

Media additives for protecting freely suspended animal cells against agitation and aeration damage

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Over the past 30 years, several different additives have been used to protect freely suspended animal cells in culture from agitation and aeration damage. These include pluronic polyols, various derivatized celluloses and starches, protein mixtures, polyvinyl-pyrrolidones, dextrans, and, more recently, polyethylene glycol (PEG) and polyvinyl alcohol. The protective mechanisms of these additives are not yet fully understood, but recent studies suggest both fluid-mechanical and biological mechanisms of protection.

The search for chemical additives to protect cells from fluid-mechanical damage started in the 1950s with the use of methylcelluloses¹. Several other additives were introduced in the early 1960s by the pioneering work of Swim and Parker², Runyan and Geyer³, and Kilburn and Webb⁴. Among the additives that have been used, the best known are serum and the pluronic family of non-ionic surfactants; others include several derivatized celluloses and starches, and several protein mixtures.

All evidence so far shows that damage of suspended cells in agitated and/or aerated bioreactors is usually due to the interactions of cells with bubbles and the rearrangement of gas-liquid interfaces. Working with bubble-column reactors, Handa-Corrigan *et al.*⁵ and Tramper *et al.*^{6,7} have shown that cell injury is due to shear forces generated either by film drainage around bubbles (such as in unstable foams), or by bubble breakup. For agitated bioreactors, Kunas and Papoutsakis⁸ have shown that when freely suspended cells are cultured in an agitated bioreactor, two fluid-mechanical mechanisms can cause cell damage and growth retardation:

- The first of these occurs only when there is a gas phase, and it is due to bubble breakup, either because of direct sparging or because of gas entrainment.
- The second mechanism causes cell damage in the absence of a gas phase (and, therefore, the absence of bubbles) only at very high agitation rates – above, for example, ~700 rpm in the 2 l bioreactor, with a 7-cm diameter four-pitched-blade impeller⁸ – by stresses in the bulk turbulent liquid. With this second mech-

anism, cell damage correlates with Kolmogorov eddy sizes similar to, or smaller than the cell size (9–15 μm).

The Kolmogorov eddy size can be readily calculated in agitated and air-lift reactors, and is a measure of the intensity of micromixing (details will be presented, in a future issue of *TIBTECH*, in an article by E. T. Papoutsakis discussing aerated bioreactors). Most bioreactors employ direct sparging or surface aeration, and when membrane oxygenation is used, the agitation intensity rarely exceeds 300–500 rpm, independent of impeller size and design. So, in practical terms, cell damage in agitated reactors is due exclusively to the first mechanism (i.e. bubble breakup), since the second mechanism becomes important only in the absence of bubbles and at very high agitation intensities. Since bubble breakup is also the predominant mechanism of cell damage in bubble-column (and presumably air-lift) bioreactors^{5–7}, the mechanism of cell injury in these reactors is essentially the same as that in agitated reactors. Based on this thesis, it can be assumed that additives that protect cells from fluid-mechanical injury in one type of reactor will also do so in the other, under conditions where the cells are exposed to a quantitatively similar level of shear. Whether cells are damaged in viscometric well-defined laminar flows (see below), or due to bubble breakup and film drainage, or due to interactions with eddies, damage is caused by shear forces acting on the cells through the surrounding fluid layer (boundary layer), which is always in a state of laminar flow. Cell damage occurring in all the above cases may therefore be referred to as either shear or fluid-mechanical damage.

All additives that protect freely-suspended cells from fluid-mechanical injury must either decrease the fragility of the cells (by nutritional uptake or other

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biological mechanisms altering their composition) or affect the forces on the cells due to their interactions with gas-liquid interfaces (by physicochemical mechanisms). To facilitate discussing the protective nature of the various additives, Michaels *et al.*⁹ proposed the following terminology:

- A *biological* protection mechanism or effect implies that the additive changes the cell itself to make it more shear resistant. This may be the result of alterations that take place quickly and without requiring metabolic events (such as the suggested effect of F68 by its physical incorporation into the plasma cell membrane^{10,11} (i.e. a *fast-acting biological mechanism*); or may require changes that demand metabolic events brought about by the additive, thus requiring a longer exposure (at least a substantial fraction of the cell-doubling time) to the additive (i.e. a *metabolic biological mechanism*).

- A *physical* protection mechanism means that the cell resistance to shear remains unchanged, but that the factors that affect the level or frequency of transmitted shear forces to the cell in a given culturing system have changed so that less cell damage is observed.

This review discusses first the issue of assessing the protection from fluid-mechanical damage, then the most widely studied shear-protective additives, and, finally, the less-known additives.

Methodology for assessing the protective effect of an additive

Two problems need to be resolved in order to study the protective effects of various additives: (1) the ability to assess quantitatively and reproducibly the protective effect of various additives, and (2) (less easy to address) the ability to assert that an additive's effect constitutes protection from fluid-mechanical damage. Both of these difficulties derive from the fact that there is substantial variability in the bioreactor and cellular parameters¹² that affect cell damage. We have proposed a way of solving these problems¹³. The variability in the shear sensitivity of cells due to their physiological state, medium composition, pH and metabolite concentration¹² can be overcome by carrying out simultaneous experiments using identical inocula, under the same, controlled bioreactor conditions, one bioreactor with the additive, the second without. Additional control experiments in static culture and/or in low agitation-intensity culture must establish that the additives do not affect the cellular physiology, at least in terms of the growth rate and cell viability which are the most commonly used parameters to assess these effects. Few studies to date satisfy all these requirements. We used two identical 2 l bioreactors (with pH and dissolved oxygen control, a 7-cm diameter four-pitched-blade impeller), run in parallel, such that one reactor is serving as the control for the other^{8,9,13-15}. Static-culture controls were also carried out using the multiple T-flask method⁹. The two reactors were inoculated simultaneously and agitated at 60 rpm (a rate that is non-detrimental to cells) until

cell densities reached $\sim 5 \times 10^5$ cells ml⁻¹. The agitation was then increased to 230 rpm (a rate that is lethal to the cells in the absence of the additives, and no net growth is observed above 200-210 rpm in this reactor with the unsupplemented medium). Agitation was then increased to progressively higher rates until the end of the exponential growth phase. The specific growth rates and cell viabilities obtained at each agitation rate were compared against the 60 rpm rate (internal control) and against each other (reactor with additive against reactor without additive; external control). The degree of protection afforded by an additive is related to the agitation rate that the cells can tolerate without apparent damage in its presence (i.e. the higher the agitation rate, the greater the protection). A variation of this method allows a direct comparison of the protective effect of two additives. Once it is established that the additives do not affect the growth rate of the cells at low agitation or in static cultures, the two bioreactors are run in parallel, one with each additive^{9,15}. The growth rates and viabilities at higher agitation are then compared against the 60 rpm rates and viabilities, and against each other. The parallel bioreactor method provides an excellent means for accurately and reproducibly assessing the effects of an additive because it uses both internal and external controls, but it is expensive and time-consuming.

Protective additives

Serum

Serum permits better cell growth in agitated and/or aerated cultures in a dosage-dependent fashion^{4,5,16}, though, until recently, it was not clear whether this was due to faster cell growth stimulated by higher serum concentrations or due to protection from fluid-mechanical damage by physicochemical or biological mechanisms. Several investigators have reported that low serum or serum-free cultures are more susceptible to fluid-mechanical damage. It was recently shown^{14,17} that progressively higher concentrations, up to 10%, of fetal bovine serum (FBS) reduce cell death and allow growth of cells at substantially higher agitation rates in bioreactors with surface aeration where cell damage is due to air entrainment and bubble breakup. Using the protocol discussed above, Kunas and Papoutsakis^{14,17} showed that hybridoma cells cannot grow at agitation intensities above 210 rpm even in the presence of 5% FBS, but that 10% FBS allows cell growth in the bioreactor at agitation rates up to 280 rpm (i.e. at least 6% FBS appears to be needed for cells to tolerate the higher agitation intensities). The protective effect of serum is measurable, even after short-term exposure, i.e. when serum is added just before or just after the exposure of the cells to lethal agitation (Fig. 1). The protective effect of FBS was therefore suggested to be largely physical, although the results could also be explained by a fast-acting biological mechanism. Viscometric studies⁹ complement these bioreactor studies, assessing the effect of an additive for protection against laminar,

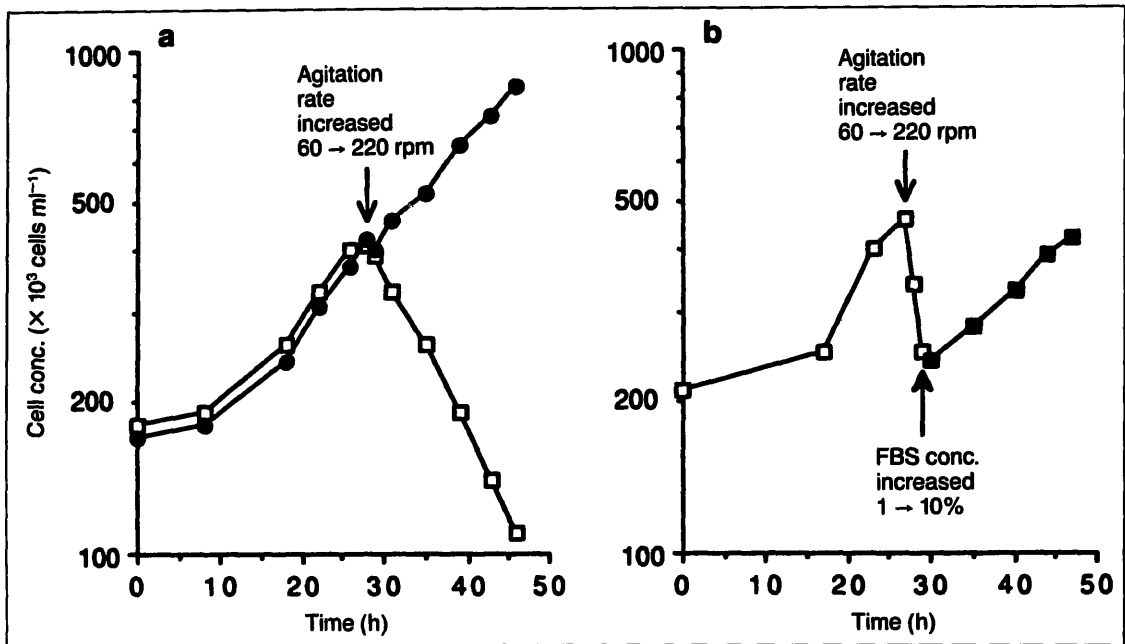


Figure 1

Fetal bovine serum (FBS) at concentrations above 6% v/v protects cells from agitation damage due to bubble entrainment and breakup, after both prolonged and short-term exposure. This is demonstrated here for 10% FBS using the hybridoma cells CRL-8018¹⁴. (a) Two identical bioreactors were run in parallel one with 1% FBS (\square) and the other with 10% FBS (\bullet). The agitation was increased from 60 rpm to 220 rpm where indicated by the arrow. (b) Cells were grown in the bioreactor with 1% FBS at 60 rpm. 110 ml of medium containing 1% FBS was added to the bioreactor at the point indicated by the open arrow, and the agitation was increased to 250 rpm 5 min later. Severe cell death results due to this agitation level as indicated by the rapidly decreasing cell concentrations. The closed arrow indicates the point at which 110 ml of FBS was added to the reactor to bring its concentration to 10% (\blacksquare). Its protective effect is immediately demonstrated by the increasing cell concentrations. Reproduced, with permission, from Ref. 14.

well-defined shear in a Couette viscometer. The viscometric studies assess only the ability of cells to resist well-defined shear of a given magnitude and duration, and thus assess only whether an additive protects cells from damage through a biological mechanism. The mechanism could be either metabolic or fast-acting, depending on the exposure time necessary for a protective effect to be demonstrated. According to the nomenclature adopted here, the fast-acting biological mechanism will include the proposed hypothesis whereby an additive protects cells from damage by means of adsorption of the additive on the cell membrane (see, for example, Ref. 18), although the cell metabolism may remain unchanged. Michaels *et al.*⁹ showed that FBS protects cells against shear damage in the viscometer after prolonged (cells grown in the presence of FBS), but not after short-term (30–40 min) exposure. From these results it can be concluded that FBS protects cells in the viscometer by a metabolic and not by a fast-acting biological mechanism. Since the biological protection requires prolonged exposure to FBS, the short-term protective effect observed in bioreactors is exclusively of physical nature. However, since (in most cases) cells in bioreactors are exposed to FBS over a prolonged time, the protective effect of serum in bioreactors is generally *both* biological and fluid-mechanical. Finally, since the short-term effect of FBS in bioreactors is almost

as strong as the long-term effect, it may be tentatively concluded that the protective effect of FBS is primarily physical in nature, but this may vary depending on the design and operational characteristics of the bioreactor, and the cells in question.

A physical protection mechanism for the effect of serum has also been suggested by Handa-Corrigan *et al.*⁵, on the basis of indirect data which show that serum changes the average bubble size and affects the draining and bursting properties in the foams formed at the top of the bubble column¹⁹. Progressively higher concentrations of FBS protect cells from bubble damage in bubble-column and spinner cultures¹⁹. Lee *et al.*²⁰ carried out experiments in spinner cultures to show the protective effect of serum on hybridoma cells and interpreted some of their findings to imply a biological protection mechanism. Their hypothesis is based mainly on the finding that cells adapted (in static T-flask cultures) to low serum concentrations cannot grow effectively at low serum concentrations at 100 rpm spinner cultures. This they interpreted to imply that serum changes the physiological properties of the cells, therefore its protective effect is biological in nature. Additional studies showing the protective effect of FBS on other hybridoma cells in spinner cultures were also reported²¹. In this case, it was found that the threshold serum concentration necessary for protection against 360 rpm agitation in spinner flasks

was only 2% (i.e. considerably lower than the threshold concentrations of 5–6% reported earlier^{14,17,20}). Finally, McQueen and Bailey²² have shown that myeloma cells grown in the presence of 10% serum could withstand turbulent fluid shear in a capillary flow (no gas–liquid interfaces) better than cells grown in the presence of 5% serum.

Pluronic polyols, polyethylene glycols and polyvinyl alcohol

It has been known for over 30 years^{2–4} that the non-ionic surfactants Pluronic F68 and F88 [which are block co-polymer glycols of poly(oxyethylene–poly(oxypropylene)–poly(oxyethylene))] protect cells from fluid-mechanical damage in agitated and aerated bioreactors. A number of investigators have used these surfactants as medium additives in static, agitated and/or aerated cell cultures^{5,10,19,23–27}. More detailed studies on the effects of various other Pluronics and reverse Pluronics [the order of the co-polymers is reversed with poly(oxyethylene) in the middle] on cell growth and/or shear protection have been published by Mizrahi²⁵ and Murhammer and Goochee¹¹.

In many of the studies involving Pluronics, it is not clear that the additive was protecting the cells from shear damage, because cells were grown either in shake flasks or low or unreported rpm spinner flasks. In other words, the enhancement of cell growth by the addition of Pluronics *could* have been irrelevant to its shear-protection capabilities. The static-culture studies of Bentley *et al.*²³ demonstrated that Pluronics have a concentration-dependent positive or negative effect on cell growth in static cultures, thus establishing that at certain concentrations these polyols affect the growth of some cells in a manner that is independent of agitation or aeration. A similar conclusion can be drawn from the shake-flask studies of Swim and Parker² and of Mizrahi^{25,26}. However, a number of studies have established that Pluronics and reverse Pluronics do also protect animal cells from fluid-mechanical damage, although the mechanism has not been firmly established, and has been the subject of considerable debate and speculation. Kilburn and Webb⁴, and Handa-Corrigan *et al.*⁵ proposed a fluid-mechanical action mechanism. Specifically, it has been suggested that these surfactants stabilize the foam, thereby reducing film drainage and bubble bursting in the vicinity of cells⁵. Mizrahi²⁵ (because he found a beneficial effect to cell growth even in the absence of fluid-mechanical damage in shake-flask experiments) suggests an additional effect through a lowered surface tension that facilitates the transport of metabolites which enhance cell growth.

Murhammer and Goochee^{10,11} proposed that F68 (and related Pluronics and reverse Pluronics) protects the cells by being inserted into the plasma membrane, thereby altering the properties of the membrane to confer increased resistance to shear forces. This action could be classified as fast-acting biological protection mechanism. Specifically, they found that in insect-cell

(Sf9) cultures, the protective effect of Pluronics and reverse Pluronics correlates well with the hydrophilic–lipophilic balance (HLB) of the additives. The polyols with the highest HLB balance were found to be the best protectants. They also found that poly(oxyethylene) glycol [more commonly known as polyethylene glycol (PEG), a potent fusogen at high concentrations] protected the Sf9 cells to some extent (but not consistently) in agitated and sparged cultures, but not at all in airlift bioreactors. Although these polyols are not metabolized by the cells²⁶, by their nature as surface-active agents, they do interact with the cell plasma membrane, and their protective effect, to some (possibly large) extent, may be due to this interaction^{10,11}. Interestingly, the correlation of the high HLB of these polyols with increased protection is also consistent with the mechanism suggested by Handa-Corrigan *et al.*⁵ and Kilburn and Webb⁴. As mentioned above, pluronic surfactants have been shown to have a concentration-dependent effect on cell growth that is independent of agitation or aeration²³. However, this does not imply that their protective effect against shear damage is of biological nature.

Ramirez and Mutharasan²¹ have used a combination of measurements of the plasma membrane fluidity (PMF) using fluorescence anisotropy (r_f) of hybridoma cells in the presence of several additives, and viscometric studies to show that a decreased PMF correlates with increased resistance to viscometric shear. For cells grown in the presence of F68, they found that the r_f increased by an average of 0.01 units. Since an r_f increase implies a PMF decrease, they suggest that F68 interacts with the plasma membrane and decreases its PMF thus making the cells more resistant to shear damage, which is consistent with the hypothesis of Murhammer and Goochee^{10,11}. However, this small increase in r_f is not sufficient to provide an increase of the cell resistance to shear damage, since, according to their data, an increase in r_f of 0.04–0.05 units is necessary for this effect.

In addition to examining the protective effect of serum against shear damage^{14,17}, we have used a similar methodology of bioreactor experiments to examine the protective effects of PEGs of various molecular masses and concentrations, and of polyvinyl alcohol (PVA, 10 kDa), and to compare these effects with the effect of Pluronic F68^{9,15}. Examining both prolonged or short-term exposure (~1 h, i.e. as fast as we can measure an effect on cell growth and viability in a bioreactor) we found that all three additives (PEG, PVA and F68) protect cells from fluid-mechanical damage, which suggests that their effect is either physical or fast-acting biological in nature. Figure 2 shows that both PEG (8 kDa) and PVA can protect cells after only a short exposure, and that PVA is a better protectant than PEG. PEGs of molecular mass >1.4 kDa, and PVA have a profound protective effect under high agitation intensities, but do not affect cell growth under static or mild-agitation conditions. PEGs in the molecular-mass range 1.4–1.5 kDa were

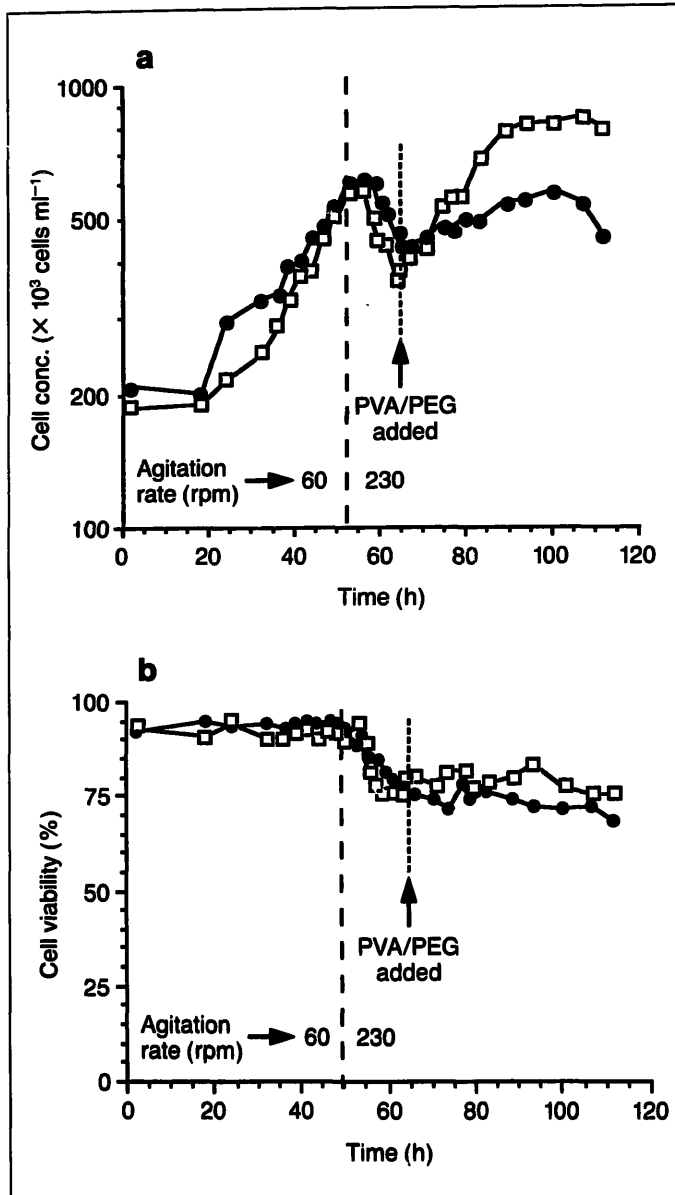


Figure 2

Comparison of the short-term protective effect of PEG and PVA in two identical agitated bioreactors run in parallel. After simultaneous inoculation, the two reactors were stirred at an agitation rate of 60 rpm until mid-exponential growth (6×10^5 cells ml^{-1}). The agitation rate was then increased to 230 rpm (a rate that is lethal to cells in the absence of additives) until cell damage was demonstrated by a reduction in viable cell concentration. 0.2% w/v PVA (□) was added to one reactor and 0.1% PEG (8 kDa) (●) was added to the other. (a) Shows viable cell concentrations; and (b) shows cell viabilities. Reproduced, with permission, from Ref. 15.

equally effective protectants. PEG concentrations of 0.05% w/v were sufficient for cell protection, and their protective effect was found to be stronger than that of Pluronic F68. PEG (10 kDa) was also found to protect TB/C3 hybridoma cells from sparging damage in a bubble-column reactor (A. Handa, PhD thesis, 1986, University of Birmingham, UK).

To elucidate the protective effects of PEG and F68 further, we carried out viscometric studies similar to the FBS studies⁹. Again, the viscometric studies assess

found to be only if an additive alters the ability of the cell to resist shear. In contrast, in the bioreactor studies, the protective effect of an additive can be due either to an increased ability of the cells to resist shear (biological mechanism) or to a reduction of mechanical stresses to which cells are exposed due to agitation and aeration (physical mechanism) or to both of these mechanisms. Unlike serum, PEG or F68 did not protect cells against shear damage in the viscometer, either after prolonged (cells grown in the presence of the additive), short-term (30 min), or intermediate (1–4 h) exposure. In fact, PEG was found to be mildly detrimental to the cells. It was therefore concluded that the shear-protective effects of these additives in the bioreactor are physical in nature, and specifically purely fluid mechanical (i.e. due to changes in the interactions between bubbles, draining films and the cells). A biological effect would have afforded protection to cells in both viscometer and bioreactor. The protective effect in the bioreactor, then, can be explained by the effect of these additives on the surface tension and rheological properties of the gas-liquid interface, since these properties affect bubble breakup and film draining, and thus the associated shear stresses¹⁵.

While the protective effects of PVA and F68 correlate well with a reduced surface tension¹⁵, surface tension alone cannot explain the protective effect of PEG and serum. Indeed, although PEG does not reduce any further the surface tension of the protein supplemented serum-free medium, it has a profound protective effect¹⁵. Similarly, although the surface tension of media supplemented with 1–10% FBS remains unchanged around 54 dyne cm^{-1} , only FBS concentrations above 5–6% have a protective effect on the cells in bioreactors^{14,15,17}. We have suggested that the effects of these additives on other surface properties of the media must be examined in order to understand their protective capabilities better¹⁵. Our experiments also raise the issue of generality of the protection mechanism of F68, PEG and serum. A recent report suggests that the protection mechanism may vary for different cell types.

Cell-type dependence

Goldblum *et al.*¹⁸ also used viscometric studies to examine the effect of various additives on the shear-resistance of insect cells. Using a cone-and-plate viscometer of 10 cm radius, they quantified cell damage by comparing the rates of lactate dehydrogenase (LDH) release per unit of shear stress. The ratio of this normalized rate with and without an additive offered a measure of the degree of protection. They used insect cells from the late-exponential growth phase of static T-flask cultures, finding that Sf9 cells grown in the presence of 0.1% F68 are 15.5 times more resistant to laminar shear, and that growth with 0.2% F68 increases shear resistance 42.3-fold when compared to cells grown without any F68. 0.1% F68 makes the TN-368 insect cells 6.3 times more shear resistant. Unless there are experimental artifacts due either to the flow in the viscometer or to the cell-damage

assays used, comparison of our viscometric study⁹ and that of Goldblum *et al.*¹⁸ shows that in the case of the hybridoma CRL-8018 cells, F68 does not affect their shear robustness, but that for the insect cells Sf9 and TN-368, its effect is profound. Goldblum *et al.*¹⁸ suggested that the protection may be due to the adsorption of the additive on the cell membrane. This could be classified as a biological mechanism. One would then expect F68 to protect these cells from agitation and aeration damage by a combination of biological and fluid-mechanical mechanisms. The biological mechanism may well be consistent with the hypothesis that F68 alters the membrane properties of the cells thus conferring increased resistance to shear¹¹. It is not yet possible to assess the significance to shear protection in a bioreactor of the large effects (6–42 times increased shear resistance due to F68) measured in the viscometric studies for the insect cells¹⁸. For example, it has been found that 0.1% F68 protects Sf9 cells in both an airlift and an agitated bioreactor equally as well as, if not better than, 0.2% F68¹¹, while Goldblum *et al.*¹⁸ showed a threefold higher protection with 0.2% F68. Clearly, the protection mechanism of F68 may well be cell-type dependent.

Derivatized celluloses and starches, dextrans, polyvinyl-pyrrolidones, proteins and cell extracts

Derivatized celluloses have been used for suspension cell-culture technology since the 1950s^{1,28,29}. Bryant¹ has reviewed the early uses of methylcelluloses (methocels) (MCs) as protectants against shear damage for the cultivation of a large variety of suspension cells, including human, mouse and monkey cells. He has also studied the effects of various MC grades on various cells of variable shear fragility in both shake-flask¹ and static cultures³⁰. Despite these (and other) studies, MCs have not been unequivocally established as reliable shear protectants (see below).

In static cultures, Bryant³⁰ found that MC (0.12% w/v of the 10 cp grade) improved the growth and glucose utilization of the human-skin cell line NCTC 3075, and of the fragile monkey-kidney cell line NCTC 3526 but did not affect the mouse-fibroblast cell line NCTC 2071-L. He interpreted his findings to imply protection against shear damage during subculturing due to 'gentle mopping with a small strip of perforated cellophane from the floor of the flask ...' If this shear damage can indeed account for the observed effects of MC, one could question the relevance of such damage to that observed in agitated and/or aerated bioreactors. However, his results may imply a physiological stimulatory effect of MC on some cells. Bryant³⁰ discussed earlier published work, some of it showing no nutritional effects by MC, but some showing that MC stimulates growth in addition to its shear-protection effect. The effect of some MCs in causing cell aggregation for some cells was also discussed³⁰. Apparently, all these possible effects are MC-grade and cell-type dependent. In his shake-flask experiments (145 rpm rotary motion in 125–500 ml

modified boiling flasks), Bryant¹, in 1966, found that all three cell lines he used could not be grown in the absence of some MC as an additive to his protein-free chemically defined medium, but they could be grown in static cultures in the protein-free medium. Use of smaller flasks (125 ml) to explore '... the possibility that slower rate of circumferential flow of fluid in the shaker flasks might lessen the stresses on the suspended cells' did not eliminate the need for MC. In today's perspective, it is hard to imagine that a 145 rpm rotary motion in shake flasks will generate damaging shear forces, but his experiments demonstrate a significant protective effect from some damage in those shake flasks. Low concentrations of the low viscosity grade (10 cp) of MC was found to be as effective as a protectant as the higher viscosity grades^{1,30,31}. Thus, it was concluded that the effect of MC was not related to the viscosity of the medium. In contrast, Hink and Strauss³² suggested that the protective effect of MC on insect cells derives from the higher medium viscosity. Higher concentrations or grades of MCs produce very viscous solutions, and some of the early work used MCs that produced media of very high viscosities.

Since that early work^{1,28–31}, MCs and other derivatized celluloses of various grades and makes have been frequently included as media additives (in combination with serum, various protein mixtures and other defined additives) in the cultivation of a large variety of cells. From existing data, it is hard to assess whether these derivatized celluloses are indeed needed as shear-protection additives in most formulations of modern serum-free media, since control experiments to demonstrate their shear-protection effect are almost uniformly missing. Telling and Elsworth³³ used sodium carboxymethyl cellulose (CMC, Edifas B50) together with tryptose phosphate broth and 10% bovine serum as medium additives of a large-scale culture of suspension baby-hamster kidney (BHK) cells at high agitation intensities (330 or 460 rpm). Although no static-culture controls were used, the agitation intensity is very high, so one assumes that without these additives, severe cell damage would occur. There is no way, however, to assess which additive offered the most shear-protection. Mizrahi and Moore¹⁶ tested a large number of synthetic polymers [hydroxyethyl starch (HES), CMC (Edifas B50), two types of polyvinyl-pyrrolidones (PVP), modified gelatin (Haemacel) and several dextrans] in 500 ml spinner flasks at 100 rpm for improved growth of human lymphocyte and lymphoblastoid cell lines in agitated cultures with low serum concentrations. They found that the dextrans had a positive effect on the growth of two cell lines (RPMI 1788 and 8098) but no effect on a third line (RPMI 1348). HES, CMC, and the two types of PVP had a positive effect on the growth of all cells tested, but the modified gelatin had a uniformly negative effect on growth. There were no static-control cultures presented to test that their agitation intensity (100 rpm) is indeed damaging to cells. Most suspended cells, with the exception

perhaps of some insect cells, do not experience shear damage under such low agitation conditions. So, it is not possible to judge if the effects they observed suggest protection from shear damage or are due to physiological reasons.

Mizrahi²⁶ subsequently reported additional tests on the effects of CMC and HES. Using radiolabelled polymers, he reported that CMC and HES are not metabolized by the cells. His experiments were carried out in 100-ml spinner flasks, but the agitation intensity was not reported. The presence of CMC and HES was found to improve cell growth and result in better cell yields on glucose accompanied by reduced glucose utilization. A mechanical protection effect was again suggested, although no static-culture control experiments were shown to substantiate this.

A. Handa (PhD thesis, op. cit.) tested several additives for protection against sparging damage in a bubble-column reactor using TB/C3 hybridoma cells. She found that a mixed-molecular-mass PVP protected the cells from sparging damage, one type of CMC (BDH) inhibited cell growth, and another two types of CMC (Blanose by Hercules and Edifas A by ICI) offered no protection against sparging damage. Similarly, dextran (488 kDa) offered no protection.

More recently, the use of MCs (in combination with other shear-protecting additives, such as serum, yeastolate or protein hydrolysates) appears to be restricted to the cultivation of insect cells (see, for example, Ref. 32), probably because of the more widespread use of F68, which does not cause any of the cell-aggregation problems often associated with the use of MCs and CMCs.

Goldblum *et al.*¹⁸ have studied the effect of various methylcelluloses and (hydroxypropyl)methylcelluloses (methocells, all made by the Dow Chemical Co.) and dextran in protecting insect cells (Sf9 and TN-368) in viscometric flows. Using similar experimental conditions (all media contained 10% FBS in addition to the additive) and the same measure to assess shear protection as for their F68 studies (see above), they found that the best protection (58–76 times less cell lysis compared to the unsupplemented medium) was offered by higher concentrations (0.5% w/v was more beneficial than 0.25%) and higher molecular-mass MCs. The best protection was offered by the additives resulting in the highest medium viscosity (4–25 times the unsupplemented medium viscosity). Similarly, dextran (476 kDa) offered substantial shear protection only at high concentrations (4.5%), where the medium viscosity is also high (6.6 times higher than the unsupplemented medium). Again, it is not possible to evaluate the significance of these findings in terms of protection in bioreactors. The fact that the protective effect of MCs and dextran was found to be medium-viscosity dependent is undesirable (it affects the power requirements for agitation and aeration as well as the mass transfer characteristics of the gas-liquid suspension) and in contrast to Bryant's studies^{1,30}. It is also difficult to understand the need for these relatively

high additive concentrations and media viscosities if the protective effect is thought to result from coating of the cells by the additive adsorbed on the cell membrane^{1,18}. Nevertheless, these studies show that both the MCs and dextran increase the shear robustness of these insect cells through a biological mechanism.

More questions than answers

In summary, the effect of MCs and CMCs as additives to protect animal cells from shear damage in agitated, bubble-column or otherwise mixed cultures may be cell-type, MC-grade and MC-make dependent. In addition, MCs and CMCs may elicit biological responses from cells, either positive or negative; these responses appear to be also cell-type and MC (or CMC)-grade and MC (or CMC)-make dependent. Even after almost 40 years of research we understand very little about the mechanism and generality of the protective effect of MCs and related additives. Unfortunately, even the phenomenology of their effect is not very well established in view of the missing control experiments and some conflicting reports.

Dextrans were found to have a protective effect on insect cells in viscometric studies¹⁸, and a positive effect on human lymphocyte and lymphoblastoid cell lines in agitated spinner cultures (100 rpm) at low serum concentrations¹⁶. In contrast, A. Handa (PhD thesis, op. cit.) found that dextran offered no protection against bubble damage in a bubble-column reactor. In my laboratory, extensive studies have established that 1–3% w/v dextrans (229 kDa) do not protect hybridoma cells from damage due to bubble entrainment and breakup in an agitated bioreactor, but instead increase cell death under intense agitation¹³. Control static or low agitation cultures showed that dextran does not affect the cells, at least in terms of growth rates. So its detrimental effect is due to the viscosity increase. The dextrans we used increase the viscosity up to threefold compared to the medium without dextran, but do not affect the surface tension of the medium. The effect of dextrans can be positive or negative depending on the cell-type, bioreactor, or even dextran grade and make used.

Finally, several protein mixtures (in addition to serum) and a protein have been used as shear protectants for the cultivation of various cells. Again, their protective effect is not unambiguously established and there is a lack of proper control experiments. Peptone (0.5% w/v)³⁴ and lactalbumin hydrolysate³⁵ have been used as media additives since the early days of suspension animal-cell culture, presumably for nutritional reasons as well as for protection against shear forces. Addition of tryptose phosphate broth was used successfully to protect against fluid-mechanical damage by Handa-Corrigan *et al.*⁵ A similar additive (yeast extract) was used in insect-cell culture, but without any explanation as to its possible role as a shear protectant²⁴. Yeastolate and lactalbumin hydrolysate are frequently used in insect culture media³², although their shear-protecting role is not explicitly acknowl-

edged. Similarly, low concentrations (0.1%) of Primatone RL, a peptic digest of animal tissue, has been used successfully as a serum substitute presumably for protection against fluid-mechanical damage^{36,37}.

Bovine serum albumin (BSA), a major component of bovine serum, is a widely used additive in serum-free media, and has been frequently used as a shear protectant by several investigators (see, for example, Ref. 38), although its protective effect has not been always clearly demonstrated. BSA was recently shown³⁹ to be an effective additive against fluid-mechanical damage of hybridoma cells in an airlift bioreactor, although it has no effect on the cells in static or spinner cultures.

Processing aspects

While several choices are now available regarding shear-protecting additives, it is not clear yet if the use of these additives is suitable for all cell culturing and processing needs. First, for each and every cell type, the physiological and/or product expression effects of an additive must be assessed carefully under both static and bioreactor growth conditions. If there are no obvious detrimental effects, the effect of the additive on cell aggregation must be evaluated, in case cell aggregation is an undesirable processing property. As discussed above, some derivatized celluloses have been found to promote cell aggregation, but this is probably a cell-type dependent effect. None of the other commonly used additives has been generally found to promote cell aggregation. In addition, the effect of the additive on the stability or possible modification of the cell-culture protein product as well as on the purification of this product must be carefully assessed. I am not aware of any reports regarding the effects of any of the aforementioned additives, at the low concentrations commonly used, on the stability or modification of proteins. An additive's effect on protein purification is likely, however, to dictate judicious choices. All of the additives discussed above are likely to complicate membrane, adsorption, precipitation and chromatographic processes to some extent. So, on the basis of a combination of chemical structure, molecular mass and protective-effect concentration, some additives may be more advantageous than others from the downstream-processing point of view. It is known that several additives are currently used in industrial processes (see, for example, Ref. 24), and one must therefore conclude that the downstream-processing complications that may arise from the use of shear-protecting additives are manageable. Nevertheless, it appears desirable to expand the repertoire of available additives in order to be able to minimize more effectively any downstream-processing difficulties.

Conclusions and future directions

Several additives have been investigated for their ability to protect freely suspended animal cells from agitation and aeration damage. I have argued that, on the basis of current knowledge, the protection is most

likely from forces associated with the interaction of cells with breaking or coalescing bubbles, or with unstable gas-liquid interfaces, such as those encountered near unstable vortex surfaces in rapidly agitated bioreactors. It is likely that certain fragile cells (such as insect or human cells that have not been adapted for culture in intensely mixed fluid environments) may be further injured by milder fluid forces encountered even in the absence of gas-liquid interfaces. It is very likely that fundamental knowledge gained from the use of shear-protecting additives in animal-cell culture will also be useful in plant-cell culture. Plant-cell damage in bioreactors may well be quite similar to animal-cell damage.

Among synthetic additives, pluronic polyols and various PEGs are the most widely tested and used. Their protective effect requires relatively low concentrations of 0.05–0.2% w/v, or even lower. PEGs, in particular, deserve an even wider testing with more cell types, and over a wider range of molecular masses and concentrations, in order to offer a larger range of choices for the processing needs of more cell types and products. Bioreactor and viscometric studies suggest that the protective effect of these additives is fast acting, probably largely fluid-mechanical, but possibly also biological, at least for certain cell types, such as insect cells. The fluid-mechanical component of the protection mechanism is likely due to the ability of these additives to reduce the magnitude and frequency of forces encountered during the interaction of cells with gas-liquid interfaces. Understanding of the protective effect of these and other additives deserves further investigation, as it may suggest more effective and simpler means of cell protection. Since it appears very likely that the protective effect of these additives is cell-type dependent, a wider use of viscometric and bioreactor studies, in combination with more sophisticated cellular and interfacial measurements, will be crucial in elucidating the contribution of fluid-mechanical and biological mechanisms to the overall protection effect.

Other promising but less tested additives include PVA, and polyvinyl-pyrrolidones, both of which deserve wider testing with more cells, and over a wider range of molecular masses and concentrations. The protective effect of PVA, based on a limited set of studies, appears to resemble that of PEGs and pluronic polyols. Derivatized celluloses, such as CMCs, were the earliest used additives, but their shear-protecting effect has not yet been unambiguously established. For historical (or other, unknown) reasons, they are more widely used in insect-cell culture, and their 'protective' effect may partially derive from biological mechanisms (such as growth stimulation) and from the increased medium viscosity. Derivatized celluloses and starches also deserve wider investigation of their protective capabilities, as well as a better understanding of the nature of their protective effect. In addition, dextrans of various molecular masses and makes have been investigated for their

possible protective effect. At this point, their effect is at best ambiguous and inconsistent; in most bioreactor studies under intense mixing conditions, their effect is in fact negative or neutral.

As well as these synthetic polymers, several protein mixtures have been found beneficial as protectants against fluid-mechanical damage. From the practical point of view, protein-containing additives are less desirable due to higher cost and/or to complications they introduce in downstream processing. Serum is the most widely studied protein-containing additive. Its effect is, to a large extent, dose-dependent above a threshold concentration (this concentration is apparently cell-type dependent). Its effect is largely fast acting, and viscometric studies suggest that for certain cell types, at least, prolonged exposure to FBS offers additional protection by a metabolic biological mechanism. So it appears that its protective effect has two possible components, a fluid-mechanical (similar possibly to that of PEGs and pluronic polyols) and a biological component. Additional studies would be valuable to assess the generality of these findings. It is not unlikely that the protective effect of serum derives, in part, from some of its non-protein components, such as lipids. Other proteins or protein mixtures that have been found beneficial for shear protection include BSA, lactalbumin hydrolysate, peptone, tryptose phosphate, yeastolate and a peptic digest of animal tissue. While a broader study and understanding of the effect of these and other proteins would be beneficial, in my opinion, such studies are of lesser urgency and lower priority in view of the likely disadvantages of protein additives as discussed above.

A key difficulty that remains to be resolved is the ability to compare the results from various additives obtained from studies carried out with different agitated, bubble-column and airlift bioreactors. Several studies have shown different effects of certain additives depending on the cells used, bioreactor type and operational characteristics of the reactors^{9,11}. In addition, it is not yet possible to use results obtained in viscometric studies to predict the additive's effect in various bioreactors.

In conclusion, much progress has been made in expanding the repertoire and understanding the effect of shear-protecting additives for culturing shear sensitive animal cells. However, both the phenomenology and the mechanistic aspects of this problem require additional sustained effort. There is little doubt that this effort will produce substantial benefits for the ever growing needs of cell-culture technologies.

Acknowledgements

I am thankful for the financial support under Grant EET-8896100 by the National Science Foundation (USA), and matching funds from the Monsanto Corporation and the Eli Lilly Company.

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