achieved is so low, in bench scale bioreactors, the oxygen demand of the cells can be met without sparging by passing air/CO_2 or air/CO_2/O_2 mixtures through the headspace. In addition, by also introducing nitrogen gas into the flow, it is possible to control the dissolved oxygen concentration at a specific level regardless of the agitator speed or $(\bar{\varepsilon}_T)_I$ value. This technique has shown that with $(\bar{\varepsilon}_T)_I$ values from 0.01 up to 0.25 W kg^{-1} in baffled bioreactors with different impellers, including a Rushton turbine, a wide range of cells could be grown equally well. The cell lines in question were TB/C3 mouse hybridomas producing monoclonal antibodies against human IgG (Oh et al. 1989, 1992; Kioukia et al. 1992) grown between 5% to 100% of air saturation and with EBNA cells (Oh et al. 1989), a non-producing hybridoma and with HPV cells, producing antibodies against human papilloma virus (Oh et al. 1989) grown at greater than 20% dO_2. In addition, a CHO320 cell line grew and produced γ-interferon equally well with dO_2 between 20% and 80% (Nienow et al. 1996).

A popular theory of damage due to turbulence suggests that if the biological entity is smaller than λ_K as given by Eq. (3), then the entity will not be damaged. Thus, if $(\varepsilon_T)_I = (\bar{\varepsilon}_T)_I = 0.25$ W kg^{-1}, $\lambda_K = 45$ μm or assuming $(\varepsilon_T)_I = (\varepsilon_T)_{I\max} = 30(\bar{\varepsilon}_T)_I$ near the impeller for Rushton turbines based on the literature (Nienow 1998) (Fig. 1), then λ_K=19 μm. Since animal cells are of the order of 15–18 μm, the lack of 'shear damage' fits in with this concept. However, it does not prove it.

In other work by Zhang and Thomas (1993) in a closed (i.e., no air/liquid interface), baffled 2 L bioreactor agitated by a Rushton turbine at 1500 rpm ($(\bar{\varepsilon}_T)_I = 4$ W kg^{-1}) under non-culture conditions, hybridoma cells were found to remain

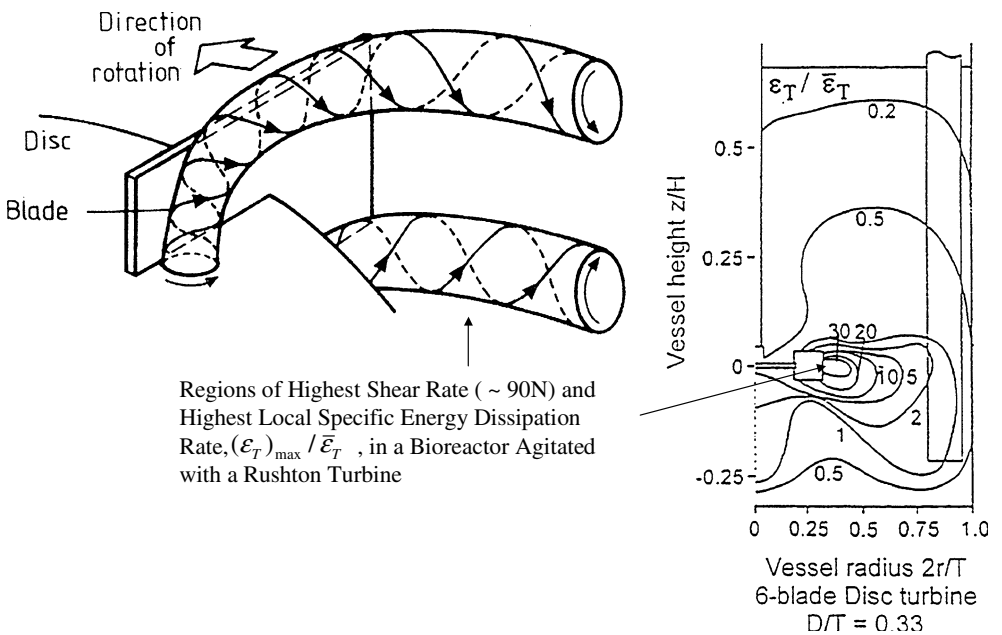

Fig. 1 A Rushton turbine and its flow characteristics (Nienow 1998)

viable. Indeed, in related studies in which TB/C3 hybridoma and NS1 myeloma cells were passed in turbulent flow through capillary tubes, mean specific energy dissipation rates as high as 10^5–10^6 W kg^{-1} were shown to be required to totally disrupt the cells (Zhang and Thomas 1993). Thus, these studies suggest that there is a strong possibility of satisfactory operation at higher $(\bar{\varepsilon}_T)_I$ values as higher cell densities are reached, leading to higher levels of oxygen demand and therefore potentially k_La.

One of the parameters which has generally been missing when trying to be more quantitative when analysing the impact of stresses due to fluid flow and turbulence on cells has been the lack of knowledge of the mechanical properties of the cells. These properties have been measured extensively by Thomas and Zhang, using micromanipulation in which cells are squeezed (see Fig. 2) (Zhang et al. 1992; Zhang and Thomas 1996). On the basis of these measurements and models developed for turbulent flow, they found good agreement between the high $(\bar{\varepsilon}_T)_I$ values that were found experimentally to be required to disrupt the cells and the model (Zhang and Thomas 1993). Again, these studies would suggest that higher levels of $(\bar{\varepsilon}_T)_I$ (and $(\varepsilon_T)_{I\max}$) might well be usable in stirred bioreactors without sparging.

In passing, because 'shear sensitivity' has been such an issue in bioprocessing, it is worth commenting briefly on another criterion that has often been invoked as a measure of this parameter, namely tip speed ($=\pi ND$) (Oh et al. 1989). Varley and Birch (1999) state ''it is now accepted that tip speed has a critical effect in terms of shear''; and that ''above a value of 1.5 m s^{-1}, it is generally thought that cell damage may begin''. However, these statements are made without any referenced work to support them. From a fluid dynamic perspective, the tip speed (m s^{-1}) does not even have the correct dimensions. For shear rate, the dimensions are s^{-1} and for shear stress, Pa (or N m^{-2}). When Oh et al. (1989) grew TB/C3 hybridomas in the laboratory at the high $(\bar{\varepsilon}_T)_I$ of 0.25 W kg^{-1}, the tip speed was still only 1.4 m s^{-1}. Indeed, low values at equal $(\bar{\varepsilon}_T)_I$ are always found on the small scale and with increasing vessel diameter at constant $(\bar{\varepsilon}_T)_I$, they increase $\propto$ (scale)$^{1/3}$, i.e., T$^{1/3}$ (Nienow 1998). Thus, maintaining tip speed <1.5 m s^{-1} is a major constraint on scale-up with severe implications for mass transfer as well as homogeneity (see below); and there is little or no evidence to support it. Hoeks et al. (2004) reported keeping $(\bar{\varepsilon}_T)_I$ constant across the scales for agitated free suspension cell culture from 5 L to 20000 L and an estimate of the tip speed at the largest scale gives values well in excess of 1.5 m s^{-1}. Retrofitting high power number impellers at constant speed (N), power and torque (Nienow 1998) with larger diameter, low power ones, as has often been done for improved mixing and air handling ability (Nienow 1998) with other organisms than animal cells, also leads to higher tip speeds. Yet, problems of mechanical damage ('shear') have not been reported to the author's knowledge. Indeed, in the only case where mechanical damage has been clearly shown to occur under 'normal' operating conditions of agitation intensity, namely with mycelial fermentations at equal $(\bar{\varepsilon}_T)_I$, that damage went down on scale-up in spite of the increase in tip speed (Amanullah et al. 2003)! On balance, tip speed does not appear to be an important parameter to consider.

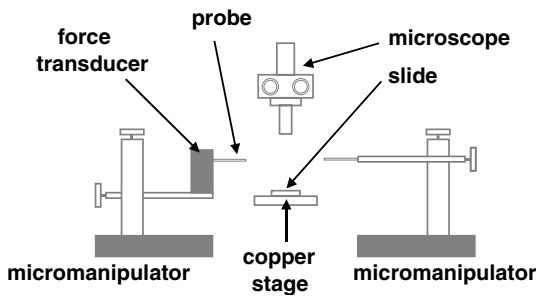

Fig. 2 Schematic diagram of micromanipulation rig for measuring cell mechanical properties (Zhang and Thomas 1996)

In the presence of sparging (and bubbles)

The bench scale studies reported above without sparging only gave sufficient oxygen transfer because of the relatively large surface area to volume ratio at this scale. In the same study, even though sparging was not essential for mass transfer purposes, it was introduced in order to

investigate its impact. Even at low agitation intensity and low rates of sparging, cells numbers were dramatically reduced (Oh et al. 1989) (Fig. 3). Similar results were reported for insect cells (Kioukia et al. 1996). However, whilst such work clearly shows that mechanical damage to cells occurs due to sparging, it does not indicate how it happens or where. Figure 4 identifies four possible regions of damage. They are: (1) bubble formation at the sparger; (2) bubble coalescence and break-up in the impeller trailing vortices; (3) during bubble rise through the bioreactor; and (4) bubble bursting at the medium–air interface.

Work in bubble columns first indicated that damage associated with rising bubbles alone was not the cause as introducing air at greater depths did not cause more damage whilst high aspect ratios caused less (Handa-Corrigan et al. 1989; Jöbses et al. 1991; Kioukia et al. 1992). Most workers therefore concluded that damage occurred due to bursting bubbles, though distinguishing it from potential damage at the sparger or in the impeller region is not easy. A particularly interesting experiment in this regard was conducted by Kioukia et al. (1992). They grew TB/C3 mouse hybridoma cells in a 2.5 L bioreactor with headspace aeration with agitation by a Rushton turbine under both baffled and unbaffled conditions. Under baffled conditions at all speeds up to 450 rpm, the cells grew similarly and satisfactorily, giving results essentially identical to Oh et al. (1989). However, when the baffles were removed (early work used such a configuration (Telling and Stone 1964; Telling and Elsworth 1965) and they were historically, often recommended because it was anecdotally said to 'reduce shear'), central vortexing occurred which increased markedly with increasing speed (Fig. 5). Though the mean specific energy dissipation rate in the absence of baffles was estimated to be 1/5th of that in their presence (Kioukia et al. 1992), with increasing speed, cells grew progressively less well (Fig. 6). As can be seen in Fig. 5c, at the highest speed without baffles, bubble entrainment occurred dynamically, i.e., bubbles both entered and left the medium through the upper interface. However, the bubbles never entered the impeller vortices; nor, of course, was the air sparged into the bioreactor.

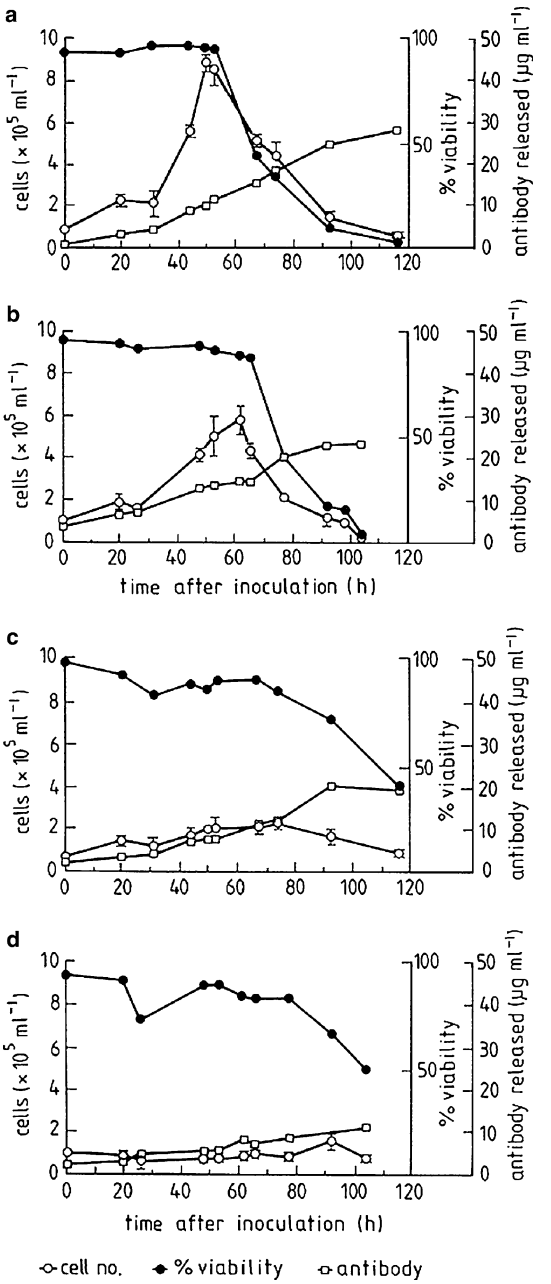

Fig. 3 The culture of TB/C3 mouse hybridoma cells in a 1.4 L bioreactor (agitated with a Rushton turbine) under different operating conditions: **a**) With headspace aeration, at 400 rpm (0.25 W kg^{-1} or ~ 1.25 HP (1000 US gal)$^{-1}$). Similar results were obtained at 100 rpm (0.004 W kg^{-1}) (data not shown); **b**), **c**) and **d**) With sparging at 100 ml min^{-1} air/5% CO 100 rev m^{-1}, 200 rev m^{-1} and 300 rev m^{-1} respectively (Oh et al. 1989)

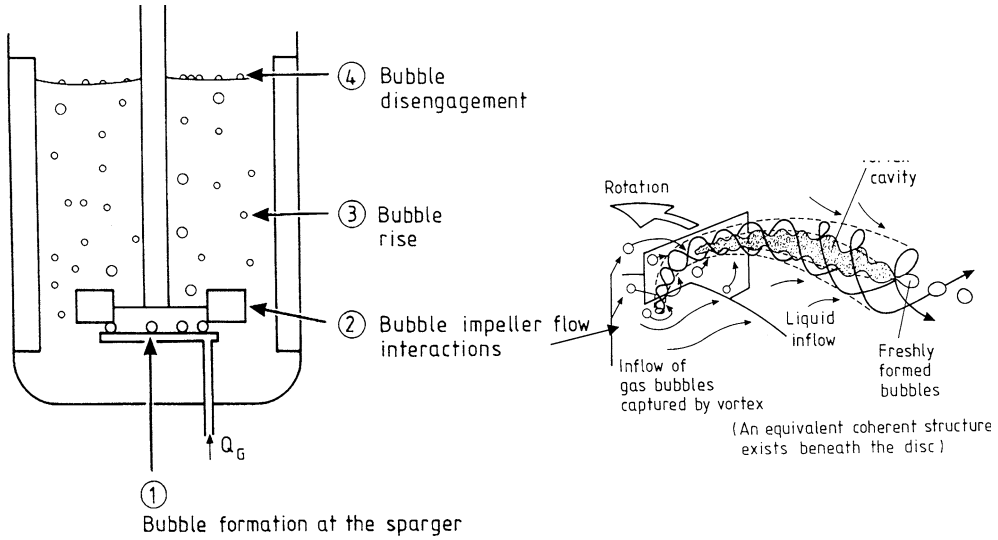

Fig. 4 Zones of potential bubble–cell interactions that may cause cell damage (Oh et al. 1992)

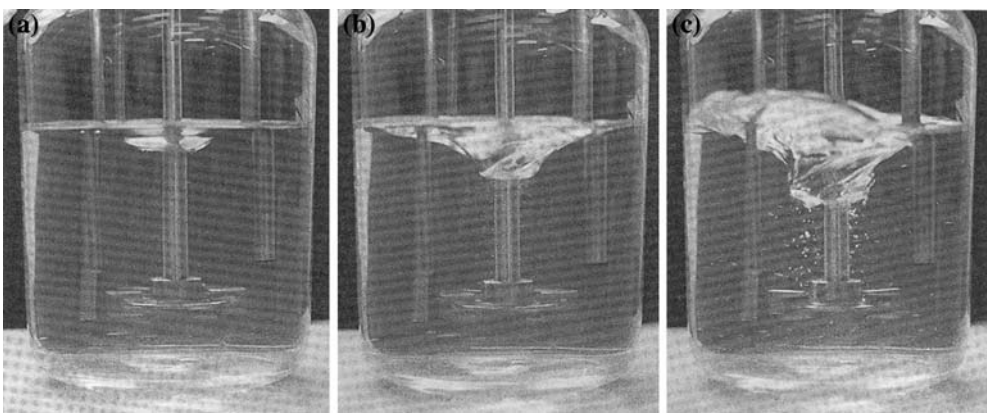

Fig. 5 Increasing gross vortexing in an unbaffled 2 L animal cell bioreactor: (**a**) at 200 rpm; (**b**) at 300 rev m^{-1}; (**c**) at 400 rev m^{-1} (Kioukia et al. 1992)

This simple experiment therefore strongly suggests, along with the conclusion of others that damage occurs when bubbles burst at the medium–air interface. It also shows that removing baffles, though it reduces the agitation power put into the medium, can cause cell death if it allows bubble entrainment due to the swirling flow that can then occur, leading to gross central vortexing.

Considerable progress has been made in mathematical modelling of the flow close to a bubble as it bursts (Boulton-Stone and Blake 1993; Boulton-Stone 1995) giving results very similar to those obtained experimentally by high-speed cinephotography. Figure 7 compares the experimental results and theoretical predictions of the bubble burst phenomena from both of which velocities can be determined. For example, the receding film at the top of the bubble is estimated to retreat at ~8 m s^{-1}. From the modelling, it is also possible to calculate the stresses generated and the maximum local specific energy dissipation rates, $(\varepsilon_B)_{max}$, associated with different sizes of bursting bubbles (Boulton-Stone and Blake 1993). Figure 8 gives the latter predictions indicating specific energy dissipation rates many orders of magnitude higher than those associated with impeller-driven mixing, the absolute value increasing rapidly with smaller bubbles. A similar

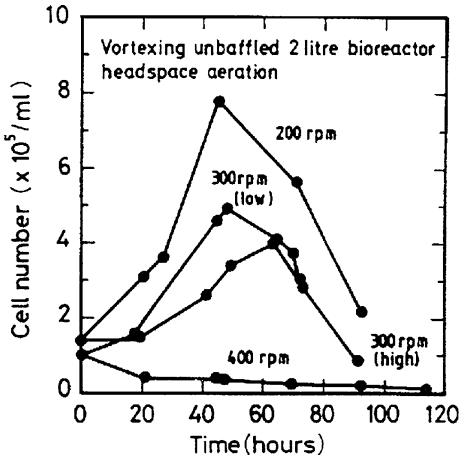

Fig. 6 Viable cells during a batch culture using headspace aeration under the agitation conditions shown in Fig. 5. (Note: In the same bioreactor with baffles to prevent air bubble ingestion, cells grew as well at speeds up to 400 rev m^{-1} as indicated here at 200 rev m^{-1}) (Kioukia et al. 1992)

functionality between size and local maximum pressure was also found, giving values of the latter greater than the bursting pressure of cells measured by the technique of micromanipulation (Zhang et al. 1993) discussed above.

Other early work related to carryover in distillation columns determined the amount of fluid ejected in association with bursting bubbles (Newitt et al. 1954). This study showed that greater quantities were ejected into the headspace by smaller bubbles, in a very similar way to the specific energy dissipation rates and pressures seen in Fig. 8. Many workers also found experimentally that smaller bubbles were more damaging to cells than larger ones at the same volumetric air flow rate in bubble columns using different spargers (Tramper et al. 1987; Handa-Corrigan et al. 1989; Jöbses et al. 1991). Similar effects were observed in stirred bioreactors by sparging at different places relative to the impeller, thus giving a range of bubble sizes under identical aeration and agitation conditions with smaller bubbles again causing more damage (Oh et al. 1992). Particularly clever was the way that Orton and Wang (1990) showed how cell death rates (Jöbses et al. 1991; Tramper et al. 1987) and liquid carryover rates (Newitt et al. 1954) were similarly related to bubble size.

The literature reviewed above points very clearly to bursting bubbles as the most damaging of the possible mechanism leading to loss of cell viability and total cell numbers in animal cell bioreactors. It is ironic in the light of these studies that in the early days of commercial scale animal cell culture, airlift and bubble columns were proposed as a replacement for stirred bioreactors because of the reduced level of 'shear' (Boraston et al. 1984: Varley and Birch 1999).

Use of surfactants to reduce cell damage due to bursting bubbles

At the scale at which many of the above studies were done, oxygen demands could probably have been met by surface aeration alone, i.e., without sparging. However, on the commercial scale, as the surface area (available for oxygen transfer)/volume of medium (proportional to oxygen demand) is inversely proportional to scale, such a possibility becomes decreasingly likely. The use of silicone tubing, porous to oxygen, submerged in the medium was initially considered as a way of getting to larger scales without sparging, but this technique brings its own problems associated with membrane fouling and carbon dioxide build-up (see below) and certainly would not be practical at the largest scales now in use. However, as long ago as 1968, Kilburn and Webb (1968) showed that Pluronic F 68 (a block co-polymer of propylene oxide and ethylene oxide) was effective in decreasing cell damage. Such effectiveness was subsequently shown in many of the above studies, involving both stirred (Oh et al. 1989, 1992; Kioukia et al. 1996; Nienow et al. 1996) and bubble column bioreactors (Handa-Corrigan et al. 1989; Kioukia et al. 1992; Meier et al. 1999).

The reasons for the effectiveness of Pluronic F 68 have not been fully elucidated and there are probably more than just one. For example, at a simplistic level, the extra damage caused by small bubbles can be related to the excess Laplace pressure, Δp (N m^{-2}), inside the bubble given by

$$\Delta p = 4\sigma/d_p \tag{8}$$

where σ is the surface tension (N m^{-1}) and d_p is the bubble size (m). Thus, smaller bubbles have

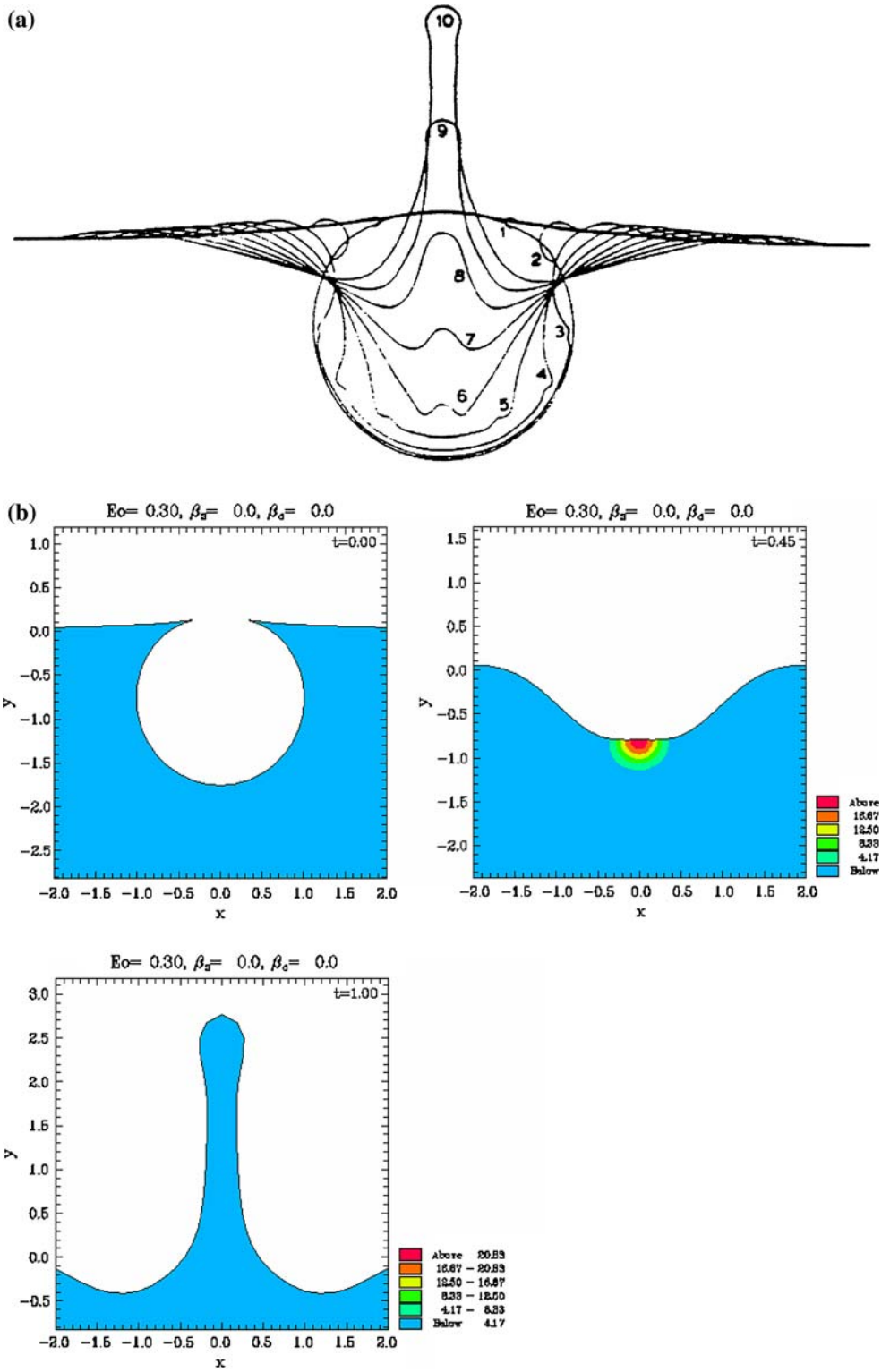

Fig. 7 Visualisation of a small bursting bubble: (**a**) From cinephotography of a 1.7 mm bubble in sea water; profiles are ~ 1/6000 s apart (Macintyre 1972); (**b**) from a mathematical model (Boulton-Stone and Blake 1993)

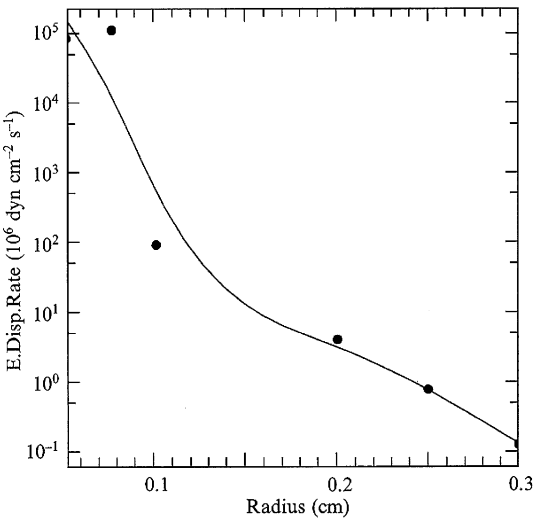

Fig. 8 Maximum specific energy dissipation rate due to bursting versus bubble radius (10^4 dynes cm^{-2} s^{-1} = 1 W m^{-3} ≡ 10^{-3} W kg^{-1} giving 10^4 W kg^{-1} with a 1 mm bubble) (Boulton-Stone and Blake 1993)

a higher excess pressure, which increases the speed of recoil of the bubble lip as it bursts (Fig. 7) and enhances the other significant hydrodynamic stresses discussed above. However, in addition, if the surfactant lowers the surface tension, this simple equation shows how it also lowers the pressure in the bubble and hence the other parameters. Table 3 shows the surface tension in various medium formulations (Dey 1998) and the effectiveness of Pluronic F68 is immediately apparent in that it lowers σ very significantly even at low concentrations.

Another possibility is that the surfactant is incorporated into the cell membrane. Ramirez and Mutharasan (1990) showed this to be so and Zhang et al. (1992) using micromanipulation showed a significant increase in cell membrane strength in cells grown in the presence of 0.5 g/l Pluronic F68 compared to cells grown without it. However, given the huge local values of $(\varepsilon_B)_{max}$ around a small bursting bubble, even a combination of the above changes (increasing cell strength and lowering $(\varepsilon_B)_{max}$) cannot be shown to account for the success of Pluronic F68 in preventing cell damage (Dey 1998).

However, Chalmers and Bavarian (1991) clearly showed using video microscopy that in the presence of Pluronic F68, the cells did not attach to bubbles whilst without it they did. This difference in behaviour was ascribed to changes in the hydrophobic interactions between bubbles and cells. Thus, in the presence of Pluronic F68 cells are not close enough to the bubbles for them to experience the very high but very localised $(\varepsilon_B)_{max}$ values. Subsequently, Meier et al. (1999), by comparing cell culture with and without Pluronic F68, and Wu (1995), both concluded that the effectiveness of the latter was associated with its ability to reduce the hydrophobicity of the cell surface (or even make it hydrophilic) thus preventing cell-bubble attachment and encouraging cell drainage into the aqueous media. The latter aspect also encourages cells to drain out of any foam that is formed (Handa-Corrigan et al. 1989; Dey 1998).

Homogeneity issues

General physical aspects

As already mentioned, many bioreactors have been designed with the starting premise that cells are very 'shear sensitive'. Therefore there has been a tendency to keep agitation and aeration intensity to a minimum. Mostly, oxygen demands have been considered to be the most important consideration. As a result, the use of enriched air or pure oxygen to satisfy this requirement has often been considered. However, this strategy can give rise to other problems especially with CO_2 in solution (see below). Consider for the moment that large-scale oxygen demand is to be met by keeping k_La constant. If the vvm is also kept

Table 3 Surface tension (σ mN m^{-1}) of various media (Dey 1998)

RPMI	70
RPMI + 0.2% Pluronic F68	38
RPMI + 5% FCS	53
RPMI + 0.06% Pluronic F68 + 100 ppm antifoam	35
RPMI + 0.4% Pluronic F68	37
RPMI + 0.05% FCS + 100 ppm antifoam	43

constant to satisfy the stoichiometry, even though it is very low (e.g. typically 0.005–0.01 vvm (Nienow et al. 1996)) because cells have a low oxygen demand, then the superficial air velocity will increase ($\propto\sim T$) (Nienow 1998), so that from Eqs. (6) and (7), $(\bar{\varepsilon}_T)_g$ could be reduced. What is the impact of these considerations on scale-up to commercial bioreactors on other important mixing parameters?

Under turbulent flow conditions, the time required to homogenise the contents of a bioreactor, the mixing time, θ_m (s), is related to agitation parameters by the equation (Nienow 1998),

$$\theta_m = 5.9(\bar{\varepsilon}_T)_g^{-1/3}(D/T)^{-1/3}T^{2/3} \quad (9)$$

for bioreactors for H=T. For bioreactors with an aspect ratio, AR (=H/T)>1, then (Nienow (1998),

$$\theta_m \propto (H/D)^{2.43} \quad (10)$$

Equation (10) was originally developed for multiple impellers but it has been shown also to indicate the great sensitivity of mixing time to fill height in 8 m^3 animal cell culture bioreactors (Nienow et al. 1996; Langheinrich et al. 1998) of aspect ratio 1.3 with a single impeller working in the draw and fill mode. Thus, if agitation conditions are chosen, via a dO_2 process control loop, for example, to satisfy the oxygen demands of the system across the scales as discussed above, i.e., $(\bar{\varepsilon}_T)_g$ is held constant or reduced, the degree of homogeneity as indicated by the mixing time gets significantly worse, especially at high aspect ratios.

Dissolved oxygen variations

This poor degree of homogeneity at the large scale can impact on a number of parameters. One is the dissolved oxygen concentration. This has usually been considered via the characteristic times of mixing compared to mass transfer and oxygen uptake (also known as regime analysis (Van't Riet and Tramper 1991)). Such an analysis was undertaken for an 8 m^3 bioreactor agitated by a Rushton turbine of 2/9 of the vessel diameter (Nienow et al. 1996) (Table 4). Based on the measured OUR for CHO 320 cells (~4.5×10^{-15} mol O_2 $cell^{-1}$ min^{-1} at 4×10^5 cells ml^{-1}) and NSO cells ((~5×10^{-15} mol O_2 $cell^{-1}$ - min^{-1} at 2×10^6 cells ml^{-1}), the characteristic times for mixing, mass transfer and oxygen uptake are given in Fig. 9. A characteristic time for mixing much longer than that for oxygen uptake suggests that significant spatial differences in oxygen concentration should occur. A time for oxygen transfer greater than that for oxygen uptake indicates potential oxygen transfer limitations. Figure 9 suggests that for the higher cell density in the NSO culture, there is a potential oxygen transfer problem at these energy dissipation rates and that spatial dissolved oxygen variations may also arise. For the lower cell density case, neither parameter is significant.

However, the agitation and aeration conditions were very gentle because the bioreactor studied had been designed during the 1960's (Pullen et al. 1985), a period when cell fragility had been a major concern. As can be seen from Fig. 9, the use of $(\bar{\varepsilon}_T)_I$ values of 0.25 W kg^{-1} ($\equiv$ 250 W m^{-3}), which as discussed above has been shown not to damage cells, would essentially eliminate the potential problems for the NSO cells. Clearly, higher cell densities would bring the issue back into consideration but, for each case, given suitable k_La

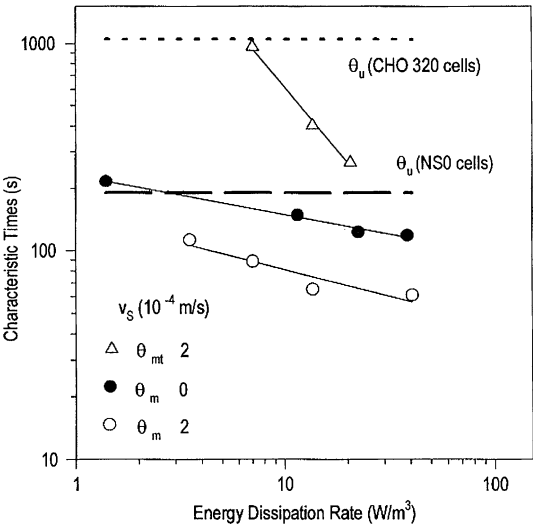

Fig. 9 Characteristic times of mixing, mass transfer and oxygen uptake at the 8 m^3 scale with 2/9T Rushton turbine for NSO and CHO 320 cells (θ_m, mixing time; θ_{mt}, mass transfer time; θ_u, oxygen uptake) (See also text and Table 4 for further details) (Nienow et al. 1996)

Table 4 Experimentally based characteristic times (Nienow et al. 1996) for mixing, mass transfer and oxygen uptake for Fig. 9 (C_L is dO_2 concentration; C_L^* is dO_2 at saturation; C_c is the cell concentration and q_{O2} is the specific OUR)

Process	Measurement
Mixing	Θ_m: Time required to achieve a homogeneity of 90%
Mass transfer	Θ_{mt}: Time required for a step change from $C_L = 0.15\ C_L^*$ to $C_L = 0.3\ C_L^*$
Oxygen uptake	Θ_u: Time required for a step change, ΔC_L, from $C_L = 0.3C_L^*$ to $C_L = 0.15C_L^*$ $(= \Delta C_L/C_c q_{o2})$

data and its dependence on agitation and sparging conditions together with the specific oxygen demand of the cell line, a similar analysis can be undertaken.

pH control

Another aspect is the homogenisation of the chemicals for pH control. First, the use of alkaline solutions to prevent excessively high values of pH due to pCO_2 in the latter stages of a fed-batch cultivation will be considered. Again, data from work on the 8 m^3 bioreactor using a 2/9 T Rushton turbine at a clearance off the base of 2/9 T, as discussed above, are the only ones available from a commercial scale vessel (Nienow et al. 1996; Langheinrich et al. 1998) though similar results from a 2 m^3 airlift bioreactor have also been reported (Wayte et al. 1997). Mixing time measurements alone were found to fit quite well with Eqs. (9) and (10), though the energy dissipation from the sparged air did have to be taken into account (Langheinrich et al. 1998) in order to correlate the data.

Measurements of mixing time are a rather crude way of considering homogenisation considerations. Also, all the above correlations for mixing time are based on feeding tracer onto the top surface of the liquid/media. θ_m values indicate the end point of mixing but not the local spatial and temporal variations in concentration associated with the whole time that it takes to achieve mixing. Probes can do so and therefore pH homogeneity was investigated in the same 8 m^3 (T = 2 m) bioreactor and in a transparent, geometrically similar, scaled-down vessel of 0.61 m diameter (Langheinrich and Nienow 1999). In addition, other work on chemical reactors has shown distinct advantages from feeding close to impellers when chemical reactions, especially involving acid or base, are under consideration (Nienow et al. 1997; Assirelli et al. 2005). Therefore, since it was found that pH excursions from the mean value with addition to the top surface were very large, additions close to the impeller were also made and both sparged and unsparged runs were studied.

The actual experimental procedure involved adding 2 M Na_2CO_3 (pH≈11.7) either onto the top surface or sub-surface close to the impeller with the medium buffered by $NaHCO_3$. One pH probe was placed at the level of the impeller (position 3 in Fig. 10), simulating the one used for pH control in the actual 8 m^3 bioreactor; and an additional probe was placed near the top surface of the medium (position 1 in Fig. 10). Figure 10a for unsparged conditions and top addition shows a smooth pH trace close to the impeller as was found in the actual commercial scale equipment. However, a very ragged and at times much higher pH is indicated by the probe near the top. Addition of phenolphthalein as an indicator showed extensive regions of pH >8. Because at the low agitation intensities used for cell culture, the air is not well dispersed, the bubbles rise in a plume and cause significantly enhanced fluid movement near the top surface that reduces the mixing time (Langheinrich et al. 1998). Figure 10b is for this condition with a top feed of alkali and it can be seen that the pH excursions near the top are less, in accord with the mixing time studies. Finally, Fig. 10c shows the result of sub-surface feeding near the impeller with both traces following each other very closely without any major excursions.

Based on these observations of spatial and temporal pH fluctuations, Osman et al. (2002) of Lonza Biologics used a well-established technique (Amanullah et al. 2003) to simulate them at a small scale during actual cell culture (Fig. 11). Two small stirred bioreactors were used in which one was run at a controlled pH for optimum

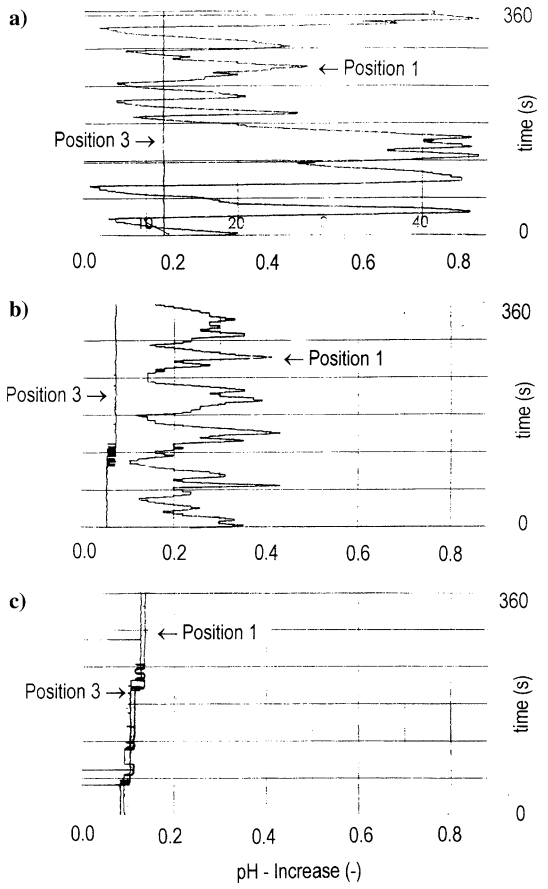

Fig. 10 Chart recordings of pH at two positions (3 near the impeller; 1 near the top surface) for three operating conditions: (**a**) surface addition, unsparged; (**b**) surface addition, sparged; (**c**) impeller addition, unsparged (Langheinrich and Nienow 1999)

operation (pH = 7.3) whilst a second smaller one had predetermined pH variations imposed on it using 2 M NaOH pulse with CO_2 sparging, mimicking approximately those fluctuations shown in the earlier study (Langheinrich and Nienow 1999). This work showed that when cells were subjected to 100 perturbations of 200 s at a cycle time of 6 min up to pH 8, cell death was increased by about 30% compared to a control with similar changes only in osmolarity. Similar results were found after only 10 excursions to pH 9. It was concluded that 'during scale-up, agitation rate should be increased to minimise the magnitude and duration of pH perturbations as much as possible and alkali should be added to a well-mixed region of the vessel'. Another way of reducing the magnitude and duration of pH perturbations in addition to agitation intensity and feed position is to use a lower concentration solution for pH control, a well-known strategy when handling fast chemical reactions (Assirelli et al. 2005).

As a measure of how carefully pH control must be carried out even at the bench-scale, Ozturk (1996) showed a picture taken of a poorly agitated bioreactor in which a viscous 'snow-ball' close to the addition point of concentrated alkali had formed. It is clearly the result of cell lysis and resembles the rheologically complex lysate generated by alkaline lysis during plasmid DNA production (Nienow et al. 2000)

Nutrients for fed-batch operations

Work on inhomogeneities due to nutrient feeding has not been reported for animal cell culture. On the other hand, it has been clearly shown to be an issue associated with high nutrient concentrations in the feed plume and related localised low dO_2 during bacterial fermentations (Enfors et al. 2001), much like that accompanying the alkali addition during pH control discussed above. It may yet emerge as requiring consideration if cell densities in animal cell culture get high enough and again sub-surface feeding of nutrients should help.

Insect culture post-infection

Post-infection with baculovirus, *Spodoptera frugiperda* (Sf9) insect cells have been reported to have an enhanced level of specific oxygen uptake some 40% above that found prior to infection (Kioukia et al. 1995). Optimum dO_2 levels for cultivation (Klopinger et al. 1990) from 40% to 60% potentially give a reduced driving force for oxygen transfer. Both these aspects suggest that insect cells may require higher agitation and/or aeration intensity. Other work (Kioukia et al. 1996) also showed a modestly enhanced sensitivity to sparging compared to animal cells in the absence of Pluronic F 68 and this finding matched the slightly lower cell strength when measured by micromanipulation (Zhang et al. 1993). Thus, some concern for cell damage would seem to be justified.

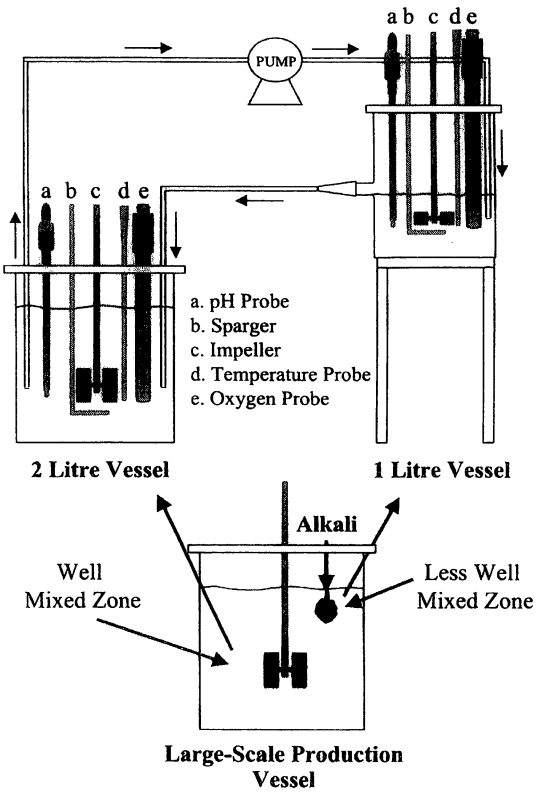

Fig. 11 Schematic representation of how a 2 L and a 4 L bioreactor were used to simulate pH inhomogeneities in a large-scale vessel (Osman et al. 2002)

However, cells grew well with agitation up to ~0.25 W kg^{-1} at 50% dO_2 using headspace aeration (Kioukia et al. 1996). Also, as with mammalian cells, addition of Pluronic F 68 at 0.1% by weight essentially eliminated any reduction in performance pre-infection under sparged conditions, even leading to a modestly higher growth rate compared to the best available with only headspace aeration. In addition, the use of Pluronic had no deleterious effects when the cells were infected. Changes in agitation speed had no effect up to about 0.25 W kg^{-1} on total cell numbers, maximum infectivity, extra-cellular viral titres and β-galactosidase expression. Nor was the rate of infection affected by agitation with or without Pluronic, though infection rates with *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV), averaged over the first 24 h, were linearly dependent on the multiplicity of infection (MOI from 0.1 to 100) (Kioukia et al. 1996). It was suggested that these findings imply infection due to a diffusion mechanism controlling the viral particles contact with the Sf9 cells.

The use of microcarriers

Very little work has been reported of a quantitative nature with respect to the operation of bioreactors for adherent cells such as VERO. It has been recognised for some time (Clark and Hirtenstein 1981; Sinskey et al. 1981) that the use of relatively small particles (of the order of 150–200 μm), either solid or macroporous, enables a large surface area for cell growth to be available in the bioreactor and hence increases the cell density that can be achieved. However, all of the concerns discussed above with respect to oxygen transfer, homogenisation and 'shear' sensitivity also have to be considered once again. In addition, the microcarriers have to be kept in suspension, at least during most of the batch time, if the area they offer is to be effectively used to grow cells. Thus, there is an additional minimum agitation intensity requirement, namely solid suspension since commercial microcarriers are slightly denser than culture media. Though many papers have considered this issue, virtually none have used agitator/vessel configurations that represent state-of the art for the latter agitation requirement.

There are two factors which make the question of 'shear sensitivity' more significant in microcarrier culture. First, the minimum agitator speed that can be used, N_{js}, and associated mean specific energy dissipation rate, $(\bar{\varepsilon}_T)_{js}$, has to be sufficient to keep the microcarriers suspended. However, unlike oxygen transfer rates, which as implied by Eq. (7), are independent of agitator type, N_{js} and $(\bar{\varepsilon}_T)_{js}$ are very sensitive to agitator type and precise vessel configurations. Thus, agitation with cylindrical bars (Croughan et al. 1987) that produce 'radial flow patterns' would be expected to require relatively high values of $(\bar{\varepsilon}_T)_{js}$ to suspend solids (Ibrahim and Nienow 2004), thus enhancing the possibility of damage to cells. Secondly, since the cells are on the microcarriers, it is important to consider the interaction between the turbulent eddies and

the cell layer growing on the microcarriers, which are of course much larger than the cells, potentially of a size that would be commensurate with the Kolmogoroff eddy size (Aunins et al. 1986). Indeed, if it is assumed that λ_K must be greater than the microcarrier size to prevent damage to cells growing on them from turbulent eddies (Aunins et al. 1986), then, even at 0.001 W kg^{-1}, λ_K is still only ~180 μm.

However, there are also other mechanisms that may lead to damage to cells on the surface of microcarriers, very similar to the way secondary nucleation occurs with crystals in stirred crystallisers (Nienow 1997a). The microcarriers may impact with each other or with the impeller (or other parts of the bioreactor) (Croughan et al. 1988; Nienow 1997a). In both cases, work in agitated systems on particle–particle and particle–impeller impacts suggests that the potential damage increases rapidly with $\bar{\varepsilon}_T$, with increasing microcarrier size (for example, particle abrasion has been shown to be proportional to the sixth power of the particle size!) and also with increasing solids concentration (Nienow 1997a). It is therefore interesting to note that the use of smaller microcarriers has been reported to reduce cell death and increase growth rates (Cherry and Papoutsakis 1989).

Clearly, it is important therefore to consider the choice of geometry for anchorage-dependent cell culture that will enable microcarriers to be suspended with low values of $(\bar{\varepsilon}_T)_{js}$. The use of a bioreactor with a hemi-spherical base has been recommended (van Wezel 1985) but in fact such a shape is not at all suitable (Nienow 1997b) when particles are to be suspended at low values of $(\bar{\varepsilon}_T)_{js}$. Aunins et al. (1993) proposed a large and deep axial flow impeller designed in-house placed off-centre and angled at 17° to the vertical for use in a 200 L unbaffled vessel. However, though its suspension characteristics were reported, power was not measured and therefore the effectiveness of the configuration in terms of $(\bar{\varepsilon}_T)_{js}$ cannot be assessed. Recently, the suspension of microcarriers under realistic and quantifiable agitation conditions was studied (Ibrahim and Nienow 2004), using impellers and vessel shapes shown previously to be able to suspend particles at very low values of $(\bar{\varepsilon}_T)_{js}$ compared to other impellers (Ibrahim and Nienow 1996). Such studies on particle suspension on the relatively small scale have been shown to be suitable for scale-up calculations (Nienow 1997b).

Hydrated Pharmacia Cytodex 3 microcarriers (size 140–210 μm and density 1040 kg m^{-3}) at ~ 20% by wt. were suspended in phosphate buffered saline solution in a baffled vessel of 0.29 m height and liquid depth with various bottom shapes (Fig. 12a) and agitators (Ibrahim and Nienow 2004). It was found that the HE3 impellers (Fig. 12b), at all D/T ratios from 0.44 to 0.35 at $C/T = 0.25$ with all the three base shapes, were able to suspend the microcarriers at $(\bar{\varepsilon}_T)_{js} < \sim 0.0005$ W kg^{-1}, though it took some time for full suspension to be reached (as also reported by Aunins et al. (1993)). In order to reduce operating times at low $\bar{\varepsilon}_T$ values, a higher agitation intensity could be used to bring solids into suspension before cell growth, followed by a subsequent reduction once cell attachment commences. Scale up via the Zwietering approach should be possible (Nienow 1997b). For further details, the original literature should be consulted (Nienow 1997b; Ibrahim and Nienow 2004). However, it should be pointed out that at $(\bar{\varepsilon}_T)_{js} = 0.0005$ W kg^{-1}, $(\varepsilon_T)_{js\max} >> 0.001$ W kg^{-1}, so even these low $(\bar{\varepsilon}_T)_{js}$ values, based on optimising the geometry for microcarrier suspension, may still cause mechanical damage problems.

Of course, it is quite possible that such low levels of agitation intensity will cause oxygen transfer and homogenisation concerns. Obviously, higher values of $\bar{\varepsilon}_T$ can easily be imparted by raising the agitator speed and feeding into the impeller zone can enhance homogenisation. However, to the present authors knowledge, these issues have not been discussed in the open literature. Therefore, choosing a geometry that minimises $(\bar{\varepsilon}_T)_{js}$ whilst ensuring higher speeds and $\bar{\varepsilon}_T$ values are available offers the best approach to large scale culture on microcarriers at the present time.

Cell recycle/cell retention

An excellent review of this aspect of animal cell culture has been produced by Voisard et al. (2003) of Serono. They conclude that the biotech

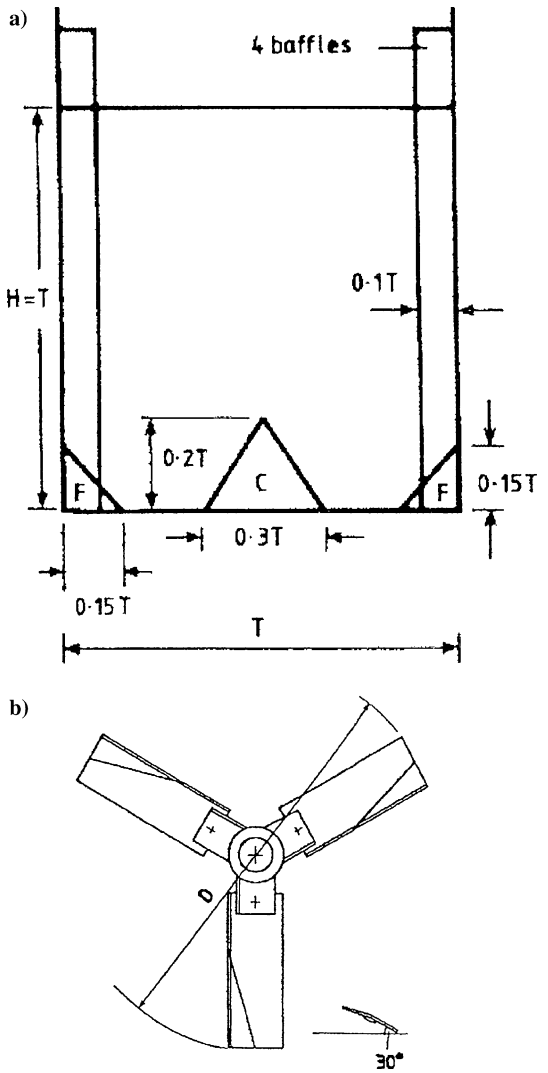

Fig. 12 Details of (**a**) the vessel and base (C, cone; F, fillet-see text) and (**b**) the axial hydrofoil HE3 impeller which was the optimum studied in order to minimise $(\bar{\varepsilon}_T)_{js}$ for the suspension of microcarriers (Ibrahim and Nienow 2004)

industry has converged on using free suspension cultures in large scale stirred bioreactors to produce recombinant proteins and antibodies. Such bioreactors can be operated in batch, fed-batch or perfusion mode, the latter two being used to reach higher cell densities. In both cases, medium is fed during the cell propagation and production phases but in the perfusion mode, metabolites are also removed. For this to be done successfully, a good cell separation device is needed either inside the bioreactor or in an external recirculation loop.

From the point of view of the scale-up of the bioreactor, all but one of the issues have already been discussed above, i.e., oxygen transfer, homogenisation of chemicals for pH control and of medium feed, and mechanical stress. Higher agitation and aeration intensities have to be used in order to meet the higher oxygen demand via the enhanced k_La as given by Eqs. (6) and (7). The higher rate of oxygen uptake leads to higher rates of CO_2 evolution and therefore pH control chemicals. Can these higher agitation and aeration intensities be imposed without damage to the cells? The above discussion and the success of companies scaling-up to 20,000 L using batch and fed batch technology suggests that they can. However, does the additional need to produce a good separation device with possibly an external circulation loop pose additional problems on scale up over and above those already discussed?

Voisard et al. (2003) identify two mechanisms by which cells can be separated at the large scale, namely by particle size and/or by density. If particle size is used, it generally implies some form of filtration. The major drawback is that, in every version of that basic idea, there is a tendency to block and foul. They also point out that in many cases, and particularly at larger scales, the flow rates and pressure drops may be damaging to cells e.g. in cross-flow filtration using hollow fibre modules whether internally or in the external circulation loop. Spin filters have been used successfully at moderate scales and do have the advantage of not requiring an external circulation loop. They are often specified by screen tangential speed (range 0.3–0.7 m s^{-1}) with higher speeds giving higher capacities ($\propto$ speed^2), and reduced fouling and cell attachment to the screen. On the other hand, there are concerns associated with the need to decouple the filter speed from the agitator speed since, in general, the optimum speed will not be the same for both. Hence, there is an inherent increase in complexity. In addition, the higher the cell density, the greater the fouling tendency.

However, perhaps, the biggest problem is that the perfusion rate must scale with the volumetric capacity of the bioreactor (proportional to the cube of the bioreactor diameter) to maintain the same effectiveness across the scales; but the

actual rate is proportional to the area of the filter (proportional to the square of the bioreactor diameter for geometrically similar configurations). In an example given in Voisard et al. (2003), for an active bioreactor volume of 2500 L, the 'dead' volume inside the spin filter would also be ~2500 L to give a total volume of ~6000 L. As the scale increases, this trend gets worse and it also impacts on the bioreactor design itself. Thus, there is a major issue concerning the use of spin filters at the larger scales where fed-batch is now being employed.

Retention by density and/or size implies settling under gravity or in a centrifugal flow field (Voisard et al. 2003). Because the cells are small and only slightly denser than the medium, they settle slowly following Stokes law:

$$v_s = \Delta\rho G d_p^2/18\mu \qquad (11)$$

where v_s is the settling velocity, G is the acceleration (equal to g (9.81 m^2 s^{-1}) under the action of gravity), $\Delta\rho$ is the difference in density between the cell and the medium, d_p is the cell diameter and μ is the medium viscosity (all in SI units). Gravity settlers can be made more effective by using multiple inclined plates but even then they would need to be used in external loops on the large scale. Thus the cells would be exposed to a less than optimum environment (especially dO_2 but also pH, etc.) for periods of time greater than 1 h (Voisard et al. 2003). Settling velocities can be enhanced by increasing the size of the particles to be separated in accordance with Eq. (11) and acoustic waves have been shown to do this. They have been used successfully on the small scale and preliminary results have been reported of rates of 1000 L day^{-1} (Gorenflo et al. 2005). However, it is considered (Voisard et al. 2003) that such a size is probably the biggest that can be utilised because of the difficulty of the removal of the heat dissipated by the acoustic field.

Equation (11) also shows that the settling velocity can be enhanced by using a centrifugal flow field which means either a hydrocyclone, possibly internally, or a centrifuge in an external circulation loop. The former is reported to be damaging to cells (Voisard et al. 2003), though the mechanism of damage has not been studied fundamentally. Centrifuges are clearly very effective at separating cells and they are reported to do so at quite high perfusion flow rates (rotation at 20 rev s^{-1} (320 g) to handle rates up to 2800 L day^{-1} (Voisard et al. 2003)). Somewhat surprisingly, they are reported to do so without damaging the cells. It would be reassuring if more results were available for large scale, long duration runs to support the latter claim.

Overall, the review by Voisard et al. (2003) suggests that for industrial manufacturing processes, the use of retention techniques requires extra development time compared to fed-batch and therefore extra time to market. For high perfusion rates, only centrifuges are able to achieve rates >~1000 L day^{-1} and though they are reported not to cause damage to the cells, there is little work to substantiate that claim. Overall, perfusion with retention and recycle is considered generally not to be being adopted because: (i) they are considered new and unproven except at the smallest scale; and (ii) high cell densities can be achieved with much greater certainty in fed-batch operations.

In addition to the retention devices themselves, the largest scales will require circulation loops which give rise to the need for the cells to spend significant periods of time away from their optimum environment, especially of dO_2, pH and CO_2 at the very high cell densities that will be produced. The loop also requires additional sterility considerations. Finally, there is also the possibility of the flow field in the loop itself causing mechanical damage to the cells due to pumping, extensional flow regions, etc. Indeed, 'shear damage', its source undefined as usual, has been reported at the industrial pilot scale, the amount of damage increasing with increasing recirculation rates (Moran 2004). The retention technique was not reported. It would appear that the use of such techniques at the large commercial scale is still some way off.

Carbon dioxide/osmolality

At the initial stages of cultivation, it is necessary to control pH by the use of CO_2 sparged in with the air, essentially along the lines first proposed by Telling and Stone (1964). However, with

increasing cell density, whether from fed-batch or perfusion techniques, there is a proportionate increase in oxygen demand. Therefore, since the respiratory quotient, RQ (mol CO_2 produced per mol O_2 consumed) ranges (Ozturk 1996; de Zengotita et al. 2002) from about 0.9 to about 1.3, the amount of CO_2 generated increases at essentially the same rate. Thus if a constant $(\bar{\varepsilon}_T)_I$ and superficial air velocity are used on scale-up (leading to a fall from 0.04 to 3×10^{-3} vvm between the 5 L and the 20,000 L scale) as suggested by Hoeks et al. (2004) to give a constant k_La, the concentration of CO_2 in the exit air and therefore in solution increases. If a low enriched air flow rate or pure oxygen are used to enhance the driving force and thereby reduce the flow rate and potential for damage due to bursting bubbles, high levels of pCO_2 of 150–200 mm Hg are predicted to be reached (Gray et al. 1996) and such levels have been measured in 1800–2500 L bioreactors and a high cell density perfusion bioreactor (Gray et al. 1996; de Zengotita et al. 2002). Detrimental effects have been reported for growth and productivity of a recombinant myeloma process (Aunins et al. 1993), hybridoma, NSO, BHK, (de Zengotita et al. 2002), CHO (Gray et al. 1996; Mostafa and Gu 2003) and insect cells (Garnier et al. 1996) at these high levels of pCO_2, though these cells grew well in the range ~ 35 to ~ 80 mm Hg pCO_2, comparable to the physiological range of 31–54 mm Hg pCO_2 (de Zengotita et al. 2002).

The dissolved CO_2 in solution can be simply considered to cause acidification by (de Zengotita et al. 2002)

$$CO_2 + H_2O \longleftrightarrow H^+ + HCO_3^- \qquad (12)$$

Inhibition by CO_2 is therefore partly due to medium acidification if pH is not controlled. If controlled by base addition, the equilibrium will be driven further to the right and thus increase the osmolality, which also leads to growth inhibition (de Zengotita et al. 2002). Thus, pH control strategy is very important for two reasons, one, as set out above, associated with temporal and spatial pH excursions associated with the feed strategy; and secondly, because of its impact on medium osmolality. Clearly, the latter aspect is also intimately connected with the measurement of dissolved CO_2 (for which an on-line instrument is now available (Chu and Robinson 2001)) and its control.

The problem of high levels of pCO_2 and associated high acidity plus increased osmolality associated with pH control is considered to be the major difficulty encountered in scaling up mammalian cell culture to achieve high cell densities (Chu and Robinson 2001; Meier 2005). All these concerns are related to the perceived need to enhance oxygen mass transfer (to cope with the higher cell densities now being achieved) by increasing the driving force and/or interfacial area by using sintered spargers, rather than k_La by agitation or aeration rate. This strategy is because to enhance k_La, based on Eqs. (6) and (7), by agitation intensity ($(\bar{\varepsilon}_T)_g$ W kg^{-1}), or air sparge rate (v_s m s^{-1}), is perceived to be more damaging to the cells. Higher values of both $(\bar{\varepsilon}_T)_g$ and v_s also increase the possibility of foaming. However, if scale up is maintained at equal vvm, then the proportion of the sparged oxygen transferred to the medium and of CO_2 transferred into the sparged air on the small scale and the large should be the same. Thus, satisfactory operation on both scales with respect to CO_2 concentration, acidity and osmolality should be achievable along with a higher k_La if the same vvm and $(\bar{\varepsilon}_T)_g$ are also used.

The large-scale problem has been addressed in some depth by Meier (2005). Using pure oxygen through sintered spargers will lead to smaller bubbles (with the greatest potential for damage when bursting) and hence locally higher k_La per bubble and approximately five times higher driving force. Thus, they will greatly enhance the rate of oxygen transfer to the medium but at the same time, they will be very poor at stripping out CO_2 since they rapidly become saturated with it. In this case, the task of CO_2 stripping is left to the main air sparge with relatively larger bubbles (Meier 2005) for which the driving force will be less than on the small scale because the stoichiometric ratios will be less suitable. Since the level of CO_2 in atmospheric air is essentially zero, using a separate nitrogen flow (Meier 2005) does not significantly enhance the driving force for stripping CO_2 compared to air. On the other hand, it adds to the potential for damage due to bursting

bubbles since there is no evidence to suggest that damage is related to gas composition, just mechanical stresses associated with bursting. On the other hand, nitrogen sparging does give some extra flexibility if there are concerns that dO_2 may become too high. Clearly, there are distinct possibilities for clever process control strategies here.

It would appear that, if k_La values can be enhanced at the larger scale by increasing superficial gas velocity (equal vvm) and keeping $(\bar{\varepsilon}_T)_g$ as at the small scale, the problems associated with pCO_2 would be eliminated. However, more work is required to substantiate this possibility. If it cannot be achieved, monitoring of pCO_2 and using a judicious combination of air, nitrogen and oxygen flow rates to control pCO_2 and dO_2 independently should allow levels of both to be achieved satisfactorily for cell culture at the 20000 L scale (Meier 2005). This approach, using higher air flow rates, was essentially used successfully by Mostafa and Gu (2003) to grow CHO cells at the 1000 L scale. Interestingly, they ascribed the success of the approach to larger bubble sizes with a pipe sparger (at 0.01 vvm) compared to a sintered sparge stone (at 0.002 vvm) rather than to the five times higher air flow rate used with the open pipe. This increase in flow rate reduced pCO_2 from 180 to 70 mm Hg. In this case, the tendency for excessive foaming with the sparge stone even at low air flow rates was also successfully overcome with the open pipe in spite of the higher air flow rate; nor was any indication of increased mechanical damage to the cells reported. Overall, Mostafa and Gu (2003) reported an improved productivity (40%), culture time (the fed-batch time was extended from 10 to 14 days) and final titre (2-fold, equivalent to that at the 1.5 L scale). However, they also noted that at constant pH, higher pCO_2 is also associated with higher osmolality and that therefore the improved performance at lower pCO_2 might also be due to reduced osmolality (Garnier et al. 1996; de Zengotita et al. 2002) but it was not monitored. In passing, it should be mentioned that the use of antifoam enabled the use of higher air flow rates (0.004 vvm) with the sparge stone without excessive foaming and again enabled the culture duration to be extended from 10 to 14 days (Mostafa and Gu 2003).

The larger the scale and the higher cell densities and associated oxygen demand, the greater the problems related to CO_2 concentration, acidity and osmolality will become unless higher gas flow rates can be shown not to be damaging to the cells. The successful use of an open pipe with 20 ppm anti-foam (Mostafa and Gu 2003) is encouraging in this respect. However, if such an approach cannot be used, there will be a limit either to the scale of operation and/or the maximum cell density attainable at the larger scale.

Optimum geometry, operational strategy and further work

Free suspension culture

Despite our increases in understanding of many aspect of large-scale animal cell culture, it is still not possible to make an optimal a priori design. Oxygenation is still the main requirement for agitation as set out above, though clearly CO_2 stripping is also of major importance. Both depend on k_La and this is much the same for both species and is related to agitation and aeration (via Eqs. 6 and 7) across the scales if pipe or ring spargers are used. It is independent of impeller type and many agitator types have been used successfully in large scale systems, e. g., Rushton turbines (Meier 2005; Langheinrich et al. 1998), Lightnin' A320s (Meier 2005), and more recently ABEC 'elephant ear' impellers (Meier 2005). The first two have been well characterised, especially the Rushton turbine though it has recently been largely superseded by so-called 'hollow-blade' agitators in bacterial culture because of their enhanced air handling ability and aerated power characteristics (Nienow 1996). This advantage has not been very significant to date for animal cell culture but could become so because of the steadily increasing cell density being achieved and hence mass transfer and aeration rate requirements. Wide blade, axial flow hydrofoil impellers compared to Rushton turbines have lower power numbers and provide good axial flow, hence enhancing bulk blending (Nienow 1996) (halving mixing time compared to radial types) when used in pairs with aspect ratio vessels >1. However,

when they are operated in a down pumping mode, large flow instabilities occur at higher aeration rates.

Studies of 'Elephant ears' impellers have not been reported in the open literature yet and thus there are no data available for engineering calculations. They are claimed to be 'low shear' impellers but in other studies, the 'low shear' concept has been shown not to have any validity and so-called 'low shear' impellers may have $(\varepsilon_T)_{I\max}$ values greater than those found with Rushton turbines (Kresta and Brodkey 2003). As a rough guide, if $(\varepsilon_T)_{I\max}$ has not been measured, it has been proposed that $(\varepsilon_T)_{I\max}$ can be estimated from $P/\rho V_{\text{swept}}$ where V_{swept} is the volume swept out by the impeller (Kresta and Brodkey 2003). As can be seen in Fig. 13a, an 'elephant ears' impeller has a large swept volume so in that respect, it does have a 'low shear' characteristic. On the other hand, so do wide blade hydrofoils whether down or up pumping (Fig. 13b) and they have been well studied (Nienow 1996; Nienow and Bujalski 2004). Up-pumping wide blade hydrofoils also give good axial blending, good air dispersion and aerated power characteristics with low power numbers (~ 0.85). Thus, larger diameter hydrofoils can replace high *Po* agitators, thereby further enhancing all these beneficial agitation characteristics (Nienow and Bujalski 2004).

Overall, therefore, based on the above analysis, the following general guidelines for the design and operation of large scale bioreactors for freely suspended cells should consider the following:

(a) Use dual, up-pumping, wide-blade axial flow hydrofoil impellers of diameter of 0.4 to 0.5 of the vessel diameter with clearance between them of 0.33 to 0.5 T with a sparger below the lower impeller. Here, the lower impeller ensures good air dispersion and the upper one efficient liquid blending close to the top. Use baffles to ensure required power input, good vertical mixing and air dispersion are all achieved.

(b) Design and operating conditions for pH control should be chosen to avoid pH excursions, preferably with sub-surface addition. 'Cleaning-in-place' can perhaps be resolved by adding some CIP fluid through the sub-surface feed pipe (though if it can not, the use of an up-pumping hydrofoil should help mixing (Nienow and Bujalski 2004) if, in spite of the obvious problems it causes, surface addition is employed). Also, the use of less concentrated reagents can be considered (Assirelli et al. 2005).

(c) Install an over-powered variable speed motor with respect to oxygen demand and control speed on dissolved O_2. Air/nitrogen should be introduced through a pipe or ring sparger and the flow rates balanced against the required CO_2 ventilation rates by measuring pCO_2. More work is required on this

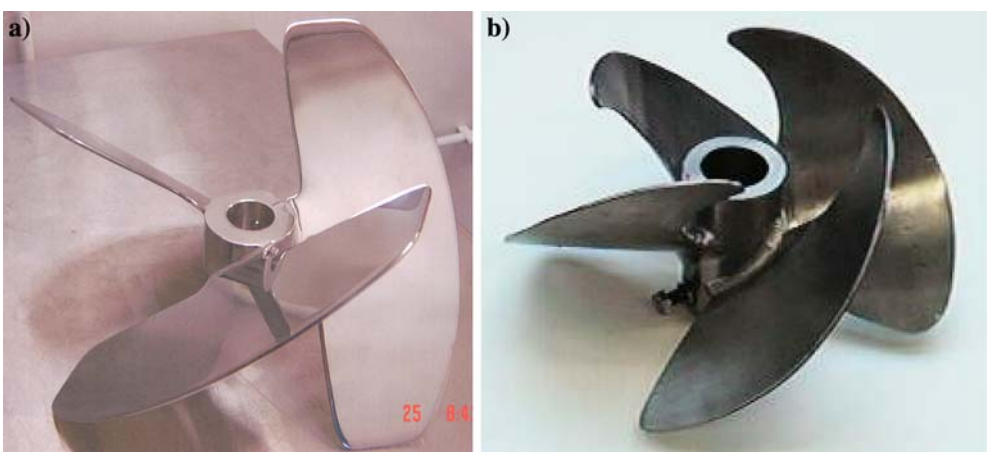

Fig. 13 (**a**) An ABEC 'elephant ear' impeller (used down-pumping); (**b**) Hayward Tyler up-pumping B2 hydrofoil

topic and the control of osmolality. Such work should also address the possibility of the existence of spatial and temporal variations of these parameters at the large scale and their impact on culture performance because of the poorer mixing.

(d) Use Pluronic F68 at concentrations >0.5 g l^{-1} to minimise cell damage due to bubble bursts from aeration.

(e) Use antifoam to control foaming as appropriate. In addition, if an up-pumping impeller is used at the top as recommended in (a), it may be useful if higher air flow rates are employed for reducing the tendency to foaming, as it has been shown to do with bacterial fermentations (Boon et al. 2002).

(f) Use an aspect ratio at around 1.0–1.3, thus reducing the zone of potential bubble burst damage as a proportion of the total volume of the bioreactor without compromising good blending.

Microcarrier culture

Very little has been published directly related to the large scale of operation being discussed here. Work at the small scale and theoretical considerations would suggest that because of the much larger size of the microcarriers, cell on their surfaces are more easily damaged by agitation than cells in free suspension. Recent work has, for the first time, realistically addressed the issue of optimising the agitator/vessel geometry to achieve suspension of the beads with the minimum values of $(\bar{\varepsilon}_T)_{js}$. By using down pumping hydrofoil impellers such as the Lightnin' A310 with a shallow dished base, $(\bar{\varepsilon}_T)_{js}$ is less than that shown to damage cells. However, the other processing aspects connected with agitation in animal cell culture, and addressed above for free suspension culture, may well be more demanding and have not really been addressed. It is possible that microcarrier culture will be limited to smaller scales than free suspension when all agitation issues are taken into account. It really depends on how sensitive to damage cells on microcarriers really are. Experience suggests that 'shear damage' has often been overemphasized in the past and perhaps it has been again. More work is required on the engineering of large scale microcarrier culture for those cases where free suspension is not possible.

Conclusions

The literature on animal cell culture is copious and increasing all the time. However, few papers realistically address the problem of scale-up of free suspension culture in agitated bioreactors, which have become the system of choice at the largest commercial scale now being employed (up to 20,000 L). Even fewer papers give quantitative details of actual work at this scale. This paper has tried to integrate the information in these few publications with relevant small-scale culture studies and physical studies of mixing in agitated bioreactors. It has particularly addressed issues of 'shear sensitivity', homogenisation, oxygen transfer and demand, and carbon dioxide stripping combined with pH control and osmolality as presently perceived. In these conclusions, aspects that need to be further studied are highlighted.

Higher cell densities (up to 10^7/ml) are regularly being achieved by using fed-batch or cell recycle techniques at the pilot and smaller commercial scale, leading to higher oxygen demands and therefore to the need for higher rates of oxygen transfer. This higher rate is generally being met at the large-scale by using enriched air and oxygen sparging, often through sintered spargers to give smaller bubbles and higher k_La's. This strategy has been adopted because, in spite of much work to show that cells are less sensitive to fluid dynamic generated stress than originally thought, especially if Pluronic F68 is used to prevent damage due to bursting bubbles (and which is generally accepted to a limited extent), there are still concerns regarding these issues. With this strategy, especially because of the reduced 'air' flow rate, there is insufficient flow to strip out the CO_2 produced (RQ$\approx$ 1) and as a result, dissolved CO_2 levels (>~80 mm Hg) damaging to cells build up, the pH tends to fall; and in trying to control it by alkali addition, damaging osmolality levels arise. Because stresses on cells

(which are of the order of 15 μm in size) results from turbulent flow structures of a similar size, it is possible to study such aspects in flows at the laboratory scale (≤5 L) and much work at this scale has already suggested that even higher agitation intensities (W/kg) than those currently in use could be utilised. The question of higher air flow rates (ideally constant vvm across the scale for effective stripping, which means superficial velocity, v_s, proportional to the linear dimension of the reactor) is more difficult to study at the bench scale because the equivalent v_s cannot be used at that scale. However, studies at the pilot scale should offer enough flexibility to establish the upper limits on v_s in the presence of Pluronic that can be used without damage. Foaming studies should also be undertaken at this scale. Such work is very important for establishing the cultivation of the highest cell densities at the present large commercial scales, which will no doubt be pushed even further in the near future.

Homogeneity inevitably decreases with increasing scale, especially in fed-batch bioreactors. However, recent work has established the use of a laboratory scale-down model to assess the impact of temporal and spatial pH fluctuations on cell culture. At the highest cell densities, the related problem of nutrient homogeneity will probably need addressing by this technique, as it has been with bacteria. Finally, studies using the same method should also be possible in order to fill in the matrix of questions posed by the pCO_2–pH—osmolality versus vvm—v_s-bubble burst conundrum.

Ideally, all these future studies would also be accompanied by more fundamental studies at the cellular level. How do cells respond to rapid changes in their environment, chemical and physical, over long periods of time? Advanced computational fluid dynamics (CFD) such as large eddy simulation may be able to give some reasonable estimate of these environmental questions at the large scale, though there is still significant work required in this area before that situation exists for stirred multiphase bioreactors. However, no matter how precise and accurate CFD predictions are, until the cells response to such changes is known and understood, the a priori design of very large-scale animal cell culture bioreactors will not begin to be possible. To move effectively towards an understanding of the behaviour of cells in very large-scale animal cell culture bioreactors and their a priori design, initially biochemical engineers with expertise on agitation in bioreactors should work closely with appropriate biologists and in the longer term, in conjunction with leaders in the development of CFD.

References

Amanullah A, Nienow AW, Buckland BC (2003) Mixing in the fermentation and cell culture industries. In: Paul EL, Atiemo-Obeng VA, Kresta SM (eds) Handbook of industrial mixing; science and practice, ch. 18. Wiley-Interscience, N.Y, pp 1071–1157

Assirelli M, Bujalski W, Eaglesham A, Nienow AW (2005) Intensifying micromixing in a semi-batch reactor using a Rushton turbine. Chem Eng Sci 60:2333–2339

Aunins JG, Croughan MS, Wang DIC, Goldstein JM (1986) Engineering developments in homogeneous culture of animal cells: oxygenation of reactors and scale up. Biotechnol Bioeng Symp 17:699–723

Aunins JG, Glazomitsky K, Buckland BC (1993) Cell culture reactor design: known and unknown. In: Nienow AW (ed) Proc. 3rd int. conf. on bioreactor and bioprocess fluid dynamics. Mechanical Engineering Publications Ltd., London, pp 175–190. ISBN 0 85298 873 7

Boon LA, Hoeks FWJMM, van der Lans RJM, Bujalski W, Wolff MO, Nienow AW (2002) Comparing a range of impellers for ''stirring as foam disruption'' (SAFD). Biochem Eng J 10:183–195

Boraston R, Thompson PW, Garland S, Birch JR (1984) Growth and oxygen requirements of antibody producing mouse hybridoma cells in suspension culture. Develop Biol Standard 55:103–111

Boulton-Stone JM (1995) The effect of surfactant on bursting gas bubbles. J Fluid Mech 302:231–257

Boulton-Stone JM, Blake JR (1993) Gas bubbles bursting at a free surface. J Fluid Mech 254:103–111

Chalmers JJ, Bavarian F (1991), Microscopic visualisation of insect cell-bubble interactions. ii: The bubble film and bubble rupture. Biotechnol Prog 7:151–158

Cherry RS, Papoutsakis ET (1989) Growth and death rates of bovine embryonic kidney cells in turbulent microcarrier bioreactors. Bioproc Eng 4:81–89

Chu L, Robinson DK (2001) Industrial choices for protein production by large scale cell culture. Curr Opin Biotechnol 12:180–187

Clark JM, Hirtenstein MD (1981) Optimizing culture conditions for the production of animal cells in microcarrier culture. Ann NY Acad Sci 369:33–46

Croughan MS, Hamel JFP, Wang DIC (1987) Hydrodynamic effects on animal cultures grown in microcarrier cultures. Biotechnol Bioeng 29:130–141

Croughan MS, Hamel JFP, Wang DIC (1988) Effects of microcarrier concentration in animal cell culture. Biotechnol Bioeng 32:975–982

Dey D (1998) Cell–bubble interactions during bubble disengagement in aerated bioreactors. PhD Thesis, The University of Birmingham, UK

de Zengotita VM, Schmelzer AE, Miller WM (2002) Characterisation of hybridoma cell responses to elevated pCO_2 and osmolality: intracellular pH, cell size, apoptosis and metabolism. Biotechnol Bioeng 77:369–380

Enfors S-O, Nienow AW, 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, Kamen A (1996) Dissolved carbon dioxide accumulation in a large scale and high density production of TGFβ receptor with baculovirus infected Sf-9 cells. Cytotechnology 22:53–63

Gorenflo VM, Ritter JB, Aschllman DB, Druin H, Bowen BD, Piret JM (2005) Characterisation and optimisation of acoustic filter performance by experimental design methodology. Biotechnol Bioeng 90:746–753

Gray DR, Chen S, Howarth W, Inlow D, Maiorella BL (1996) CO_2 in large-scale and high-density CHO cell perfusion culture. Cytotechnology 22:65–78

Handa-Corrigan A, Emery AN, Spier RE (1989) Effects of gas–liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles. Enzyme Microb Technol 11:230–235

Hoeks, FWJMM, Khan M, Guter D, Willems M, Osman JJ, Mommers R, Wayte J (2004) Industrial applications of mixing and mass transfer studies. Alvin. W. Nienow's Day; Stirred, Not Shaken Symposium, The University of Birmingham, Birmingham, UK

Ibrahim S, Nienow AW (1996) Particle suspension in the turbulent regime: the effect of impeller type and impeller/vessel configurations. Chem Eng Res Des (Trans I Chem E, Part A) 74:679–688

Ibrahim S, Nienow AW (2004) The suspension of microcarriers for cell culture with axial flow impellers. Chem Eng Res Des (Trans I Chem E, Part A) 82:1082–1088

Jöbses I, Martens D, Tramper JJ (1991) Lethal events during gas sparging in animal cell culture. Biotechnol Bioeng 37:484–490

Kilburn DG, Webb FC (1968) The cultivation of animal cells at controlled dissolved oxygen partial pressure. Biotechnol Bioeng 10:801–814

Kioukia N, Nienow AW, Al-Rubeai M, Emery AN (1992) The impact of fluid dynamics on the biological performance of free suspension animal cell culture: further studies. Food Bioprod Proc (Trans I Chem E, Part C) 70:143–148

Kioukia N, Al-Rubeai M, Zhang Z, Emery AN, Nienow AW, Thomas CR (1995) A study of uninfected and baculovirus-infected *Spodoptera frugiperda c*ells in T- and spinner flasks. Biotech Lett 17:7–12

Kioukia N, Nienow AW, Al-Rubeai M, Emery AN (1996) The influence of agitation and sparging on the growth rate and infection of insect cells in bioreactors and a comparison with hybridoma culture. Biotech Prog 12:779–785

Klopinger M, Fertig G, Fraune E, Miltenburger HG (1990) Multi-stage production of *Autographa californica* nuclear polyhydrosis virus in insect cell bioreactors. Cytotechnology 4:271–278

Kresta SM, Brodkey RS (2003) Turbulence in mixing applications. In: Paul EL, Atiemo-Obeng VA, Kresta SM (eds) Handbook of industrial mixing; science and practice, ch. 2. Wiley-Interscience, N.Y, pp 19–87

Langheinrich C, Nienow AW, Eddleston T, Stevenson NC, Emery AN, Clayton TM, Slater NKH (1998) Liquid homogenisation studies in animal cell bioreactors of up to 8 m^3 in volume. Food Bioprod Proc (Trans I Chem E, Part C) 76:107–116

Langheinrich C, Nienow AW (1999) Control of pH in large scale, free suspension animal cell bioreactors: alkali addition and pH excursions. Biotechnol Bioeng 66:171–179

Langheinrich C, Nienow AW, Eddleston T, Stevenson NC, Emery AN, Clayton TM, Slater NKH (2002) Oxygen transfer in stirred bioreactors under animal cell culture conditions. Food Bioproduct Proc (Trans I Chem E, Part C) 80:39–44

MacIntyre F (1972) Flow patterns in breaking bubbles. J Fluid Mech 77:5211–5228

Meier SJ, Hatton TA, Wang DIC (1999) Cell death from bursting bubbles: role of cell attachment to rising bubbles in sparged reactors. Biotechnol Bioeng 62:468–478

Meier SJ (2005) Cell culture scale-up: mixing, mass transfer, and use of appropriate scale-down models. Biochemical engineering XIV. Harrison Hot Springs, Canada

Moran E (2004) Recombinant protein production from mammalian cell culture: technologies and scale-up. Biochemical Engineering Research Network (BERN) Meeting, University College Dublin

Mostafa SS, Gu X (2003) Strategies for improved dCO_2 removal in large-scale fed-batch cultures. Biotechnol Prog 19:45–51

Newitt DM, Dombrowski N, Knelman FH (1954) Liquid entrainment. 1. The mechanism of drop formation from gas or vapour bubbles. Trans I Chem E 32:244–261

Nienow AW, Lavery M (1987) Oxygen transfer in animal cell culture medium. Biotechnol Bioeng 30:368–373

Nienow AW (1996) Gas–liquid mixing studies: a comparison of Rushton turbines with some modern impellers. Chem Eng Res Des (Trans I Chem E, Part A) 74:417–423

Nienow AW, Langheinrich C, Stevenson NC, Emery AN, Clayton TM, Slater NKH (1996) Homogenisation and oxygen transfer rates in large agitated and sparged animal cell bioreactors: some implications for growth and production. Cytotechnology 22:87–94

Nienow AW (1997a) The mixer as a reactor – liquid/solid systems. In: Harnby N, Edwards MF, Nienow AW (eds) Mixing in the process industries, 2nd edn (paperback revision, chapter 17. Butterworth Heinemann, London, pp 394–411

Nienow AW (1997b) The suspension of solid particles. In: Harnby N, Edwards MF, Nienow AW (eds) Mixing in the process industries, 2nd edn (paperback revision), chapter 17. Butterworth Heinemann, London, pp 364–393

Nienow AW, Drain SM, Boyes AP, Carpenter KJ (1997) An experimental study to characterise imperfect macromixing in a stirred semi-batch reactor. Ind Eng Chem Res 36:2984–2989

Nienow AW (1998) Hydrodynamics of stirred bioreactors. In: Pohorecki R (ed) Fluid mechanics problems in biotechnology. App Mech Rev 51:3–32

Nienow AW, Chamsart S, Patel H, Hitchcock AG, Hanak JAJ (2000) The development of a cell lysis reactor of 60 litres capacity for plasmid DNA production for gene therapy AIChE Annual Meeting, Los Angeles, Nov., Paper 284 g

Nienow AW (2003) Aeration-biotechnology. In: Kirk Othmer encyclopaedia of chemical technology, 5th edn. Wiley, New York, published in the on-line edition of the Encyclopaedia on April 18th

Nienow AW, Bujalski W (2004) The versatility of up-pumping hydrofoil agitators. Chem Eng Res Des (Trans I Chem E, Part A) 82:1073–1081

Oh SKW, Nienow AW, Al-Rubeai M, Emery AN (1989) The effect of agitation intensity with and without continuous sparging on the growth and antibody production of hybridoma cells. J Biotechnol 12:45–62

Oh SKW, Nienow AW, Al-Rubeai M, Emery AN (1992) Further studies of the culture of mouse hybridomas in an agitated bioreactor with and without continuous sparging. J Biotechnol 22:245–270

Orton D, Wang DIC (1990) Effects of gas interfaces on animal cells in bubble aerated bioreactors. The 1990 Annual AIChE Meeting, Chicago, USA, Nov., paper 103b

Osman JJ, Birch J, Varley J (2002) The response of GS-NSO myeloma cells to single and multiple pH perturbations. Biotechnol Bioeng 79:398–407

Ozturk SS (1996) Engineering challenges in high-density cell culture systems. Cytotechnology 22:3–16

Pullen KF, Johnson MD, Phillips AW, Ball GD, Finter NB (1985) Very large-scale suspension cultures of mammalian cells. Dev Biol Std 60:175–185

Radlett PJ, Telling RC, Whiteside JP, Maskell, MA (1972) The supply of oxygen to submerged cultures of BHK21 cells. Biotechnol Bioeng 34:437–445

Ramirez OT, Mutharasan R (1990) The role of the plasma membrane fluidity on the shear sensitivity of hybridomas grown under hydrodynamic stress. Biotechnol Bioeng 36:911–920

Sinskey AJ, Fleischaker RJ, Tyo MA, Giard DJ, Wang DIC (1981) Production of cell-derived products: virus and interferon. Ann NY Acad Sci 369:47–59

Telling RC, Elsworth R (1965) Submerged culture of hamster kidney cells in a stainless steel vessel. Biotechnol Bioeng 7:417–434

Telling RC, Stone L (1964) A method of automatic pH control of a bicarbonate - CO_2 buffer system for the submerged culture of hamster kidney cells. Biotechnol Bioeng 6:147–158

Tramper JJ, Joustra D, Vlak JM (1987) Bioreactor design for growth of shear-sensitive insect cells. In: Webb C, Mavituna F (eds) Plant and animal cell cultures: process possibilities. Ellis Horwood, Chichester UK, pp 125–136

van Wezel AL (1985) Monolayer growth systems: homogeneous unit processes. In: Animal cell biotechnology. Academic Press Inc., London, pp 265–281

Van't Riet K, Tramper J (1991) Basic bioreactor design. Marcel Dekker, N.Y

Varley J, Birch J (1999) Reactor design for large-scale suspension animal cell culture. Cytotechnology 29:177–205

Voisard D, Meuly F, Ruffieux PA, Baer G, Kadouri A (2003) Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells. Biotechnol Bioeng 82:751–765

Wayte J, Boraston R, Bland H, Varley J, Brown M (1997) pH effects on growth and productivity of cell lines producing monoclonal antibody: control in large-scale fermentors. Cytotechnology 22:87–94

Wu J (1995) Mechanisms of animal cell damage associated with gas bubbles and cell protection by medium additives. J Biotechnol 43:81–94

Zhang Z, Al-Rubeai M, Thomas CR (1992) Effect of Pluronic F68 on the mechanical properties of mammalian cells. Enzyme Microb Technol 14:980–983

Zhang Z, Ferenczi MA, Thomas CR (1992) A micromanipulation technique with theoretical cell model for determining mechanical properties of single mammalian cells. Chem Eng Sci 47:1347–1354

Zhang Z, Thomas CR (1993) Modelling of animal cell damage in turbulent flows. In: Nienow AW (ed) Proc. 3rd int. conf. on bioreactor and bioprocess fluid dynamics. Mechanical Engineering Publications Ltd., London, pp 475–482. ISBN 0 85298 873 7

Zhang Z, Al-Rubeai M, Thomas CR (1993) Comparison of the fragilities of several cell lines. Biotechnol Tech 7:177–182

Zhang Z, Thomas CR (1996) Micromanipulation – a powerful technique for measuring cell mechanical strength. In: Microsystem technology for chemical and biological microreactors, DECHEMA Monographs, VCH, 132, 163–176, ISBN 3-527-10226-4