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Animal-cell damage in sparged bioreactors

Yusuf Chisti

The gas sparging of culture broth causes damage to suspended animal cells. However, despite this, sparged bioreactors remain the preferred means of cell culture because sparging is a robust method of supplying oxygen, especially on a large scale. This article examines the underlying mechanisms involved in bubble-associated cell damage and the methods available for controlling such damage.

Animal cells cultured in bioreactors are widely used to produce various therapeutic proteins, vaccines and diagnostic monoclonal antibodies[1,2]. Cells need oxygen to grow and thrive, and many schemes have been developed to ensure that the cells receive sufficient levels of oxygen[2–4]. Although many options are available for supplying oxygen, sparging of the culture broth with a gas mixture remains the most practicable method of supplying oxygen[4,5], especially in large-scale culture[6–8]. (Sparging, or submerged aeration, is the process of bubbling air or another gas through a relatively deep pool of the culture broth, usually inside a bioreactor. A sparger is the device through which the gas enters the bioreactor.)

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Of the many kinds of cell-culture system available[2], stirred tanks and air-lift bioreactors are the most commonly used in commercial processes, and they both rely on sparged aeration. This situation is not likely to change in the foreseeable future because direct sparging is effective and simple. However, sparging damages animal cells. This article examines the underlying mechanisms of cell damage in sparged bioreactors and the methods that might be used to protect the cells.

Bubble-associated damage

Cell lines differ tremendously in their sensitivity to aeration[9]. In bubble-free media, mouse-cell lines are more sensitive to turbulence than human and insect cells[4]. However, in sparged bioreactors, mouse hybridomas are generally more robust than insect cells (e.g. the Sf9 cell line from *Spodoptera frugiperda*[10]). In all

cases, small bubbles (e.g. <2 mm diameter) are more damaging to cells than large bubbles[8,9,11,12] (e.g. ~10 mm diameter). Bubbles as large as 10–20 mm in diameter have been chosen for use in the commercial culture of several mouse hybridomas[8]. Large bubbles have mobile interfaces and, because they rise faster, they carry fewer attached cells to the surface. Also, larger bubbles (unlike smaller ones) do not remain on the surface as a stable foam that permanently retains any cells carried into it[8].

However, the effect of bubble size on the extent of cell damage might not be the same across cell types[13], and cell damage is also directly affected by the aeration rate[9,14,15]. Although mechanical agitation under typical culture conditions does not damage freely suspended cells in the absence of sparging[8,16,17], sparging-associated damage can be enhanced by impeller agitation, especially if the impeller disrupts gas bubbles issuing from the sparger[12]. For example, in one study using a Rushton turbine to agitate serum-free batch cultures of Sf9 cells[14], the specific growth rate was constant and independent of agitation speed until the speed reached 270 rpm (Fig. 1a). Higher speeds reduced the growth rate but, even at the highest speed, >97% of the cells remained viable for approximately 24 h into culture[14]. At a constant agitation rate of 200 rpm, aeration rates of >0.04 vvm reduced the specific growth rates (Fig. 1b). In all cases, there was enough dissolved oxygen and the specific growth rate was not sensitive to the oxygen concentration over the conditions of the experiments. In this instance, sublethal levels of hydrodynamic forces clearly affected cell growth and reproductive processes without either destroying the cells or causing other obvious physical damage.

Mechanisms of damage

The specific events that are responsible for the bubble-associated damage (e.g. bubble formation at the sparger, bubble detachment, rise through the fluid, break-up within the culture broth or events at the disengagement surface) and the mechanism(s) of damage are only now being clarified. Cell damage in a sparged system typically follows first-order kinetics; thus, the rate of cell loss (dX/dt) or damage depends on the cell concentration X at time t (Eqn 1),

$$\frac{dx}{dt} = -k_d X \qquad (1)$$

where k_d is the constant specific death rate.

Exactly how the k_d value is influenced by the operating conditions of the bioreactor and the properties of the cell are important questions that continue to occupy biochemical engineers. Using an elegant method of mechanistic analysis, Tramper *et al.*[11,18] related the first-order rate constant for cell death owing to all causes to the aeration rate in a bubble column (Eqn 2),

$$k_d = \frac{24 Q V_K}{\pi^2 d_B^3 d_T^2 h_L} \qquad (2)$$

where Q is the volumetric aeration rate, h_L is the height of fluid, d_T is the column diameter, d_B is the bubble diameter and V_k is a hypothetical killing volume surrounding the bubbles (the total volume of fluid
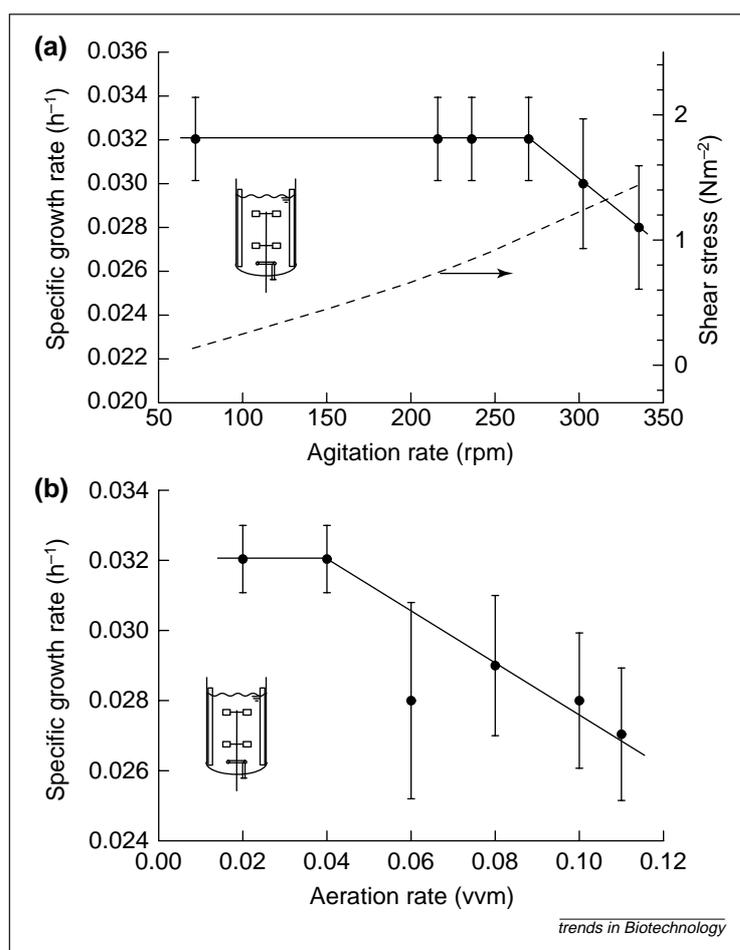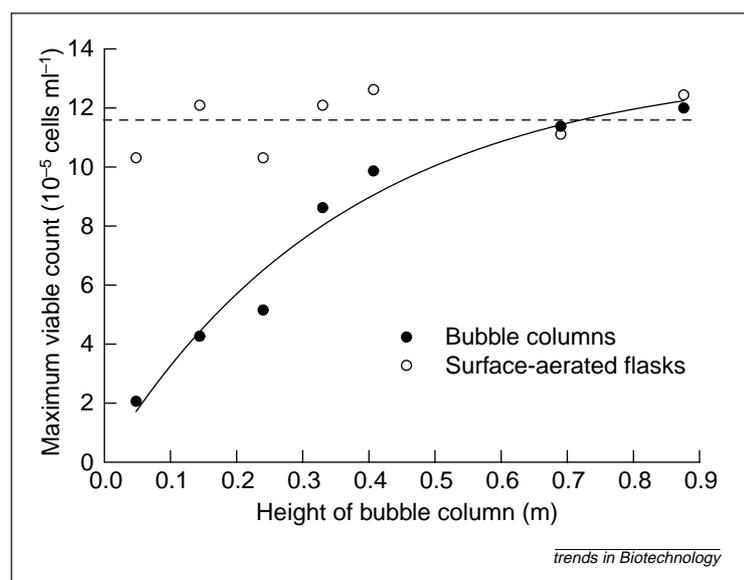

Figure 1

(**a**) Effect of Rushton-turbine agitation speed on the specific growth rate of *Spodoptera frugiperda* Sf9 cells in serum-free batch culture. (The vessel had two turbine impellers.) The aeration rate was constant at 0.03 vvm (volume per volume per minute). The variation of shear stress with impeller speed is also shown (dashed line). (**b**) The effect of aeration rate on the specific growth rate of *S. frugiperda* Sf9 cells in serum-free batch culture. The Rushton-turbine agitation rate (two turbines) was constant at 200 rpm ($\tau = 0.66$ N m^{-2}). (Modified, with permission, from Ref. 14.)

associated predominantly with the bubble wakes – the cells trapped in the circulating wake are carried with the bubble to the surface, where most of the damage takes place). According to Eqn 2, the specific cell-death rate is proportional to the frequency of bubble generation ($6Q/\pi d_B^3$). Alternatively, because the bubble-generation frequency at a hydrodynamic steady state is equal to the frequency of rupture at the surface, the death rate can be interpreted as depending on the frequency of bubble rupture and the killing volume associated with the bubbles.

Based on Eqn 2, the death-rate constant should decline with increasing height of fluid, as experimentally confirmed for insect cells[11,18], myelomas[19] (Fig. 2) and hybridomas[13,19,20]. This supports the view that cell death occurs predominantly in the bubble-disengagement zone that exists at the surface[9,13]. For a myeloma grown in serum-supplemented medium, the minimum culture height that provided a performance equivalent to that of surface-aerated flasks was ~0.7 m (Fig. 2), corresponding to a bubble-column aspect ratio of ~14 (Ref. 19). If the bubble rise was the main contributor to cell death, the specific cell-death rate would be

Figure 2

Effect of the bubble-column height on the maximum viable cell concentration achieved during culture of a myeloma in medium supplemented with 5% v/v foetal calf serum. The cells were inoculated at 2×10^5 cells ml^{-1}. The specific power input in bubble columns was ~0.42 W m^{-3}. The dashed line is the mean value of the maximum cell count in surface-aerated-flask controls. (Reproduced, with permission, from Ref. 19.)

independent of the height of fluid[21]. Because this is contrary to experimental observations, rise events are unlikely to be the principal contributors to cell damage[21].

If gas entry at the sparger was the main contributor to cell damage, decreasing the number of sparger holes at a given gas-flow rate in the reactor would increase the specific cell-death rate constant[21]. In bubble columns, Jöbses et al.[21] noted that increasing the sparger–hole gas velocity from 0.6 m s^{-1} to 2.5 m s^{-1} at a constant volumetric gas flow in the reactor had no effect on the first-order rate constant for cell death. Therefore, events at the sparger can be regarded as not contributing to cell damage. This process of elimination has thus identified bubble break-up at the culture surface as the principal cause of damage to suspended cells in sparged bioreactors such as bubble columns[21].

Equation 2 also applies to air-lift devices[15], especially those in which the gas is not carried into the down-comer. Because most of the agitated tanks used in animal-cell culture operate at relatively low mechanical-power inputs and the impeller does not usually serve as a bubble-breaking device, Eqn 2 should provide a lower limit on k_d in such tanks[17]. As confirmed for Chinese hamster ovary (CHO) cells in mechanically agitated reactors, cell–bubble interactions within the bulk fluid do not appear to affect cells significantly even in the absence of protective additives[16]. In particular, bubble coalescence and break–up within the bulk culture cause no significant damage[16].

Although data do suggest that the bubble-rise event can be disregarded as a substantial contributor to cell damage, most of the supporting evidence was obtained in fairly short columns whose heights did not vary by more than 2 m. Other reasoning indicates that cells adhering to rising bubbles are, in fact, damaged by the rise event, even though the damage is not easily revealed in the relatively short columns that are typically used to quantify bubbling-associated cell death[22]. In bioreactors that are more than 1 m tall, the rise event can contribute more substantially to the observed damage. Clear evidence on the effects of bubble rise is lacking. However, the turbulence in the circulating fluid carried behind a rising bubble can be sufficiently intense that cells might be damaged when the bubble is less than ~0.7 cm in diameter. For small bubbles only, the microeddy length scale approaches the dimension of the cell (Box 1).

Using a hybridoma culture, van der Pol et al.[20] showed that the first-order death-rate constant increased with increasing $1/d_T^2$ in bubble columns, as predicted from Eqn 2. This equation also suggests that the cell damage increases in direct proportion to the superficial gas velocity in the column or to the specific power input. Increasing the liquid level at a constant molar aeration rate in a column of fixed diameter decreases the specific power input[23–25]. Equation 2 further suggests that, for a given V_k, large bubbles should be less damaging than small bubbles, which agrees with a substantial body of empirical evidence[8,11–13,18,26].

Equation 2 is perhaps best interpreted as follows: at a fixed volumetric flow rate (Q), the specific power input in a bubble column with a uniform diameter is independent of the culture height. However, as the height increases, the hydrostatic pressure increases and the mass–flow rate of the gas must increase to maintain a constant Q. With an unchanged specific power input but an increased mass–flow rate of the gas, the mean bubble size must increase in the upper region of the column. Not only would this change in bubble diameter reduce the frequency of bubble rupture but it would also cause the damaging effect per bubble to decline greatly, because larger bubbles release less energy on rupture[17]. These two effects – a lower rupture frequency and a reduced energy of the rupture event – cause a reduction in the death-rate constant, k_d. Equation 2 disregards the properties of a cell (e.g. diameter, strength) as having no effect on sparging-associated damage but such influences might exist. Indeed, for a given cell line in unaerated turbulent capillaries[27] and aerated, agitated fluids[28], cell size has been shown to affect the ease of cell rupture.

The cell-death rate has been shown to increase linearly with increasing aeration rate[11,18,26]. In addition, the killing volume (V_k) has been shown to be independent of the aeration rate and the height of fluid, provided that the bubble diameter is not affected[11,18]. The killing volume is thought to depend on the bubble diameter, increasing with diameter but in such a way that the ratio V_k/d_B^3 declines as the bubble diameter increases. This has been confirmed by Wu and Goosen using S. frugiperda insect cells in bubble columns that were sparged with bubbles of different mean diameters while maintaining a constant volumetric gas flow rate[26]: increasing bubble diameters reduced the specific death rate (Fig. 3). In view of the numerous contrary observations, predictions of reduced cell damage with decreasing bubble size are clearly incorrect[17].

The dimensionless specific killing volume ($6V_k/\pi d_B^3$) in Eqn 2 depends on the concentration of serum in the

Box 1. Energy dissipation in the bubble wake

The energy-dissipation rate in the wake carried behind a bubble depends on the drag force experienced by the bubble. The drag force F_D can be calculated as follows (Eqn 1),

$$F_D = \frac{C_D U_T^2 \rho_L A_p}{2} \qquad (1)$$

where C_D is the drag coefficient, U_T is the terminal rise velocity of the bubble, ρ_L is the density of the medium and A_p is the projected area of the bubble. For a bubble of diameter 0.3–5 cm, the drag coefficient is roughly constant at ~2.6. The projected area is obtained using Eqn 2.

$$A_p = \frac{\pi d_B^2}{4} \qquad (2)$$

The terminal rise velocity of an air bubble with a diameter of 0.2–8 cm in a water-like medium depends on the bubble diameter d_B, in accordance with Mandelson's equation (Eqn 3),

$$U_T = \sqrt{\left(\frac{2\sigma}{d_B \rho_L} + 0.5 d_B g\right)} \qquad (3)$$

where σ is the gas–liquid interfacial tension and g is the gravitational acceleration. The volume of the bubble wake is approximately 0.8 times the bubble volume. Most of the drag is associated with the circulating fluid wake behind the rising bubble, and the energy-dissipation rate E in the wake can be approximated using Eqn 4.

$$E = F_D U_T \qquad (4)$$

The specific energy-dissipation rate in the wake can now be calculated (Eqn 5).

$$\text{Specific energy dissipation rate} = \frac{E}{\text{wake volume}} = \frac{6E}{0.8\pi d_B^3} \qquad (5)$$

If isotropic turbulence is assumed to exist in the bubble wake, the

energy-dissipation rate can be used to calculate the length scale λ of the microeddies (Eqn 6),

$$\lambda = \left(\frac{\mu_L}{\rho_L}\right)^{3/4} \epsilon^{-1/4} \qquad (6)$$

where μ_L is the viscosity of the medium and ϵ is the energy-dissipation rate per unit mass (i.e. E/ρ_L). If the calculated length scale of the microeddy is larger than the typical dimensions of a cell, the cell is unlikely to be damaged by turbulence in the wake. However, damage can occur if the dimensions of the eddies approach those of the cell.

The calculated values of the energy-dissipation rate and the corresponding eddy-length scale are shown in Fig. I for bubbles of various diameters. As shown, the microeddy size remains greater than ~20 μm when the bubbles are between 0.7 cm and 3 cm in diameter. In comparison with this, the suspended cells such as hybridomas tend to be between 10 μm and 17 μm in diameter, or significantly smaller than the eddies. Consequently, no cell damage is expected during rise of a bubble that is ≥0.7 cm in diameter.
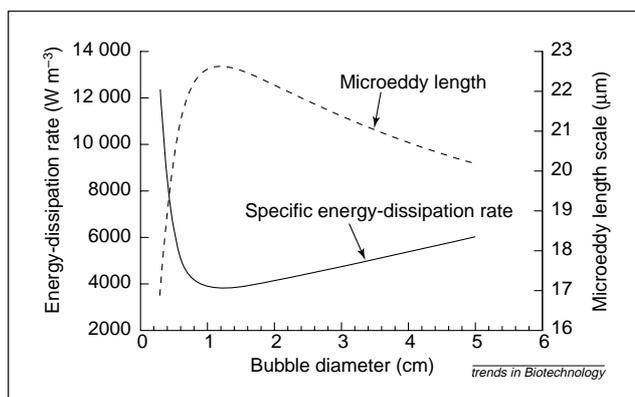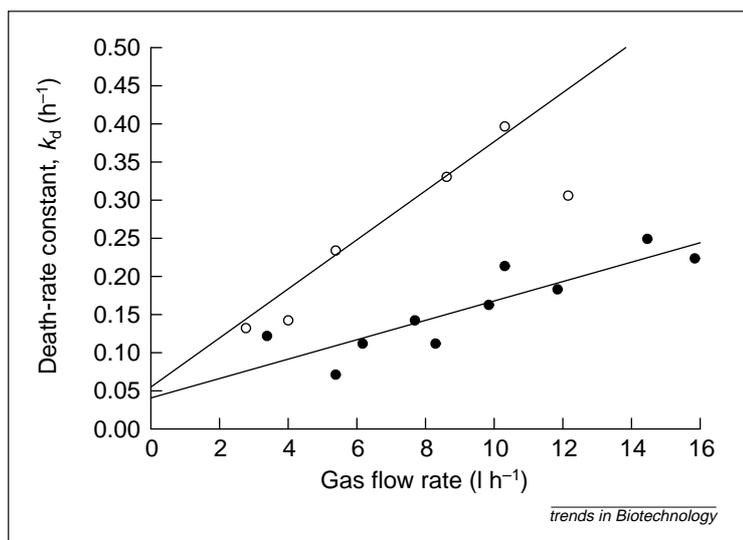

Figure I

The specific energy-dissipation rate and the microeddy length scale in the wake of a rising bubble as functions of bubble diameter.

culture medium. van der Pol et al.[20] showed that the dimensionless killing volume declined as the serum concentration was raised from 0% to 2.5% v/v; the dimensionless killing volume in the absence of serum was ~2.7 times that at 2.5% serum in a hybridoma culture[20]. This and other similar observations[15] at least partly explain the well-known protective effect of serum (Fig. 4), and the cell-protective effects of other proteins can be similarly explained[17]. Serum-free media with 1 (g protein) l⁻¹ (equivalent to supplementing the medium with 2% v/v serum) are commercially used in sparged air-lift culture vessels[7].

In an alternative approach, Wang et al.[29] modelled the aeration-associated cell damage as being proportional to the interfacial area of the bubbles in a sparged system. In view of substantial evidence[17], turbulence in the bulk liquid was disregarded as a contributor to damage. The model did not distinguish between the various potentially damaging events associated with bubbling (e.g. coalescence and break-up, formation at the sparger, rupture at the surface) and so did not identify a specific damage mechanism. Their approach was conceptually similar to the 'killing volume' model
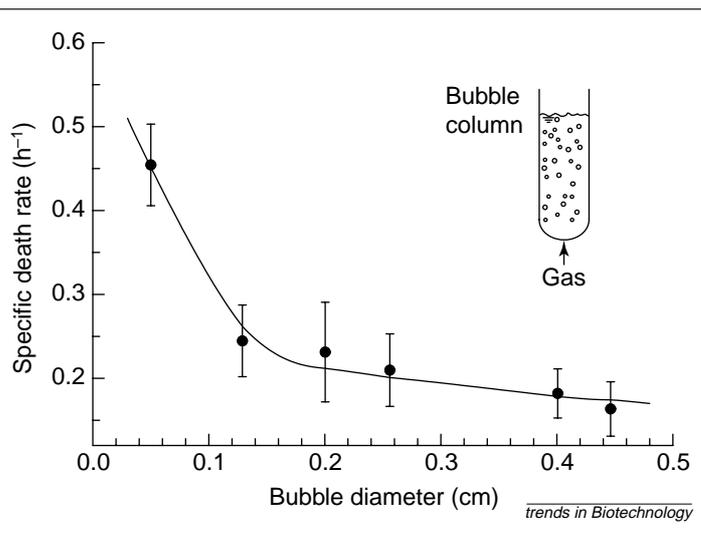
Figure 3

The effect of bubble diameter on the specific death rate of *Spodoptera frugiperda* insect cells in bubble columns aerated at a constant volumetric gas flow rate of 10 ml min⁻¹. (Adapted, with permission, from Ref. 26.)

Figure 4

The effect of foetal-calf-serum concentration and air-flow rate on the first-order death-rate constant of a mouse–mouse hybridoma in a draft-tube air-lift bioreactor. Open circle, 2% serum; closed circle, 3% serum. (Reproduced, with permission, from Ref. 15.)

proposed by Tramper *et al.*[18]: that a cell-inactivation zone exists around bubbles and that cells in this zone could be damaged or inactivated during bubble formation, coalescence, break-up or rupture[29]. The model suggested a first-order deactivation of cells in which the specific death rate k_d depended on the specific gas–liquid interfacial area (a_L) (Eqn 3),

$$k_d = \left(\frac{k_c s}{K_m} \right) a_L \qquad (3)$$

where k_c is an intrinsic cell-deactivation rate constant (cells $m^{-3} s^{-1}$) that depends on the type of cell and the culture conditions. The parameter s in Eqn 3 is the equivalent thickness (in m) of the inactivation zone around a bubble and K_m is a Michaelis–Menten-type saturation constant (cells m^{-3}). The parameter s presumably depends on the operating conditions, such as viscosity and also on bubble size. Using data for hybridomas in agitated vessels, Wang *et al.*[29] showed that k_d increased with increasing specific interfacial area.

Although cell damage is commonly found to have first-order kinetics, this does not necessarily support the inactivation mechanisms set out in the Wang *et al.*[29] model. Almost every cause of damage, irrespective of the specific cells involved, produces a first-order decline in the number of viable cells[11,18,20,21,30–37]. Wang *et al.*[29] concluded that the principal determinants of cell damage were the cell–bubble encounter rate, the rate of bubble break-up within the fluid and the bursting rate at the surface. Cell death correlated linearly with specific gas–liquid interfacial area, with the proportionality constant being 0.0125 m h^{-1} for a mouse hybridoma[29]. Interestingly, Eqn 3 suggests a declining death rate with increasing bubble diameter in a reactor with a given gas holdup, ϵ_G. This is because the specific interfacial area a_L depends on the gas holdup and the bubble size[23] (Eqn 4),

$$a_L = \frac{6\epsilon_G}{d_B(1 - \epsilon_G)} \qquad (4)$$

Thus, if s in Eqn 3 is assumed to be independent of bubble diameter, k_d should decline with increasing d_B; Eqn 4 is based on fundamental principles.
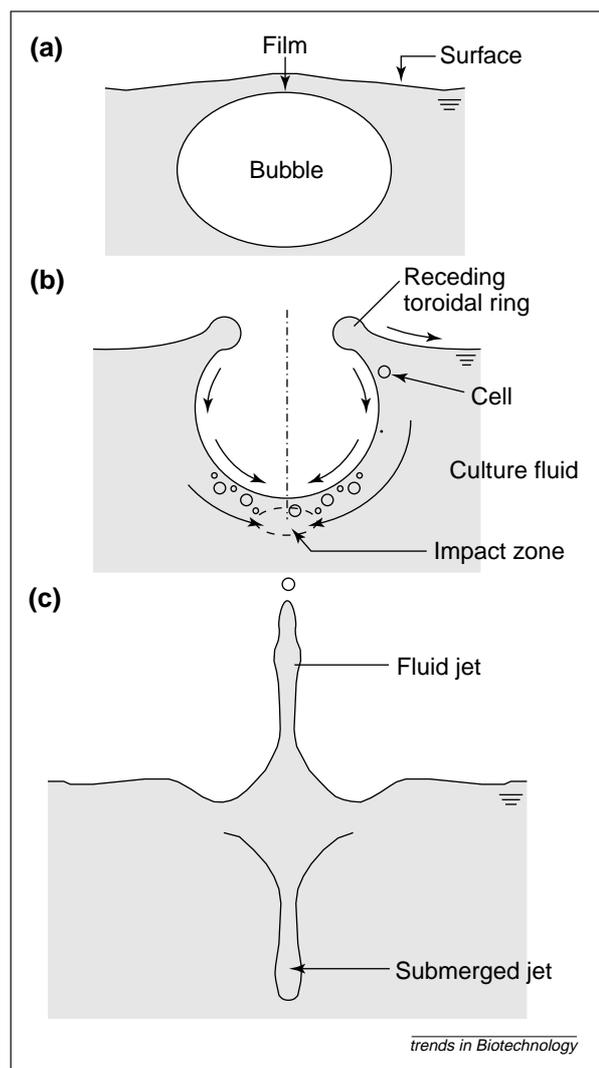
Bubble rupture

The reason for the rupture of large bubbles being less damaging to cells than the breakup of small bubbles has become clear through the analysis of the mechanics of rupture[38,39]. Numerical simulations and experimental observations of bubble rupture at an air–water interface confirm that smaller bubbles rupture considerably more violently than large ones[38,39]. Bubbles reaching the surface rise to different heights above the flat liquid surface before rupturing. For a bubble that is stationary at the surface, a portion is always submerged below the surface of the liquid; the degree of submergence is generally greater for smaller bubbles.

During rupture, the liquid drains from the film between the raised liquid surface and the bubble[40,41]. As the film thins, a hole develops at the centre of the dome and the film rapidly retreats into the bulk liquid (Fig. 5). The rupture of the film and the consequent elongational and accelerational flow have been postulated to damage cells in the film and those adhering to the bubble cavity[40]. A cell on the surface of a rupturing film would apparently not experience any motion until struck by the toroidal ring of fluid at the receding edge of the film[40]. The force experienced by the cell will depend on the film thickness at the instance of the rupture[40]: the rupture of thinner films is likely to be less damaging than that of a thicker film.

Once the bubble ruptures, fluid accelerates rapidly and moves down the interior walls of the bubble cavity (Fig. 5). The flow from around the walls impacts at a stagnation point located immediately below where the bubble had been (Fig. 5). The high pressure produced by the impacting streams forces a jet of fluid upwards into the gas phase above the liquid surface (Fig. 5); a second, opposing jet is forced into the fluid below the stagnation point. Intense accelerational flows around the bubble cavity and the formation of liquid jets occurs most during the rupture of small bubbles, with bubbles larger than ~6 mm rupturing less violently.

According to Boulton-Stone and Blake, 'one of the most important factors determining the motion following film rupture, in terms of the energy released, is the height of the top of the bubble above the equilibrium free-surface [of the liquid]'[38]. Bubbles rupturing lower down, beneath the equilibrium surface of the liquid, release more energy because of their higher internal pressure, and this energy is released as high-speed liquid jets. Bubbles larger than about 6 mm bursting at the surface do not form significant jets[38], which is consistent with numerous observations dating from the 1950s and earlier.

When a jet is produced, the speed of the jet declines as the size of the rupturing bubble increases; for example, for 1 mm air bubbles rupturing on water, a maximum jet speed of 6.4 m s^{-1} has been calculated and, for the rupture of 6 mm bubbles, the maximum jet speed declines to 0.94 m s^{-1} (Ref. 38) (these speeds are for jets ejected into the atmosphere above the liquid). The maximum pressure produced during the bursting of bubbles of various sizes declines with increasing bubble diameter; the maximum energy-dissipation rate
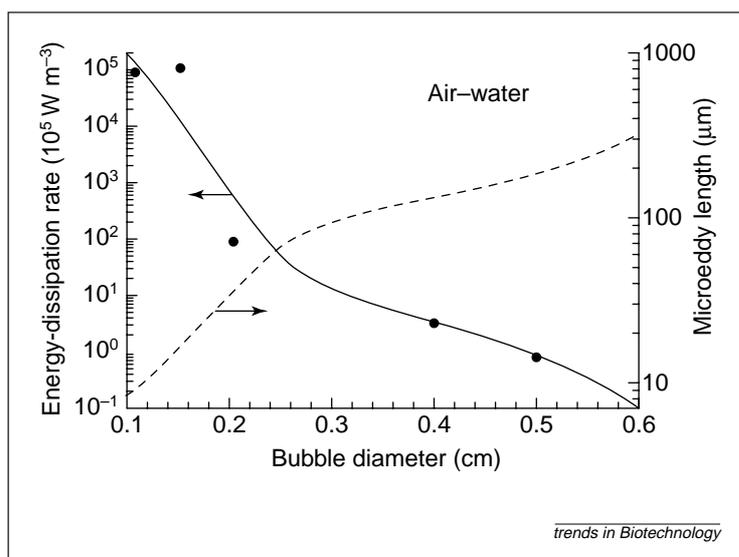
Figure 5

Events during the rupture of a bubble. (**a**) Bubble arrives at the surface and rises so that the film between the bubble and the surface thins. The height of the rise and the film thickness depend on the bubble size; smaller bubbles rupture deeper. (**b**) A hole develops in the film and the toroidal ring of the rupture vent expands and recedes into the bulk fluid. Streams of fluid draining into the bulk fluid impact beneath the bubble, generating intense local turbulence and creating a jet of fluid (**c**); a similar jet is produced underneath the liquid surface. When produced, the jet might rupture near the top to form droplets.
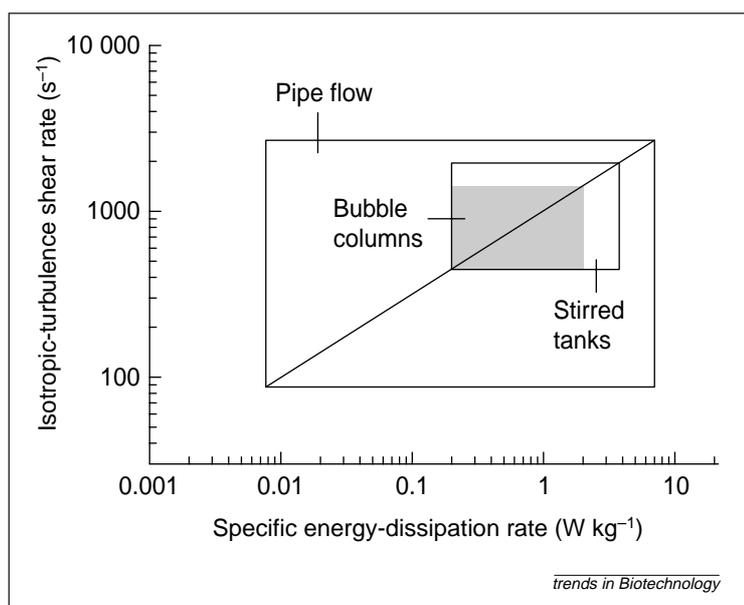

Figure 6

The maximum energy-dissipation rates produced during the bursting of air bubbles of various sizes on the surface of water. The dashed line shows the length scale of micro-eddies associated with the bubble-burst event. (Adapted, with permission, from Ref. 38.)

occurs just beneath the bubble immediately before the jet forms[38]. The calculated maximum energy-dissipation rates for the smallest bubbles are equivalent to a stress of approximately 10.4 N m^{-2} (Ref. 38). The maximum energy-dissipation rates decline with increasing bubble diameter (Fig. 6).

The Kolmogoroff eddy-size approach might also be usefully applied to bubble-rupture-associated cell damage, despite assertions to the contrary[42]. Thus, a microeddy length scale can be calculated[17] from the specific energy-dissipation rates for the rupture of bubbles of various diameters (Fig. 6). As expected, the eddy-length scale approaches typical cell diameters (10–20 μm) only for the rupture of small bubbles and it is precisely such bubbles that have been implicated in damaging cells. However, there is still no direct,

conclusive evidence for links between the dimensions of microeddies, cell diameters, bubble sizes and observed damage. Volumetric specific energy-dissipation rates as high as 10 kW m^{-3} (10 W kg^{-1}) do not occur on a global scale in the usual types of bioreactor (Fig. 7) but, locally (e.g. behind a rupturing bubble), such rates can easily be encountered. Similarly, turbulence might not be isotropic in a process equipment as a whole[24] but zones of local isotropic turbulence can be found in regions of intense energy dissipation[17].

A violent bubble-rupture event would be inconsequential if there were no cells in the vicinity. However, unfortunately, cells attach to rising bubbles, and the fluid behind bubbles is particularly rich in cells. That some animal cells will attach to rising bubbles has been shown with *Trichoplusia ni* and *S. frugiperda* insect cells[40,43,44]. The cells adhered to rising microbubbles of the type used commonly in froth flotation (diameter ⩽3 mm). Hydrophobic interactions are apparently responsible for cell attachment, a mechanism that has also been observed with ore particles attaching to bubbles during foam flotation. Very small bubbles are generally not suited to the aeration of cell cultures[8], but larger bubbles also transport cells to the surface. However, this is not predominantly as adhering cells. Instead, the cells are trapped in the circulating wake carried behind large bubbles. The fluid associated with the wakes is known to have a higher particle concentration than the bulk fluid[39,44].

More than 95% of the cells in the liquid ejected as a jet during the rupture of bubbles have been shown to be nonviable even when the cells in the bulk culture contained fewer than 10% dead cells[39,44]. Clearly, the cells carried in the bubble wake are destroyed by the rupture event. Based on simulations of bubble ruptures for diameters of 0.77 mm, 1.70 mm and 6.32 mm, Garcia-Briones *et al.* calculated maximum energy-dissipation rates of 9.52 × 10^7 W m^{-3}, 1.66 × 10^7 W m^{-3} and 9.40 × 10^4 W m^{-3}, respectively[39]. Comparing these values with energy-dissipation rates that have damaged

Figure 7

The relationship between isotropic-turbulence shear rate and the specific energy-dissipation rate is the same (solid line) in various culture devices, but the devices operate in different ranges of specific energy-dissipation rates (boxes) along the diagonal line. The plot is for a water-like culture fluid (density 10^3 kg m^{-3}; viscosity 10^{-3} Pa s).

cells in various unaerated flow devices (capillaries and viscometers), Garcia-Briones et al. realized that values of the order of 5.81×10^2 W m^{-3} did not damage the cells significantly, whereas appreciable damage occurred when the energy-dissipation rate exceeded $\sim 2.25 \times 10^4$ W m^{-3} (Ref. 39).

Clearly, therefore, a 6.32 mm bubble still has a significant potential to damage cells and a smaller bubble would produce instantaneous damage. For example, in gas-free capillary flow, more than 80% of the cells died within 0.3 s of exposure to energy-dissipation rates of about 4.80×10^7 W m^{-3} (Refs 39,45). Interpreting data from several sources, Garcia-Briones et al. further noted that average energy-dissipation rates of 2990–29 000 Wm^{-3} have generally produced a significant decline in growth rate in impeller-agitated bioreactors[39]. These dissipation rates are average values and the cells probably experienced much higher local energy-dissipation rates in the vicinity of the impellers.

If a stable foam is not allowed to form and relatively large bubbles (~10–20 mm diameter) are used for aeration so that the energy dissipated during rupture remains below the cell-damaging threshold, the cells carried in the wake will not be damaged or removed into a foam layer. For bubbles of a given size, the volume of the circulating wake can be reduced by increasing the viscosity of the culture broth: small increases will reduce the rise velocity of the bubbles and the circulation in the wakes will be slowed down. This means that slight enhancements in viscosity could reduce the transport of particles in the wake. This might be one explanation for why viscosifying additives have frequently been found to reduce cell damage. Also, for a given volumetric aeration rate, the use of larger bubbles reduces bubbling frequency and so reduces the frequency of bubble rupture.

Controlling cell damage

Various approaches have been taken to reduce bubble-rupture-associated damage to cells. Some common approaches include the use of cell-protective additives, compartmentalization of the culture into cell-free and cell-containing zones (with sparging confined to cell-free regions[46–48]), the use of bubble-free oxygenation through microporous or diffusion tubing made of silicone rubber or another polymer[4], the use of liquid oxygen vectors such as perfluorocarbons[4], and surface aeration[4].

Alternatives to bubbling are typically only suited to specific cases. Direct sparging remains the predominant method of aeration of cell cultures and is not likely to be displaced in the foreseeable future[5]. Immobilizing cells within protective capsules and macroporous spheres is another method that has been tested on many different cells[49]. However, such methods are not particularly suited to most metabolically active cells because the capsule and the immobilizing gel matrix greatly impede the mass transfer of nutrients and products[4]. Also, immobilization can be expensive and difficult, and, because of cell growth, the structure of the immobilizing beads tends to be unstable.

One adaptation of submerged aeration is the 'bubble bed' bioreactor, which can substantially reduce the surface rupture of bubbles in short-term batch cultures[50]. The bubble-bed device (Fig. 8) is similar to a concentric draft-tube air-lift bioreactor, but a downward-pumping axial-flow impeller located in the draft tube circulates the fluid. The downward liquid flow in the draft tube is sufficiently fast that gas bubbles injected into the draft tube cannot escape; hence, the bubbles do not rupture at the surface of the culture. Pure oxygen or oxygen-enriched gas is used to reduce the required gas-injection rate to very low values. The gas holdup in the downcomer increases during batch operation and a noncirculating bed of bubbles develops. The cross-sectional area of the draft tube increases towards the bottom (Fig. 8) and so the velocity of the liquid falls as it moves down; thus, the downward-flowing liquid cannot drag the bubbles out of the draft tube and into the bubble-free annular zone.

According to Sucker et al.[50], the cell growth and antibody production in bubble-bed devices is comparable to values obtained in surface-aerated spinner flasks, even when the sparged culture in the bubble-bed units is not supplemented with serum, Pluronic F68 or other protective additives. The bubble-bed bioreactor is certainly an elegant concept but it is of limited usefulness for long-term continuous culture or extended batch culture. In most large-scale operations, direct sparging of culture is unavoidable and cell damage must be controlled through a judicious choice of aeration regime (aeration rate, gas composition, bubble size and frequency, and location of sparger) and the use of suitable cell-protective agents.

Cell-protective additives

Bubble-associated damage can be significantly reduced by adding shear-protective substances to the culture medium. Many additives have been examined for shear-protective function[51,52] and some have been in use for ~40 years (Box 2). The protective effect depends on the properties of the specific additive, the

concentration used, the type of cell and the mechanism(s) of protection (with some additives, multiple modes of protection cannot be ruled out). The protective action can be purely physical or physiological, or several kinds of physical and physiological protective mechanisms can operate simultaneously[17].

Serum and serum proteins

A concentration-dependent protective effect has been widely observed for foetal calf serum, horse serum and other types of serum in aerated and bubble-free suspension cultures of many types of animal cell[13,15,20,31,51,53–56]. Typically, protection increases with increasing serum concentration (Fig. 4) up to 10% v/v serum. In one study, a reduction in foetal calf serum level from 2.5% to 0% substantially increased the death-rate constant of a hybridoma in aerated culture[20]. Investigations revealed that serum had no long-term biological effect but did have a direct, nonspecific physical protective effect[20]. Thus, cells growing in low-serum medium were immediately protected upon transfer to a medium with 2.5% serum.

The physical protective effect of serum has been claimed to originate in its ability to reduce plasma-membrane fluidity[55,56]. According to Martens et al.[15], serum has an immediate physical protective effect but also a physiological protective effect that takes longer to become effective. The latter finding concurs with the results of Michaels et al.[54] In bubble-free experiments in a Couette viscometer (shear rate = 5000 s^{-1}, shear stress = 5 N m^{-2}), prolonged exposure of cells to foetal calf serum reduced their shear sensitivity in laminar-flow studies[54]. However, a shorter (30–120 min) exposure to foetal calf serum did not affect the shear tolerance of cells. Based on these results, the protective effect of foetal calf serum was claimed to have both physical and biological (biochemical) origins[54].

The precise nature of the physical protective effect of serum is not clear. Supplementation with serum enhances the viscosity of the culture medium, but the effect is small[3,57]. Nevertheless, Croughan et al.[53] and others[58,59] have associated the observed protection with the turbulence-dampening effect of serum. By contrast, other evidence suggests that there is no relationship between the degree of protection afforded by the serum and the enhancement of viscosity[60,61]. However, supplementing the culture medium with a large amount of serum (e.g. 10% v/v) will dampen impeller-generated turbulence, as shown by the direct measurements of root-mean-square velocity fluctuations[62], and a similar effect might operate behind rupturing bubbles. In addition to dampening turbulence, the serum proteins can coat the cells so that the fluid eddies no longer penetrate to the vicinity of the cell membrane[53]. In one study, increasing the concentration of foetal calf serum from 0% to 10% v/v reduced the plasma-membrane fluidity of a hybridoma line, increasing the cells' resistance to shear damage[55,56].

In addition to whole serum, serum components such as albumin also protect cells[37]. Hülscher et al.[63] showed in an air-lift vessel that supplementing the serum-free culture medium with bovine serum albumin (BSA) at ≥0.4 g l^{-1} improved the growth of a hybridoma. Average wall-shear rates were reduced by BSA supplementation but this effect was largely independent of the
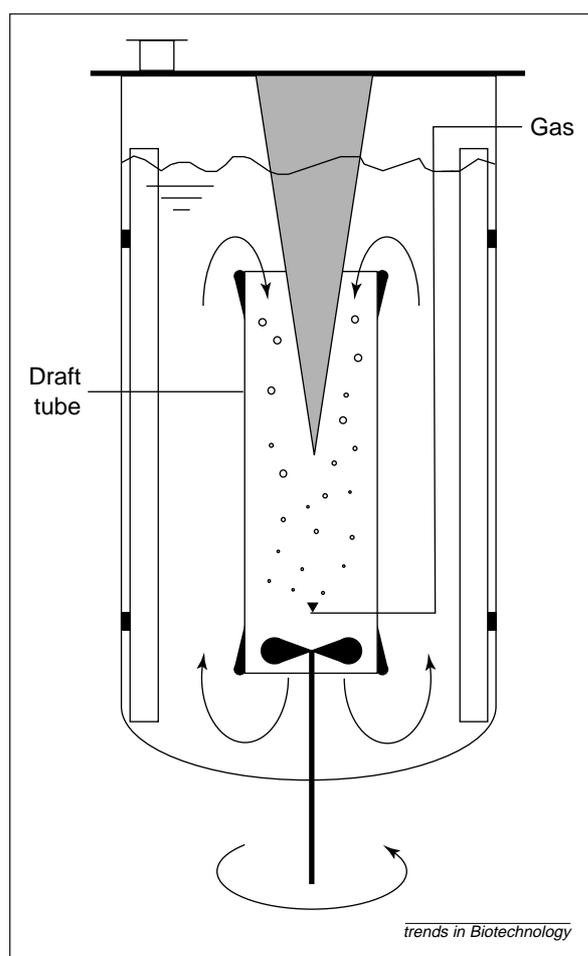
Figure 8

A bubble-bed reactor. The gas is sparged at the bottom of the draft tube. The bubbles are prevented from leaving the draft tube at the top because they cannot rise against the downward flow produced by the propeller. The bubbles do not exit at the bottom of the draft tube because the wider cross section of the tube near the bottom slows down the liquid flow sufficiently that the bubbles are not dragged out.

BSA concentration over the range 0.1–1.0 g l^{-1}, even though the cell protection occurred from 0.4 g l^{-1}. (A protein concentration of 1 g l^{-1} is equivalent to ~2% v/v serum, and so 0.2–2.0% serum should be enough to reduce turbulence in boundary layers next to a ridged surface. However, at least at this level, turbulence dampening alone might not be a sufficient explanation for the observed improved growth.)

Box 2. Media additives associated with cell protection

Derivatized celluloses (e.g. methyl cellulose)
Derivatized starches (e.g. hydroxyethyl starch)
Dextrans
Pluronics
Poly(vinyl alcohol)s
Poly(vinyl pyrrolidone)
Poly(ethylene glycol)s
Proteins (e.g. serum albumin)
Serum

At an aeration power input of ~9 W m^{-3} in the BSA-supplemented medium [1 (g BSA) l^{-1}], the mean shear rates in the downcomer zone of the reactor were ~100 s^{-1} and were independent of height[63]. For the same conditions, the mean wall shear-rates in the riser zone varied axially from ~600 s^{-1} at 0.06 m from the sparger to ~100 s^{-1} approximately midway up the riser. Although the BSA concentration over the range 0.1–1.0 g l^{-1} had almost no effect on the mean wall shear rate, the concentration affected the probability distribution of the shear rates 0.06 m above the sparger: higher concentrations produced narrower distributions[63]. These results were obtained in a concentric draft-tube air-lift device that was sparged in the draft tube. The aspect ratio of the vessel was ~7 and the ratio of the riser and downcomer cross-sectional areas was ~0.7.

Although serum and serum components might be satisfactory for use in cell cultures for producing certain *in vitro* diagnostics, they are best avoided in cultures that produce therapeutic, protective and diagnostic proteins for use *in vivo*. Serum and its components can contaminate the product with various etiological agents that are not easily detected[64–66].

Chemical agents
Pluronic® F68

Another well-known shear protectant is the nonionic surfactant polyol Pluronic® F68, a block copolymer of poly(oxyethylene) and poly(oxypropylene). Pluronic F68 has a molar mass of 8358 Da and a critical micelle concentration of ~9.2 g l^{-1}. The central poly(oxypropylene) block of the molecule is lipophilic and the poly(oxyethylene) blocks are hydrophilic[67]. In addition to Pluronic F68, several other pluronics are known to function as shear protectants[68], but Pluronic F68 is the most widely used. The various pluronics differ in the relative sizes of the lipophilic and hydrophilic blocks of the molecule[67].

Highly pure Pluronic F68 is added to cell culture media[3,9,13,21,51,54], typically at 0.5–3 kg m^{-3}. The protective effect of the surfactant is concentration dependent, increasing with concentration but levelling off at ~0.5 kg m^{-3}. Only pluronics with a hydrophilic–lipophilic balance (HLB) of >18 are effective protectants, and those with HLB values of ≤18 have been shown to lyse insect cells[67,68]; Pluronic F68 has an HLB of >24 (Ref. 67). Impurities in commercial grades of pluronics (e.g. aldehydes, formic acid and acetic acid) are toxic to animal cells[67]. These contaminants can be removed from aqueous solutions of Pluronic F68 by silica-gel column adsorption[67]. Because steam sterilization of pluronics can produce peroxide derivatives[67], media containing pluronics should be sterilized by membrane filtration.

The protective effect of Pluronic F68 is commonly associated with its ability to suppress the attachment of cells to bubbles[13]: in the presence of the surfactant, fewer cells are carried to the surface, where much of the cell damage occurs in aerated culture. However, there is evidence for other protective mechanisms. For example, a cell-protective effect of Pluronic F68 has been observed even in unaerated culture[21,55]. In one case, this effect was observed in a strongly agitated culture sample in which, although no bubbles were

present, a deep vortex was drawn into the fluid by the marine impeller used[21]. Extensive cell damage occurred but the damage was significantly reduced when the culture was supplemented with Pluronic F68 (Ref. 21).

Based on these observations, Jöbses *et al.*[21] concluded that the protective effect of Pluronic F68 was due to its direct influence on cells and to some effect of the surfactant on the gas–liquid interface. One possible explanation for this protection in the absence of bubbles is that the damage might have taken place at the rapidly moving surface of the vortex[69], and that supplementing the culture with Pluronic F68 reduced the cells' tendency to adhere to the new surface. Hence, fewer cells could have attached to the surface during its formation at the periphery of the vessel. Unlike previous observations[21,55], Kioukia *et al.*[10] saw no benefit of Pluronic F68 in mechanically stirred unaerated cultures of *S. frugiperda* cells, but the surfactant essentially eliminated bubble-associated cell damage in sparged cultures.

Using flow cytometry, Al-Rubeai *et al.* showed that the leakage of a positively charged dye (fluorescein diacetate) from mouse hybridomas increased with the intensity of agitation[70], suggesting a possible link between the permeability of the cell membrane and the shear rate. However, because intense agitation was accompanied by the entrainment of gas bubbles into the culture[70], the observed leakage could not be conclusively associated with fluid turbulence. Al-Rubeai *et al.* noted that, for otherwise fixed agitation conditions, the leakage of the dye reduced when the culture medium was formulated with 1% Pluronic F68[70]. The protective effect of the surfactant was apparently linked at least partly to its biochemical association with the cell membrane. The agitation- and vortex-associated hydrodynamic forces were also reported to affect the antigen receptors embedded in the membrane of the hybridoma[70]. The number of active surface receptors declined with increasing intensity of agitation and the accompanying increase in the size of the vortex, but the viability of the cells was unaffected. Pluronic F68 had a protective effect on the receptors.

Pluronic F68 is known to reduce the plasma-membrane fluidity of cells and this has been suggested as a possible mechanism of protection[55,56]. Other additives that affect plasma-membrane fluidity also affect a cell's shear tolerance; for example, cholesterol enrichment of the culture medium reduced membrane fluidity and enhanced the shear resistance of hybridomas, whereas supplementing the culture with benzyl alcohol increased plasma-membrane fluidity and made cells more shear sensitive than the controls[55,56]. Other protective mechanisms proposed for Pluronic F68 include: a stabilizing action on the gas–liquid interface and consequent slower film drainage during bubble rupture[13]; improved nutritional transport because of a reduced cell–fluid interfacial tension; and incorporation of the surfactant into the cell membrane, accompanied by membrane stabilization[68,71,72]. Using direct measurements of the burst tension of cells with and without Pluronic F68, Zhang *et al.* concluded that the Pluronic F68 supplementation strengthened cells[72], and suggested that the strengthening effect originated from incorporation of the surfactant into the cell membrane.

Although the protective effect of Pluronic F68 is said to be solely caused by physical factors[54], it is known to produce physiological and biochemical effects in at least some cases. Growth-suppressing and -stimulatory effects of Pluronic F68 have even been seen in some static cultures, although such effects are sometimes associated with impurities in the Pluronic F68 samples. Pluronic F68 affects the permeability of some cells; for example, exposure of *Saccharomyces cerevisiae* to 0.2% w/v Pluronic F68 for 30 min at 20°C increased the cells' permeability, and they became susceptible to otherwise nonlethal doses of certain antibiotics[73]. The mean activity of alcohol dehydrogenase in permeabilized cells was >3.5 times that in normal cells. Although Pluronic F68 could permeabilize the yeast cells, it did not inhibit growth or affect cell viability relative to controls when added to culture media at up to 1% w/v, which was the highest concentration tested[73].

Other data suggest that the cell-protective effect of Pluronic F68 might not be as general as originally thought[37]. For porcine erythrocytes suspended in isotonic buffer, Zhang *et al.* found that the addition of Pluronic F68 actually increased cell lysis in agitated environments relative to surfactant-free medium[37]. It is not known whether Pluronic F68 incorporates into an erythrocyte's membrane but cholesterol enrichment or depletion of erythrocyte membrane is reported not to affect membrane viscosity or, presumably therefore, fluidity.

Poly(ethylene glycol)

Poly(ethylene glycol) (PEG) supplementation of the medium protects some cells but not others. One study used different PEGs with molecular weights of 400–35 000 Da in the concentration range 0–2 kg m^{-3} (Ref. 36). The polymer protected sparged cultures when its molecular weight exceeded 1000 Da and the concentration exceeded 0.25 kg m^{-3}, and the extent of protection depended on the molecular weight and concentration of the PEG. Under the best conditions, PEG supplementation reduced the first-order cell-death rate constant to 0.5% of its value in the PEG-free medium[36].

The PEG additives affected the surface tension of the culture medium. However, in extreme cases the surface tension only varied between ~60 × 10^{-3} N m^{-1} and 70 × 10^{-3} N m^{-1} (Ref. 36). The pattern (but not the magnitude) of variation in surface tension with the addition of PEG was similar to the pattern of change in the death-rate constant. This led the authors to suggest that the presence of PEG at the gas–liquid interface was somehow related to the protective mechanism[36,66].

Comparing protective additives

The number of cells killed in a bubble-rupture event appears to depend on the number adhering to the bubble surface just before its rupture[44,74]. Additives that rapidly lower the gas–liquid interfacial tension prevent cell adhesion to bubbles[74] and so reduce cell damage. Michaels *et al.*[54] showed that, in sparged bioreactors, supplementing the culture medium with Pluronic F68, foetal calf serum or PEG (8000 Da, 1 kg m^{-3}) protected CRL-8018 hybridoma cells regardless of whether they were grown in the presence of the additive or whether the additive was added after the damaging levels of agitation were imposed. In single bubble-sparged

suspensions of *S. frugiperda*, Chattopadhyay *et al.*[74] observed that Pluronic F68 and methyl cellulose were better protectants than poly(vinyl alcohol) (PVA), dextran and various PEGs; PVA, dextran and 4000 Da PEG afforded similar levels of protection.

Whether or not cells attach to bubbles is determined by the free-energy change of the process. Adhesion is thermodynamically favoured if the free-energy change (ΔG) per unit area is negative. During adhesion, the cell–vapour interface replaces the cell–liquid and vapour–liquid interfaces; hence, the free-energy change of adhesion can be expressed as follows[75] (Eqn 5),

$$\Delta G = \sigma_{CV} - (\sigma_{VL} + \sigma_{CL}) \qquad (5)$$

where σ_{CV}, σ_{VL}, σ_{CL} are interfacial tensions at the cell–vapour, vapour–liquid, and cell–liquid interfaces, respectively. When adhesion occurs, ΔG is negative [i.e. $(\sigma_{VL} + \sigma_{CL}) > \sigma_{CV}$]. Additives that lower σ_{VL} sufficiently rapidly (i.e. within the mean residence time of a bubble in the reactor) should reduce $(\sigma_{VL} + \sigma_{CL})$, hence reducing the likelihood of cell–bubble adhesion. The interfacial tension (σ_{VL}) of most basal cell-culture media tends to be ~0.07 N m^{-1}. Supplementing the medium with 0.1% Pluronic F68 would typically reduce surface tension by ~1.5 × 10^{-2} N m^{-1}. The vapour–cell interfacial tension is generally (5.7 − 6.9) × 10^{-2} N m^{-1}, whereas the cell–liquid interfacial tension has been estimated to range between 1 × 10^{-3} N m^{-1} and 2.7 × 10^{-3} N m^{-1} (Ref. 75).

Additives can also alter a cell's hydrophobicity, or cell–vapour and cell–liquid interfacial tensions. At least some of the surface-active additives reduce cell adhesion to bubbles by reducing the surface hydrophobicity of cells[76]. Reduced surface hydrophobicity (attachment to hydrocarbons) has been observed for insect cells (*S. frugiperda* and *T. ni*) in the presence of Pluronic F68, methyl cellulose and foetal calf serum but not in the presence of polyethylene glycol[76].

Michaels *et al.*[77] examined the protective effects of various additives and possible mechanisms of protection on CHO cells grown in a serum-free medium. The medium was a 1:1 mix of Dulbecco's modified eagle's medium (DMEM) and Ham's F12, additionally supplemented with protein (370 μg ml^{-1}) and 10 mM penicillin–streptomycin–neomycin. Protective additives tested included poly(vinyl pyrrolidone) (PVP), PEG, 3% v/v serum, BSA, PVA, Pluronic F68 and methyl cellulose. The cell-protective effects were compared to the additives' abilities to affect cell–bubble attachment, to influence flow in thin films, to stabilize foam and to influence cell carryover into foam. Cell–bubble attachment was characterized in terms of induction time: the time taken for the film between a cell and an adjacent bubble to drain and thin so that the cell attached to the interface[77]. Induction time is a kinetic measure of hydrophobicity and is strongly influenced by the surface chemistry of cells and bubbles. Media with PVA, Pluronic F68 and methyl cellulose had high induction times (>2 s) and low attachment of CHO cells to bubbles[77]. Media with PVP, PEG, serum and serum-free medium with no additives had low induction times (<1 s).

Media with serum and BSA offered some protection to cells in sparged, agitated environments, but the

protective effects of Pluronic F68, PEG, PVA, PVP and methyl cellulose were stronger[77]. Based on froth flotation experiments, the additives increased cell removal into foam in the following order: methyl cellulose <PVA<Pluronic F68<3% serum<serum-free medium without additives<PEG<PVP[77]. Media supplemented with Pluronic F68, PVA and methyl cellulose produced more-stable foams and thinner films. In these media, fluid and cells drained rapidly during film thinning. Cells were trapped or flowed out more slowly when the films were generated with no additives, with 3% foetal calf serum, PEG or PVP[77].

Based on these observations, Michaels et al.[77] classified additives into three groups. The first contained PVA, Pluronic F68 and methyl cellulose, which reduced cell–bubble attachment relative to additive-free medium. There was less carryover of cells into the foam and, overall, cell damage was reduced. The second contained PEG and PVP, which increased cell–bubble attachment relative to additive-free medium. Apparently, there was reduced motion in thin films. Despite reduced induction times, these additives were effective cell protectants, which implies that they have a different mode of protection than group-1 additives. The third group contained foetal calf serum and BSA, which protected cells but not to same extent as the synthetic additives. Group-3 additives increased induction times. Cells were apparently carried into foam but drained out. These additives enhanced foam stability, allowing more time for cell drainage before bubble rupture. PVA, PVP and methyl cellulose are typically used at a concentration of 0.1% w/v.

Additives that protected cells affected the interfacial properties of the culture medium in various ways:

- PEG, PVP, serum and BSA increased surface viscosity at the air–medium interface (i.e. the interfacial rigidity was enhanced relative to additive-free medium)[78].
- Pluronic F68 and PVA greatly reduced surface viscosity[78].
- Methyl cellulose, PEG, PVA and Pluronic F68 decreased foam stability (i.e. its half-life)[78].
- Serum, BSA and PVP stabilized foam[78].

The impact of foaming on cell survival is a complex function of how readily the foam is produced, the stability of the foam, the rate of film drainage and the tendency of the cells to attach to the bubbles. When the cells' tendency to attach is fixed, more cells can be transported into the foam layer of easily foaming systems by a froth flotation mechanism. If the foam is stable but the cells drain out easily, the foam layer can protect cells by delaying bubble rupture until the cells have drained into the culture fluid. When the foam is stable but the cells do not drain out, the culture productivity might decline because of the permanent removal of cells into foam. Cells can be damaged when the foam is unstable (i.e. bubbles collapse rapidly), the bubbles are small and drainage is not sufficiently rapid for the cells to move away before the bubble rupture event. Changes in the cell concentration itself can also alter cell-damaging influences. For example, in CHO cell suspensions, cell concentrations $>2 \times 10^{-6}$ ml^{-1} have been reported to reduce foam stability[78].

Dextran has also been tested as a cell-protective additive[37,66,74,79] but it appears to be less widely used. In studies with agitated hybridomas and CHO cells under conditions that caused the entrainment of bubbles, the presence of dextran (229 kDa, 1–3% w/v) increased cell damage in comparison with dextran-free environments[79]. Whether this effect depended on the amount of dextran added was not clear. Dextran did not inhibit cell growth under milder agitation conditions when no bubbles were entrained[79]. When bubble entrainment was eliminated, the cells experienced no damage even under intense agitation, with or without dextran[79].

In contrast to the effect of dextran, several derivatized cellulose additives that also enhanced viscosity eliminated the damaging effect of entrained bubbles when added at 0.1–0.5% w/v. Similarly, PVA added at 0.2% w/v eliminated damage owing to bubble entrainment[79]. The derivatized cellulose and PVA presumably acted by reducing the attachment of cells to bubbles[77]. The better protective performance of methyl cellulose than of dextran was also noted by Chattopadhyay et al.[74], who observed similar protective capabilities of PVA, dextran and PEG4000. Using dextran, there is clear evidence for a consistent decline in the first-order cell-death-rate constant with increasing viscosity of the medium in a bubble column[66] (the viscosity was increased by adding dextrans of various molecular weights). The death-rate constant declined by approximately six times as the viscosity increased from ~1 mPa s to 70 mPa s.

Conclusion

Many additives are available to protect animal cells in sparged culture. Additives protect in complex and as-yet uncertain ways. Additives such as foetal calf serum and Pluronic F68 have a clear protective effect even when added in small amounts to basal media to reduce cell damage by bubble burst. However, as noted by Dey and Emery[80], the first-order rate constant for cell death does not correlate usefully with the effects of these additives on surface tension, the height of the jet produced by a bursting bubble or the speed of the jet.

Inevitably, in a production process, the choice of additives would require empirical evaluations to show their performance, optimal concentrations and combinations, best times for addition, and so on. What is eventually accepted would also depend on the additives' compatibility with the final use of the product and the needs of the downstream purification process[64]. Obviously, much remains to be learnt about the precise mechanisms by which the various additives protect cells.

Based on the studies reviewed here, several important observations can be made about freely suspended animal cells in sparged and agitated bioreactors.

(1) The aeration rate should be kept as low as possible.
(2) The mean bubble size should be greater than 7 mm, preferably 10–20 mm.
(3) The location of the sparger should be such that the rising bubbles do not interact with any impellers.
(4) With small air-lift and bubble-column reactors, the aspect ratio should be ~14 but, in larger columns (in which the sparged portion of the cross section can be a small fraction of the total cross-sectional area), more-realistic aspect ratios of about six or seven are satisfactory.
(5) When an impeller is used, the average energy dissipation (input) rate should remain below ~1.0×10^3 W m^{-3}.

A preferred practice is to minimize bubble coalescence and breakup within the culture fluid. Observations 1–3 are consistent with industrial practice[8]. The sole purpose of the impeller should be to suspend the cells and to mix the fluid gently so that the oxygen transferred from the bubbles is distributed throughout the vessel. Consistent with this purpose, the impeller should be of a type that does not produce excessively high local rates of energy dissipation. For this reason, relatively large, high-pitch, axial-flow hydrofoils are the preferred impellers[8,81]. In addition, a suitable additive such as Pluronic F68 should be used whenever feasible. In specific situations, forces other than ones associated with bubble rupture can affect culture performance[17,52,82–89]. Although the rupture of sparged bubbles damages cells, effective methods are available for limiting the adverse impact of bubbles, and submerged or sparged aeration should continue to dominate as the preferred method of supplying oxygen to cells.

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