

Mixing Theory for Culture and Harvest in Bioreactors of Human Mesenchymal Stem Cells on Microcarriers

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Abstract. The use of human mesenchymal stem cells (hMSCs) in regenerative medicine is a potential major advance for the treatment of many medical conditions, especially with the use of allogeneic therapies where the cells from a single donor can be used to treat ailments in many patients. Such cells must be grown attached to surfaces and for large scale production, it is shown that stirred bioreactors containing $\sim 200 \mu\text{m}$ particles (microcarriers) can provide such a surface. It is also shown that the just suspended condition, agitator speed N_{JS} , provides a satisfactory condition for cell growth by minimizing the specific energy dissipation rate, ε_T , in the bioreactor whilst still meeting the oxygen demand of the cells. For the cells to be used for therapeutic purposes, they must be detached from the microcarriers before being cryopreserved. A strategy based on a short period (~ 7 min) of very high ε_T , based on theories of secondary nucleation, is effective at removing $> 99\%$ cells. Once removed, the cells are smaller than the Kolmogorov scale of turbulence and hence not damaged. This approach is shown to be successful for culture and detachment in 4 types of stirred bioreactors from 15 mL to 5 L.

Key words: Mesenchymal stem cells; Bioreactors; Microcarriers; Growth; Suspension; Detachment.

INTRODUCTION

The use of stem cells for regenerative medicine is a potential major advance in the treatment of many medical conditions, especially with the use of allogeneic therapies where the cells from a single donor can be used to treat ailments in many patients. Human mesenchymal stem cells (hMSCs) are promising for such therapies given their ability to grow in stirred bioreactor which are the most likely candidate for large scale production. Many of the process requirements that the stirring must achieve are typical of all bioreactors; namely adequate homogeneity with respect to nutrients, metabolites, temperature and pH; and mass transfer of O₂ into the media and CO₂ out. There are also a number of special features; firstly, the cells are the product rather than the agent for making a product such as a therapeutic protein; and secondly, they have to be grown attached to a surface. To provide sufficient surface area to make growth in a stirred bioreactor efficient, small particles (microcarriers), typically of ~ 200 µm, are added to it. Thus, particle suspension becomes important. Also, like all animal cells, hMSCs do not have a cell wall and are potentially damageable due to agitation; and the larger the entity in suspension compared to the Kolmogorov scale of turbulence, the greater the likelihood of damage. Thus, cells which in free suspension are ~ 15-18 µm are now exposed to greater stresses due to their position on the surface of ~ 200 µm microcarriers. Cells may also be damaged or stripped off the surface of the microcarrier by microcarrier-impeller or microcarrier-microcarrier impacts. So the specific energy dissipation rate, ε_T , used during culture must be kept low. This approach suggests that operation at the minimum agitation speed for suspension should be adopted initially as a suitable strategy, especially has higher speeds lead to only small increases in mass transfer to particles [1].

Whilst there is significant focus in the literature on cell growth, there is little on the detachment or harvesting of the cells except at extremely small scales. Typically, cell detachment from microcarriers is by enzymatic digestion [2] aided by extensional flow using a pipette. At larger scales, such an approach becomes impractical and in addition, the enzymes can also damage the cells if exposure is prolonged. Thus, a new method is required. Given the sensitivity of particles of the size of microcarriers to abrasion (or if crystals, to secondary nucleation [3]), it was decided to try enhances the action of the enzyme by a short period of intense agitation at agitator speeds significantly greater than N_{JS} .

This paper reports these two approaches to culture and detachment in 4 different bioreactors, 15 mL ambrTM (TAP Biosystems) (Figure 1), 125 mL spinner flasks (Figure 2), 250 mL DASGIP bioreactor (Eppendorf) (Figure 3) and a 5 L Sartorius Stedim bioreactor (Figure 4).

Figure 1 The 15 mL ambrTM bioreactor (63 mm high x 31 mm wide x 18 mm deep)

Figure 2 The 125 mL spinner flask

Figure 3 The 250 mL DASGIP bioreactor and impeller

Figure 4 The 5 L Sartorius Stedim bioreactor.

THEORY

Damage to cells on microcarriers

During culture at N_{JS} , the mean specific energy dissipation rate, $(\bar{\varepsilon}_T)_{JS}$ (numerically equal to the specific power, $(P/M)_{JS}$, imparted to the medium) is given by:

$$(\bar{\varepsilon}_T)_{JS} = (P/M)_{JS} = P_o \rho_L N_{JS}^3 D^5 / M_{JS} \quad (1)$$

where P_o is the impeller power number (dependent on the impeller type), D is the impeller diameter and M_{JS} is the mass of medium and microcarriers in the vessel. Though there are some issues over the use of Kolmogorov's theory of isotropic turbulence because the Reynolds numbers, Re , are in the transitional regime ($\sim 10^3 < Re < \sim 10^4$), it is the standard approach for considering the impact of fluid dynamic stress on organisms [4]. It suggests that provided the size of the biological entity, d_E , which is suspended in the flow, is less than the Kolmogorov scale, λ_K , the entity should not be damaged where

$$(\lambda_K)_{JS} = (v^3 / (\varepsilon_T)_{JS \max})^{1/4} \quad (2)$$

where $\varepsilon_{T \max}$ is the maximum local ε_T close to an impeller and v , the kinematic viscosity. In addition,

$$\varepsilon_{T \max} = \Phi \bar{\varepsilon}_T \quad (3)$$

where Φ depends rather weakly on the impeller type (it is similar with both so-called 'high shear' Rushton turbines and 'low shear' hydrofoil impellers [5]) and more on impeller diameter/vessel diameter ratio, D/T [5]. For cells on microcarriers, d_E is considered to be the size of the microcarrier, d_{micro} , typically $\sim 200 \mu\text{m}$, and earlier work showed that cell growth is not compromised provided $\lambda_K \geq \sim 0.6 d_{micro}$ [4, 6].

There is little literature on cell damage from microcarriers by impact with other microcarriers or with the rotating impeller. On the other hand, much has been done in relation

to damage to crystals (secondary nucleation) from such mechanisms [3]. Details of the models are beyond the scope of this paper but in essence they suggest that ‘damage’ (whether destroying cells or just removing them from microcarriers) are relatively very sensitive to $\bar{\varepsilon}_T$. For example, for microcarrier-impeller impacts, the potential for damage, D_{M-I} is related to the agitation parameters by the functionality,

$$D_{M-I} \propto \bar{\varepsilon}_T N \quad (4)$$

whilst for microcarrier-microcarrier impacts, the potential for damage, D_{M-M} is related to agitation by;

$$D_{M-M} \propto \bar{\varepsilon}_T^{1.5} \quad (5)$$

The precise formulation of these equations is not important. What is important is that the impact mechanisms are very sensitive to agitator speed. Thus;

$$D_{M-I} \propto N^4 \quad (6)$$

and

$$D_{M-M} \propto N^{4.5} \quad (7)$$

Cell detachment

The above theoretical relationships [3] suggest that increases in agitator speed above N_{JS} should lead to substantial increases in the impact mechanisms which could remove cells from microcarriers. However, once the cells are detached and become freely suspended, damage to the cells from these mechanisms does not occur because they are too small (~15 – 18 μm) compared to the Kolmogorov scale [7]. Thus, Equ 2 becomes

$$(\lambda_K)_D = (V^3 / (\varepsilon_T)_{D_{\max}})^{1/4} \quad (8)$$

and though $(\varepsilon_T)_{D_{\max}}$ is larger, $\lambda_K >$ the cell size, so the cell should not be damaged [7, 8].

EXPERIMENTAL

Cells were cultured at 37°C with a pH of ~ 7 maintaining 100% dO₂ by headspace aeration in the four different agitated bioreactors at N_{JS} as measured visually (see Table 1). The smallest was 15 mL ambrTM, which is shown in Fig. 1 [8]. The second was a 125 mL unbaffled spinner flask (Fig. 2) [4] and the other two were both cylindrical bioreactors of 250 mL (DASGIP) (Fig. 3) and 5 L (Sartorius Stedim) (Fig. 4). Further details of the procedure are given elsewhere [7].

After carefully removing the growth media by pipette, detachment was undertaken *in-situ* by the addition of an enzyme solution to the microcarriers with cells still attached and then agitating intensively for 7 minutes at the speeds given in Table 1. Subsequently, the cells were removed from the microcarriers by using a Steriflip® 60 µm filtration unit (Millipore, UK) so that the cells and suspending fluid passed through and the microcarriers remained in the filter. Further details are again given elsewhere [9].

In all cases after both culture and harvesting, in order to identify whether there had been any change in the hMSCs as a result of their treatment, they were analysed: 1) according to the International Society of Cell Therapy (ISCT) panel of markers to ascertain their immunophenotypic expression; 2) by tissue-culture adherence and morphology; and 3) for their multilineage differentiation potential [7, 8]. All were appropriate showing that the cells maintained the desired quality attributes for therapeutic usage. In addition, the post-harvest viability of the cells as determined by a Nucleocounter NC-3000 (Chemometec, Denmark) was always $> 95\%$.

Table 1. Agitation Parameters During Culture and Detachment

RESULTS AND DISCUSSION

The Kolmogorov scale at N_{JS} during culture in each bioreactor.

Taking each bioreactor in the order of Table 1, the shape of the 15 mL ambrTM together with the flow patterns generated by the impeller [9] makes it difficult to prevent the microcarriers settling in the corners, especially those furthest away from the impeller. Cells tend to attach preferentially there, forming cell aggregates which increase as the culture progresses. The small scale leads to a high agitation speed [10] but the characteristics of the ambrTM enhance it further, leading to a high $\bar{\varepsilon}_T$. Estimating Φ as 18 [5] based on the equivalent diameter of the ambrTM vessel [9] (giving $D/T = 0.48$) gives $(\varepsilon_T)_{JS \max} = 0.14$ W/kg and the Kolmogorov scale, $(\lambda_K)_{JS} = 52$ µm. Thus, $(\lambda_K)_{JS} = \sim 0.25d_{micro}$, much smaller than has

generally been considered acceptable for satisfactory culture on microcarriers [4, 6, 7, 8]. Yet the cells grew well (data not shown) and maintained their quality attributes.

With the unbaffled spinner flasks, $\bar{\varepsilon}_T$ is low, probably because of the large D/T (~ 0.7) radial flow impeller [10]; and because the depth of the impeller means that as it rotates, it moves in a volume almost equal to that of the medium in the vessel. For this large D/T ratio, Φ has been estimated to be 10 [5], giving $(\varepsilon_T)_{JS\max} = 6.3 \times 10^{-3}$ W/kg and $(\lambda_K)_{JS} = 112$ μm . This value is much closer to the size suggested in the earlier work indicating that λ_K should be $> \sim 0.6$ times the size of the microcarrier for satisfactory culture [6].

The DASGIP is also unbaffled but has probes in it, which produce some baffling. The axial flow impeller is effective for suspension at $D/T = 0.48$ [10] giving $N_{JS} = 1.92$ rev/s. Estimating Φ as 18 [5] for this D/T ratio gives $(\varepsilon_T)_{JS\max} = 0.046$ W/kg and $(\lambda_K)_{JS} = 68$ μm , bigger than with the ambrTM but still smaller relative to the size of the microcarrier than is generally expected for good growth. Finally, for the 5 L Sartorius bioreactor [7] with $D/T = 0.43$ giving $\Phi = 25$ [5], $(\varepsilon_T)_{JS\max} = 0.049$ W/kg and $(\lambda_K)_{JS} = 67$ μm . Thus, for these two fairly similar geometry bioreactors, the agitation parameters that determine the potential for damage, namely $(\varepsilon_T)_{JS\max}$ and $(\lambda_K)_{JS}$ are very similar. Again the cells grew well [7].

The Kolmogorov scale during detachment in each bioreactor

For the 15 mL ambrTM, N_{JS} and especially $(\varepsilon_T)_{JS\max}$ were very high leading to a relatively small λ_K compared to the size of the microcarrier. Thus, a detachment speed, $N_D = 2N_{JS}$ was first chosen (Table 1), which increases the higher detachment parameter by 35 times greater than during cultivation. Detachment was successful (Figure 5) with $(\varepsilon_T)_{D\max} = 2.8$ W/kg and $(\lambda_K)_D = 24$ μm , somewhat greater than the size of the detached cells. The cells maintained the hMSC quality attributes with a viability $> 95\%$. Subsequently, the speed was reduced (see Table 1) to give $(\varepsilon_T)_{D\max}$ approximately equal to that parameter in the other bioreactors. Detachment was again successful.

Figure 5 SoloHill Plastic Microcarriers: a) with cells attached as indicated by red arrows; b) ‘clean’ microcarriers (A) after cells (B) have been detached.

For the spinner flask, because $(\varepsilon_T)_{JS\max}$ was so low, $N_D = 5N_{JS}$ was chosen, increasing the higher detachment parameter by up to ~ 1400 times that used during cultivation to give $(\varepsilon_T)_{D\max}$ approximately equal to the lower value used with the ambrTM (1.31 W/kg), to give

$(\lambda_K)_D = 30 \mu\text{m}$. Thus, again, $(\lambda_K)_D$ was greater than the size of the detached cell and detachment was successfully achieved with the cells maintaining their quality attributes.

For the DASGIP, initially an agitation speed $\sim 5N_{JS}$ as with spinner flasks was used but it caused severe surface bubble entrainment, perhaps because of the lack of proper baffling. As a result, N_D was reduced to $\sim 3.25N_{JS}$ to avoid this phenomenon, which also gave $(\varepsilon_T)_{D_{\max}} = 2.23 \text{ W/kg}$, a similar value to that in the two smaller vessels; and $(\lambda_K)_D = 26 \mu\text{m}$. Once again, successful detachment was achieved.

The volume of media used in the 5L bioreactor was too great to be treated in the Steriflip but detachment was achieved by using the spinner flasks sequentially as above.

CONCLUSIONS

These two agitation strategies offer a useful approach to the scale-up of stem cell culture, at least until much higher cell densities are achieved when $\bar{\varepsilon}_T$ required to give adequate oxygen transfer may not be sufficient at N_{JS} . Nevertheless, with cells growing at $(\lambda_K)_{JS} = \sim 30\%$ of the microcarrier size, it may be that, as with animal cells in free suspension [9], hMSCs are more robust than has generally been perceived.

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NOTATION

d — particle diameter, m

D — impeller diameter, m

M — mass in vessel, kg

N — agitator speed, rev/s

P — power input, W

Po — power number, dimensionless

Re — Reynolds number (ND^2/ν), dimensionless

T — bioreactor diameter, m

ε_T — local specific energy dissipation rate, W/kg or m^2/s^3

$\bar{\varepsilon}_T$ — mean specific energy dissipation rate, W/kg or m^2/s^3

λ_K — Kolmogorov scale of turbulence, m

ν — kinematic viscosity, m^2/s

Φ — $(\varepsilon_T)_{\max} / \bar{\varepsilon}_T$, dimensionless

ρ_L — fluid density, kg/m^3

SUBSCRIPTS

D — during detachment

E — biological entity

JS — at the just suspended condition

max — maximum

micro — microcarrier

M-M — microcarrier-microcarrier impacts

M-I — microcarrier-impeller impacts

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Culture Platform	Culture volume/ Detachment volume	Power no., P_0/Φ^1	D (m)/ T (m)	N_{JS} (s ⁻¹)	$(\epsilon_T)_{JSmax}$ (W/kg)	$(\lambda_K)_{JS}$ (μm)	N_D (s ⁻¹)	$(\epsilon_T)_{Dmax}$ (W/kg)	$(\lambda_K)_D$ (μm)
				Expansion parameters			Detachment parameters		
15 mL TAP ambr™	15 mL/ 6 mL	2.1 ² / 18	0.011/ 0.023	6.67	0.142	52	13.3/ /10.8	2.83/ 1.50	24/29
125 mL spinner flask	100 mL/ 60 mL	1.0 ³ / 10	0.055/ ~0.08	0.5	0.0063	112	2.5	1.31	30
250 mL DASGIP bioreactor	100 mL/ 70 mL	1.5 ⁴ / 18	0.030/ 0.063	1.92	0.046	68	6.25	2.23	26
5 L Sartorius bioreactor	2.5 L/ NA	1.5 ⁵ / 25	0.070/ 0.16	1.25	0.049	67	NA	NA	NA

1) Estimated from reference [5]; 2) From reference [9]; 3) From reference [4]; From reference [7] since agitators in the DASGIP and Sartorius bioreactors are similar 3 blade, pitched turbines; 5) From reference [7]

Table 1

Figure captions

Figure 1 The 15 mL ambrTM bioreactor (63 mm high x 31 mm wide x 18 mm deep)

Figure 2 The 125 mL spinner flask

Figure 3 The 250 mL DASGIP bioreactor and impeller

Figure 4 The 5 L Sartorius Stedim bioreactor.

Figure 5 SoloHill Plastic Microcarriers: a) with cells attached as indicated by red arrows; b) 'clean' microcarriers (A) after cells (B) have been detached.

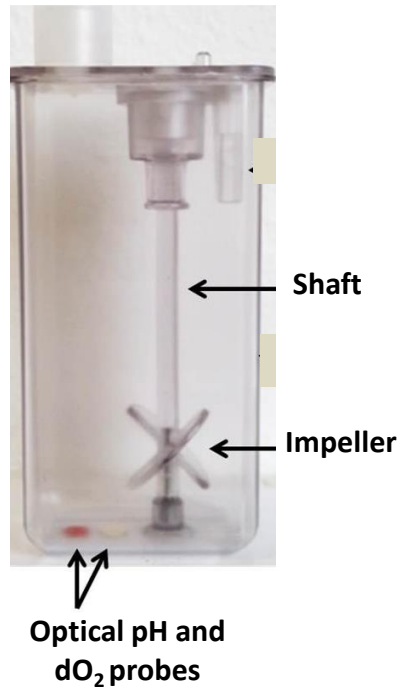


Figure 1

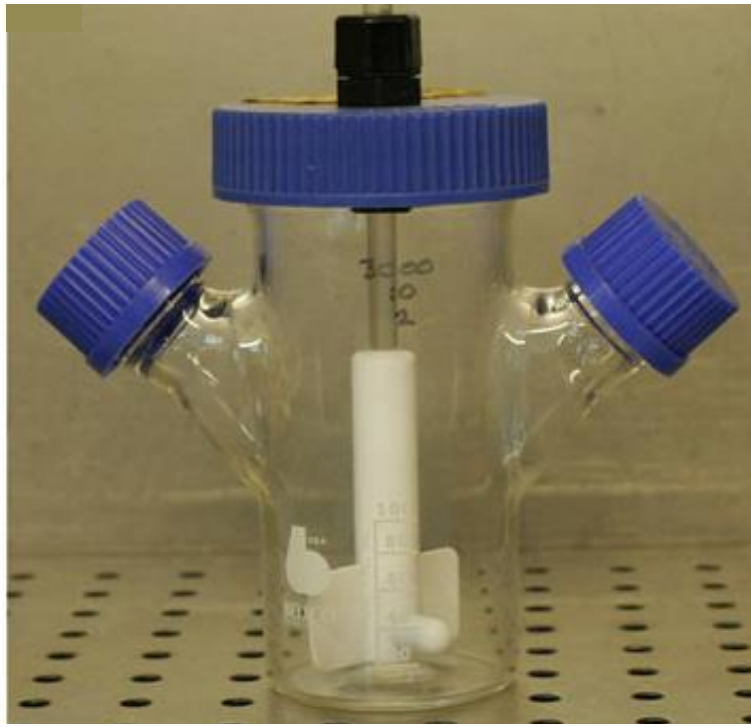


Figure 2



Figure 3

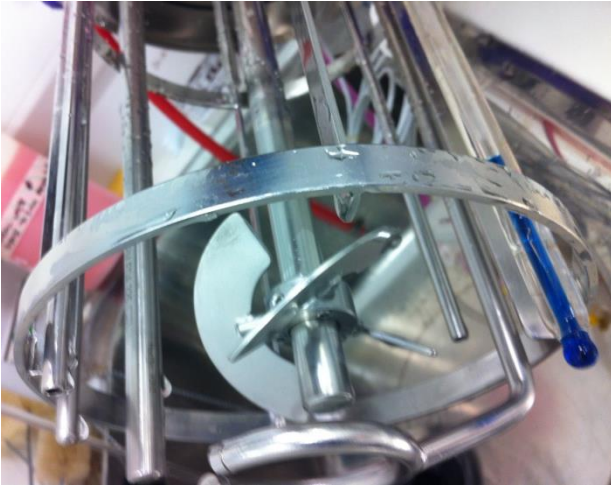


Figure 4

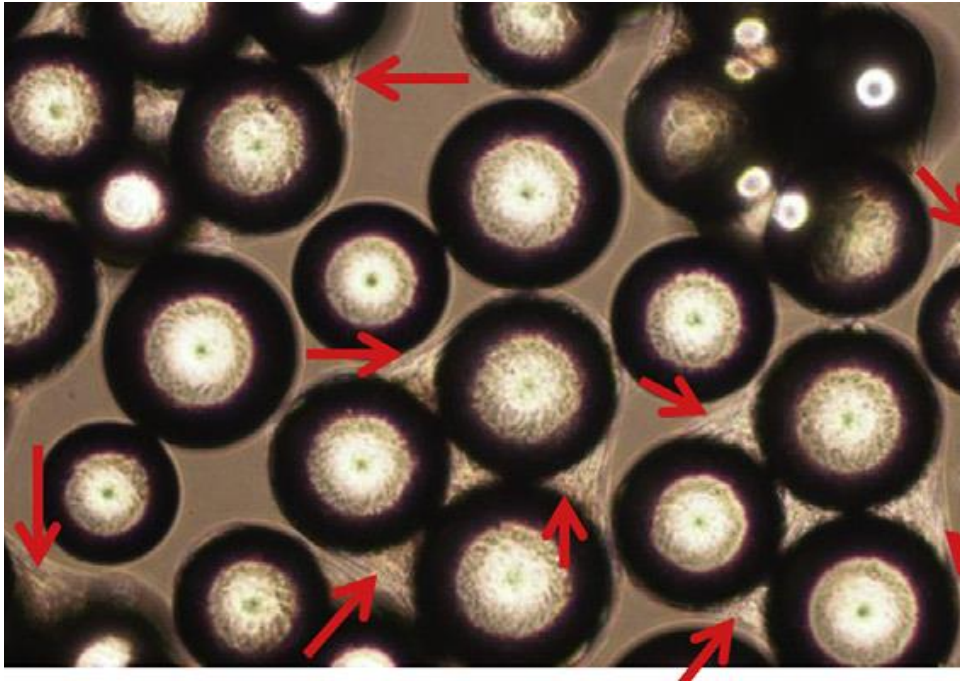


Figure 5a

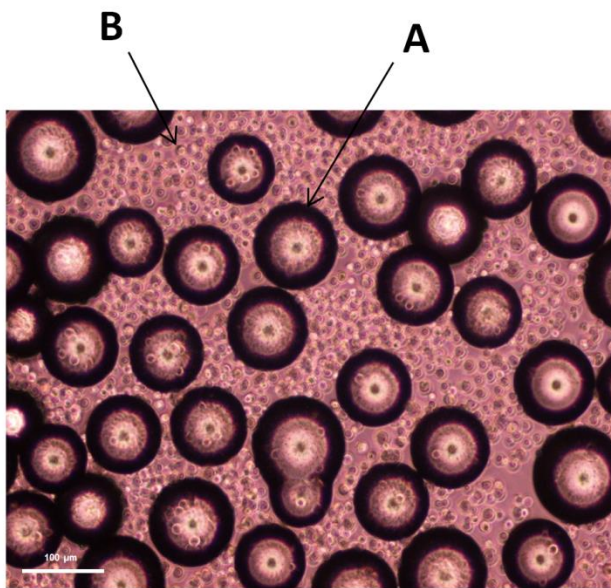


Figure 5b