

Effects of the hydrodynamic environment and shear protectants on survival of erythrocytes in suspension

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Abstract

Survival of media-suspended porcine erythrocytes exposed to various hydrodynamic environments was investigated with and without such shear protectant additives as bovine serum albumin, dextran and the non-ionic surfactant Pluronic F68. Erythrocytes provided a model cell population with cells of a uniform size, metabolic state and shear tolerance. Because the cells were non-growing, any shear adaptation effects were avoided. Cell lysis was followed by microscopic counts or release of haemoglobin. The cells were susceptible to agitation damage in un-aerated shake flasks agitated at 100 rpm or greater. Relative to additives-free operation, the presence of 0.1% (w/v) dextran or albumin prolonged cell survival, but Pluronic F68 actually enhanced cell lysis in flasks agitated at 100 rpm. The protective effect of the additives depended on the hydrodynamic conditions. The protective effect of albumin was demonstrated also in aerated conditions in a split-cylinder airlift bioreactor (aspect ratio of 8.8; riser-to-downcomer cross-sectional area ratio of 1.0; specific power input of 0.34 W m⁻³). Comparison of the cell lysis characteristics in the airlift device and the best case performance of the shake flask showed longer survival in the flask (100 rpm); however, the length of survival in the reactor (approx. 70 h) was sufficient for practical purposes. In all cases, the cell lysis pattern conformed initially to zero-order dependence in cell concentration, becoming first-order after varying degrees of exposure to hydrodynamic forces. Fatigue failure of cells was inferred.

Keywords: Erythrocyte; Animal cell culture; Shear effects; Hydrodynamics; Airlift bioreactor

1. Introduction

Suspension culture of hybridomas and other animal cells is of increasing importance for the production of therapeutic, diagnostic and protective proteins (Chisti, 1993; Lubiniecki, 1990). While all cells are sensitive to intense hydrodynamic forces (Chisti and

Moo-Young, 1986), animal cells are particularly fragile because they lack a protective cell wall and are larger than most microbial cells. Design, scale-up and operation of cell culture bioreactors demands an understanding of the mechanisms of damage to cells cultured under various hydrodynamic environments. Causes of cell damage have been investigated in devices such as capillaries (Augenstein et al., 1971; McQueen and Bailey, 1989), where shear rates are better defined, and in bioreactors where the hydrodynamic regime is more complex (Chisti and Moo-

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Young, 1989). While animal cell lines differ in susceptibility to shear stresses, Macmillan et al. (1987) have documented poorer apparent growth with increasing agitation in shake flask cultures of a hybridoma line. Compared with stationary culture, agitation in shake flasks, even at a low speed (40 rpm), significantly reduced the growth rate. Previous studies typically employed hybridomas or other cells in batch culture, with or without aeration. The cultures used contained cells at different stages of growth and, hence, the sizes and the metabolic states of the cells were different. Damage to cells was quantified through cell counts or other indicators of cell death which, in growing populations, provided only a net result of the growth and death processes.

This work reports on the effects of hydrodynamics on survival of porcine erythrocytes suspended in culture media in shake flasks and an airlift reactor. Erythrocytes provide a model cell population with cells of a uniform size and metabolic state. Significantly, erythrocytes do not multiply *in vitro*; hence, cell growth, effects of growth stages and adaptation to hydrodynamic environments do not mask the effects of damage-causing forces on the cells. The latter consideration is of especial importance in correctly assessing hydrodynamic damage because growing cells do adapt (natural selection) to mechanical forces with duration of exposure. For example, Wu et al. (1990) noted for insect cells that the beneficial effect of shear protective agents (serum, methyl cellulose and Pluronic F68) were not as important as the effect of passage number.

2. Experimental procedures

2.1. Collection and preparation of cells

A predetermined amount of blood from freshly slaughtered adult pigs was collected directly into glass containers containing a solution of sodium citrate to give a final sodium citrate concentration of 0.32% (w/v). Citrate suppressed clotting by chelating the calcium ions that are necessary for clot formation. Citrated blood was suspended in buffered isotonic saline (0.9% w/v sodium chloride solution buffered to pH 7.2–7.4 with 5mM Tris-HCl) to give an erythrocyte count of about 3.0×10^6 cells ml^{-1} .

This corresponded to the maximum cell concentration that can be typically achieved in batch suspension culture of hybridomas (Chisti, 1993; Moo-Young and Chisti, 1988). The suspension was supplemented with penicillin (50 units ml^{-1}) and streptomycin (0.05 mg l^{-1}) to control microbial growth. In some cases, the cell slurry was further supplemented with bovine serum albumin (Sigma Chemical Co., St. Louis, MO; catalog No. A-7638), dextran (71 400 daltons average molecular mass; Sigma Chemical Co., St. Louis, MO; catalog No. D-4751) or the non-ionic surfactant Pluronic F68 (BASF Wyandotte Corporation, Parsippany, NJ; catalog No. 5882704) to 0.1% w/v to examine the effects of these additives on survival kinetics of the cells.

2.2. Hydrodynamic studies

The cell slurry was placed either in shake flasks (300 ml slurry in 500-ml flask) held on a 37°C controlled temperature orbital shaker (0–400 rpm), or in a split-cylinder airlift reactor. The latter consisted of a 0.055 m diameter glass vessel (Fig. 1) partitioned into a riser and downcomer by a stainless steel baffle sealed at the walls by Teflon linings. The riser-to-downcomer cross-sectional area ratio (A_r/A_d) was unity. The vessel had a working volume of 1 l and the working aspect ratio was 8.8. The height of the baffle was 0.32 m and it was located 0.1 m above the base of the reactor. The clearance,

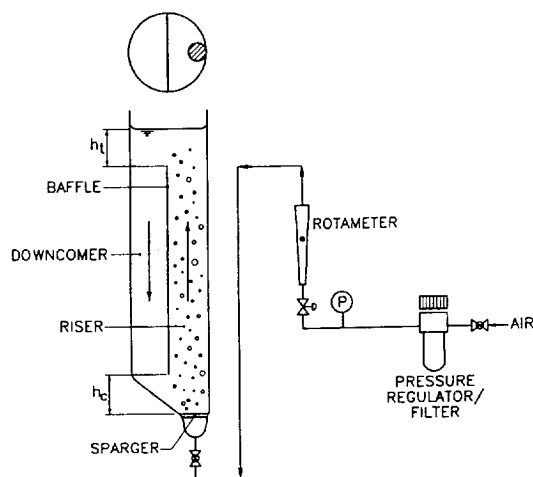


Fig. 1. Split-cylinder airlift reactor.

h_r , between the upper edge of the baffle and the static height of cell slurry was 0.065 m. The reactor was sparged with air through a sintered glass sparger (110×10^{-6} m pore size; 1.5×10^{-2} m frit diameter) located at the base of the riser (Fig. 1). Similar reactors were investigated by Ganzeveld et al. (1995) for possible use in culture of anchorage-dependent animal cells.

The air flow was controlled at a superficial gas velocity, U_{Gr} , based on cross-sectional area of riser, of 7.0×10^{-5} m s⁻¹. This corresponded to a specific power input of 0.34 W m⁻³, calculated with the equation (Chisti, 1989):

$$\frac{P}{V_L} = \frac{\rho_L g U_{Gr}}{1 + \frac{A_d}{A_r}}, \quad (1)$$

where P is the power input due to gassing, V_L is the volume of liquid and g is the gravitational acceleration. The density (ρ_L) of the cell slurry was assumed to be that of water. The reactor was jacketed and temperature was controlled at 37°C by circulation of thermostated water through the jacket.

2.3. Quantification of cell damage

Cell number and morphology were examined with a haemocytometer and optical microscope. In addition, the degree of cell lysis was quantified by spectrophotometric measurement of haemoglobin released on lysis of cells. Thus, at various times after exposure of the cell slurry to specified hydrodynamic environments, samples of the slurry were withdrawn, pelleted at 920-g in a centrifuge, and the absorbance of the supernatant was measured at 410 nm. The cell numbers could be independently estimated from the optical density measurements as follows:

$$\frac{N}{N_0} = \frac{OD_{max} - OD_t}{OD_{max} - OD_0}, \quad (2)$$

where OD_{max} was the highest optical density reading achieved upon complete lysis of cells in a batch, OD_0 was the initial optical density reading, N_0 was the initial cell count, N was the cell count at any time t and OD_t was the optical density measurement at time t .

3. Results and Discussion

Composition of the suspending medium is known to affect the permeability of the membrane of red blood cell to nutrients and metabolites (Langsdorf and Zydney, 1994). To avoid possible variations, the media used in this work differed only in the non-nutrient shear-protective additive. The media contained no nutrients other than dissolved oxygen.

Fig. 2 presents the time-course of decline in cell number in albumin-supplemented cell slurry in shake flasks agitated at 100 and 200 rpm. Cell number data obtained by direct counts and that calculated from optical density measurements are shown (Fig. 2). Excellent agreement is seen between the two techniques over most of the exposure period. Clearly, the hydrodynamic environment corresponding to an agitation rate of 200 rpm caused more rapid lysis of cells than did milder agitation at 100 rpm. Shake flasks were not bubbled with air; only surface aeration supplied the oxygen needs of the cells. The slight disparity between the two measurement techniques seen in Fig. 2 at long periods (> 80 h) of exposure was observed consistently. This behaviour was associated with oxidation of the released haemoglobin which caused high apparent optical density measurements and, hence, lower than actual cell numbers.

The fraction of cells remaining after exposure to different agitation–time combinations in albumin-supplemented slurry in shake flasks is shown in Fig.

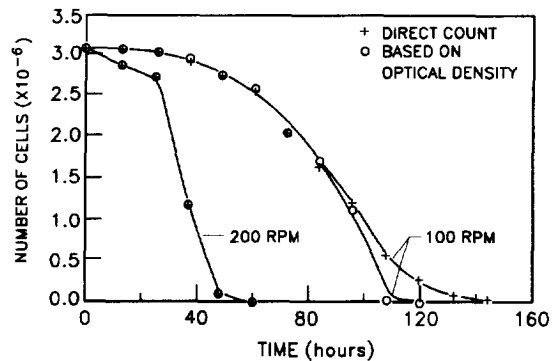


Fig. 2. Comparison of the direct count and optical density methods of evaluation of cell number in shake flasks with albumin-supplemented cell slurry agitated at different rates.

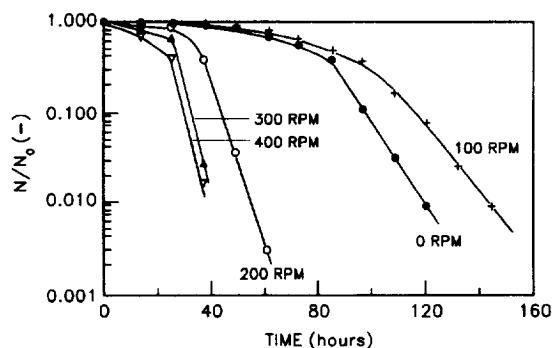


Fig. 3. Effect of agitation rate and exposure time on cell lysis in albumin-supplemented cell slurry in shake flasks.

3. Note that the cells in static shake flask ($\text{rpm} = 0$) lysed significantly faster than those agitated at 100 rpm. The faster lysis in static environment was associated with oxygen starvation of the cells. Agitation at 100 rpm enhanced oxygen supply through the surface by improving the gas-liquid mass transfer coefficient and mixing in the slurry. However, more severe agitation, above 100 rpm, caused progressively faster loss of cells. This behaviour was observed consistently also in slurry without any additives and in dextran-supplemented slurry as shown in Figs. 4 and 5, respectively. Comparison of Figs. 3–5 shows that changes in agitation rate over 0–100 rpm had the greatest survival enhancing effect in additives-containing media relative to shake flasks without additives. This suggests that, relative to additives-free operation, the additives caused some reduction in oxygen transfer from the surface. Addi-

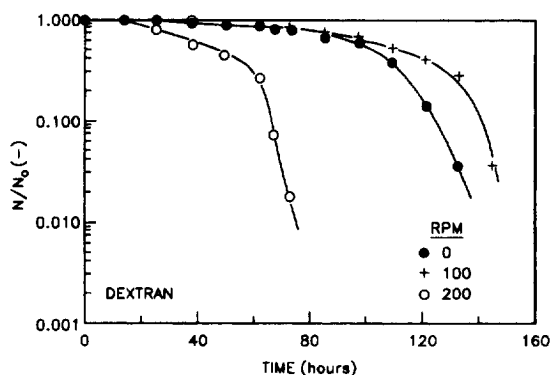


Fig. 4. Effect of agitation rate and exposure time on cell lysis in dextran supplemented cell slurry in shake flasks.

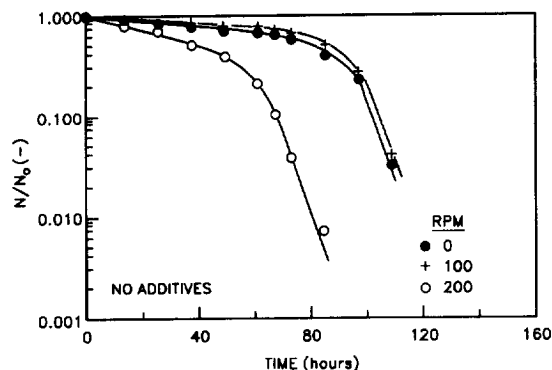


Fig. 5. Effect of agitation rate and exposure time on cell lysis in additives-free cell slurry in shake flasks.

tives that are viscosity enhancers or surfactants are known to reduce oxygen absorption in surface aeration either by suppressing turbulence in the liquid film at the gas-liquid interface or by creating a film of surfactant molecules on the surface of the liquid (Cullen and Davidson, 1956; Moo-Young and Shoda, 1973). The surfactant film may provide an additional physical barrier to oxygen transport and have a calming effect just below the surface by making it more rigid. In systems without additives (Fig. 5), the surface-aeration in stationary flasks was sufficiently close to meeting the oxygen demand that increase in agitation rate to 100 rpm had only a marginal effect on survival. Note that even in additives-free operation, agitation at 100 rpm caused no more lysis than observed in stationary flasks (Fig. 5).

The observed lysis behaviour could be analyzed in terms of a two zone model: an initial period in which the lysis rate was independent of the cell concentration, thus

$$-\frac{dN}{dt} = K_0, \quad (3)$$

and a subsequent period of faster lysis where the rate had a first-order dependence on the number of cells, or

$$-\frac{dN}{dt} = K_1 N. \quad (4)$$

Both the zero-order (K_0) and the first-order (K_1) lysis rate constants were affected by the agitation conditions (Figs. 2–5) and the type of additive (Figs. 6 and 7). In all cases, K_0 was much lower than K_1 .

This lysis behaviour suggests that the cell membrane and other structures that maintain physical integrity of the cell withstand hydrodynamic abuse for some time; failure occurs eventually, possibly due to prolonged fatigue. Note that fatigue failure of structures is well known; structural strength declines over time with frequent intermittent, or continuous, exposure to strain. Such recurrent exposure is expected in turbulent flow fields. Interactions between the cell membrane and the additive molecule seem to affect the ability of the membrane to withstand hydrodynamic forces. Depending on the type of additive, such interactions may strengthen or weaken the membrane and are a likely explanation for the observed differences in survival characteristics. Membrane-related properties of the cell – deformability, fragility and rate of aggregation, for example – are known to be affected by the composition of the medium (Langsdorf and Zydney, 1994). Because the flasks were not aerated, bubble-associated damage that has been observed for other types of animal cells (Bavarian et al., 1991; Chalmers and Bavarian, 1991; Handa-Corrigan et al., 1989; Jöbses et al., 1991) could not occur.

The *in vitro* survival times of erythrocytes as measured in this work are meaningful only in the context of the natural life span of these cells. Erythrocytes have a finite life span *in vivo*. Transfused cells may survive up to 130 d. Physical abuse in the circulation and incapability for extensive self-repair lead to ultimate destruction of the cell (Surgenor, 1974; p. 22). *In vitro*, the red cell survival time in citrated, refrigerated (4°C), blood without such other

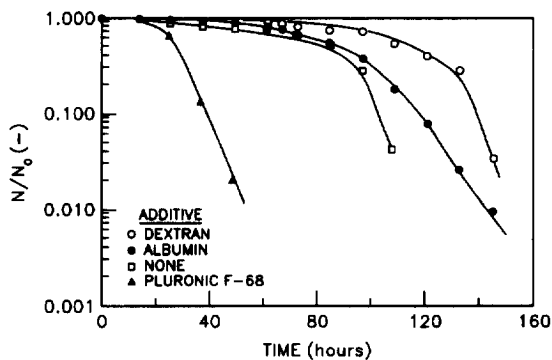


Fig. 6. Effect of additives on cell lysis in shake flasks agitated at 100 rpm.

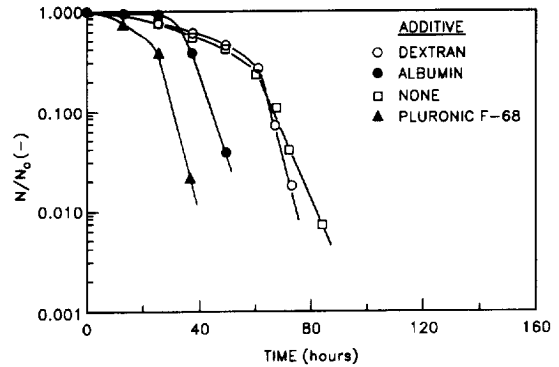


Fig. 7. Effect of additives on cell lysis in shake flasks agitated at 200 rpm.

additives as glucose, is about 6 d (Surgenor, 1974; p. 38). *In vitro* survival at 37°C is expected to be still lower even in quiescent environment with sufficient nutrients. Thus, the maximum observed survival time of about 80 h in shake flasks (100 rpm; no additives; Fig. 6) is only a little less than expected if the cells are to die a 'natural' death. Dextran supplementation increases survival to almost 5 d (Fig. 6). Dextran, a viscosity enhancer, is postulated to improve survival by dampening turbulence. If this were the sole mechanism of protection and membrane-additive interactions were not involved, the protective effect should be more widespread across cell lines. This does not seem to be the case. For example, Michaels et al. (1992) noted that dextran magnified cell death in an intensely agitated environment.

As shown in Fig. 3, for the albumin-supplemented slurry the greatest effect of agitation rate on cell loss occurred as the agitation rate increased from 100 to 200 rpm. Further increase in agitation rate to 400 rpm did not cause as dramatic an increase in cell loss (Fig. 3). This effect is clearly illustrated in Fig. 8 where the specific cell lysis rate is plotted against the agitation speed. A sharp increase in the specific lysis rate is observed over the 100–300 rpm range. The specific lysis rates in Fig. 8 were calculated from the slopes of the first-order portions of the lysis curves in Fig. 3. This suggests the existence of a 'critical' agitation-related hydrodynamic environment over which the cell are most susceptible to shear-related damage. For the shake flasks, this range seems to be over 100–300 rpm; however, this critical range may

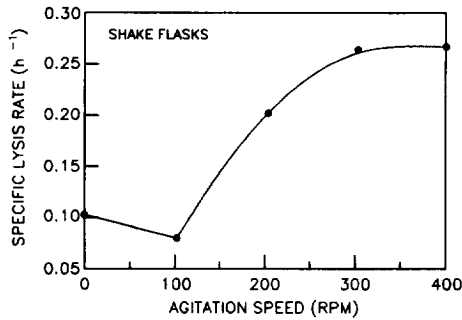


Fig. 8. Effect of agitation rate on specific cell lysis rate in albumin-supplemented cell slurry in shake flasks.

be additive dependent which is consistent with the hypothesis that different additives affect the membrane differently.

The protective effect of different additives on cells is shown in Figs. 6 and 7. The data in Fig. 6 were obtained at 100 rpm in shake flasks. Presence of albumin and dextran improved cell survival relative to additives-free operation. Pluronic F68 did not protect the cells against shear; it actually increased the susceptibility of cells to agitation. For any given additive, the magnitude of the protective effect depended on the hydrodynamic environment. Thus, while dextran and albumin had a clear protective effect at 100 rpm, these additives failed to afford any protection in the critical hydrodynamic environment at 200 rpm (Fig. 7).

For slurries with albumin and with no additive, the fraction of cells remaining after various durations of exposure to the environment of the airlift reactor is shown in Fig. 9. Albumin supplementation extended the duration of survival sufficiently that, for similar cells, a 3 d batch culture is expected to pose no lysis problems in these systems.

The cell lysis characteristics of the airlift device and the best case performance of the shake flask (100 rpm) are compared in Fig. 10 for albumin-supplemented cells. Although the cells survived longer in the shake flask, the length of survival in the airlift device was sufficient for practical purposes. As shown in Fig. 10, in the airlift device no cell loss was experienced during the first 24 h. A slow, zero-order lysis ensued and at 66 h the lysis became faster with first-order kinetics. The zero-order (K_0) and the first-order (K_1) rate constants were, respec-

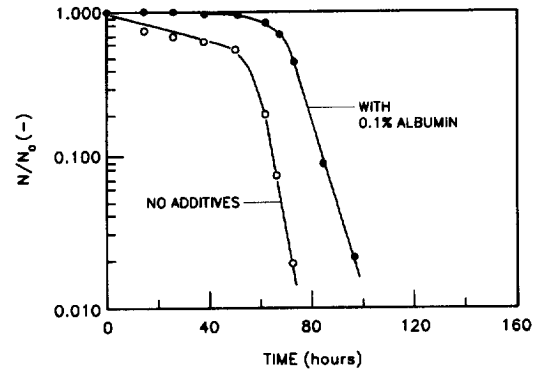


Fig. 9. Fraction of cells remaining as a function of exposure time in airlift reactor.

tively, 16.4×10^{-3} cells per ml per h and 0.119 h^{-1} . The cell loss behaviour could be summed up as follows:

$$N = N_0 \text{ for } 0 < t < 24;$$

$$N = N_0 - K_0 t \text{ for } 24 < t < 66; \text{ and}$$

$$N = (N_0 - 66K_0) \exp[-K_1(t - 66)]$$

for $66 < t < 108$, where t was in h.

In vivo, in dynamic circulation, red blood cells are deformable and take a variety of shapes (Surgenor, 1974; p. 214). In a quiescent environment, unconfined red blood cells at steady state have the biconcave circular disc configuration (Surgenor, 1974; p. 214). In comparison, most animal cells used in commercial suspension culture are spheroidal. Because of these differences, questions arise as to the

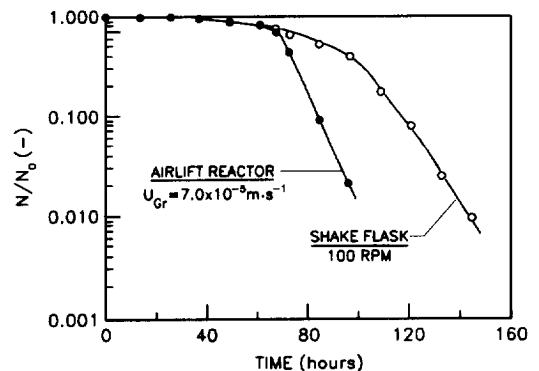


Fig. 10. Comparison of cell survival in shake flask and airlift reactor for albumin-supplemented cells.

shear susceptibility of erythrocytes relative to the more commonly used cells such as hybridomas. For a hybridoma cell line Macmillan et al. (1987) documented reduced growth rate relative to stationary culture in shake flasks agitated at 40 rpm. Because the flasks had a small size – one-tenth the total flask volume used in this study – the hydrodynamic environment must have been substantially more quiescent than in the present work. Thus, the porcine erythrocytes were significantly more robust than the unusually sensitive hybridoma line described by Macmillan et al. (1987), but apparently less so compared to the more typical hybridoma cells such as those investigated by Dodge and Hu (1986) in small spinner vessels or by Chisti (1993) in industrial stirred tank bioreactors. However, the media used by Dodge and Hu (1986) and by Chisti (1993) were supplemented, respectively, with 10% (v/v) horse serum and 5% (v/v) fetal calf serum. Because serum is a demonstrated shear protectant (McQueen and Bailey, 1989) it explains, at least partly, the higher observed shear tolerances of the hybridomas.

Because of an anisotropic geometry, red blood cells may be more susceptible to certain directional shear forces than to others. However, in circulation in airlift devices, the cells are unlikely to experience the structural deformations they undergo in veins and arteries *in vivo*. In the low aeration laminar flow environment of the airlift reactor the anisotropic cells are likely to be aligned with the flow streamlines, further reducing the hydrodynamic forces on the cells.

A non-growing and well-defined cell population such as that provided by erythrocytes is particularly useful in comparative evaluations of different bioreactors, or different modes of operation of a given reactor, for supporting culture of fragile cells. This methodology can be used to distinguish regimes that are hydrodynamically less damaging. The reactor configuration or operating regime that is less damaging to erythrocytes is likely to be less destructive to other delicate cells.

4. Conclusions

A minimal level of agitation was necessary for longest survival of erythrocytes. Beyond this level,

increasing agitation caused progressively faster lysis. The general pattern of cell lysis displayed two phases: an initial period of slow lysis that was zero-order in cell concentration and a subsequent period of faster lysis with first-order kinetics. This behaviour indicated that cellular structures withstood mechanical forces for considerable periods before failing under fatigue. Supplementation of the cell suspension with albumin and dextran improved survival, but Pluronic F68 made the cells more susceptible to lysis in flasks agitated at 100 rpm. Cell damage was greater in the airlift device than in shake flasks, but this had no adverse effect on applicability of the airlift system to animal cell culture. The qualitative behaviour of cell loss in albumin-supplemented medium was similar for the airlift and shake flasks. Albumin was shown to have a protective effect on erythrocytes in aerated and non-aerated environments. In the latter, the magnitude of protection depended on the hydrodynamic conditions.

5. Nomenclature

A_d	cross-sectional area of the downcomer (m^2)
A_r	cross-sectional area of the riser (m^2)
g	gravitational acceleration ($m\ s^{-2}$)
h_c	clearance of the baffle from the bottom of reactor (m)
h_t	clearance between upper edge of baffle and static slurry level (m)
K_0	zero-order lysis rate constant (cells per ml per h)
K_1	first-order lysis rate constant (h^{-1})
N	number of cells per ml (ml^{-1})
N_0	initial number of cells per ml (ml^{-1})
OD_{max}	optical density reading upon complete lysis (–)
OD_t	optical density reading at time t (–)
OD_0	initial optical density reading (–)
P	power input (W)
rpm	revolutions per minute (min^{-1})
t	time (h)
U_{Gr}	superficial gas velocity based on riser ($m\ s^{-1}$)
V_L	volume of liquid or slurry (m^3)
ρ_L	density of the slurry ($kg\ m^{-3}$)

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