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Agitation conditions for the culture and detachment of hMSCs from microcarriers in multiple bioreactor platforms

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ABSTRACT

In our recent work in different bioreactors up to 2.5 L in scale, we have successfully cultured hMSCs using the minimum agitator speed required for complete microcarrier suspension, N_{JS}. In addition, we also reported a scaleable protocol for the detachment from microcarriers in spinner flasks of hMSCs from two donors. The essence of the protocol is the use of a short period of intense agitation in the presence of enzymes such that the cells are detached; but once detachment is achieved, the cells are smaller than the Kolmogorov scale of turbulence and hence not damaged. Here, the same approach has been effective for culture at N_{JS} and detachment in-situ in 15 mL ambr™ bioreactors, 100 mL spinner flasks and 250 mL Dasgip bioreactors. In these experiments, cells from four different donors were used along with two types of microcarrier with and without surface coatings (two types), four different enzymes and three different growth media (with and without serum), a total of 22 different combinations. In all cases after detachment, the cells were shown to retain their desired quality attributes and were able to proliferate. This agitation strategy with respect to culture and harvest therefore offers a sound basis for a wide range of scales of operation.

1. Introduction

Unlike cell culture for traditional biopharmaceutical production, where the product of interest is usually a recombinant protein, cells expanded on microcarriers for cell therapies form the basis of the therapeutic. It is thus critical that the stem cells are successfully detached and separated from the microcarriers in a manner that does not adversely affect cell quality [1]. Therefore, successful cell recovery is determined not only by quantities of cells recovered but also by the quality of recovered cells. Indeed, effective cell recovery will reduce overall cost of goods by increasing process efficiency and enabling process intensification. However, few studies have harvested greater than millilitre samples of the microcarrier culture. This lack of publications on harvesting is somewhat surprising given that only successfully recovered cells can be used as part of any therapeutic. As such, an integral component to our work is the cell recoverability in all culture platforms we have considered. In addition, though there is substantial literature recognizing that during culture, gentle agitation is desirable, this condition is generally left rather vague.

In our 2011 paper on cultivating hMSCs on microcarriers in a stirred bioreactor, we used 125 mL spinner flasks (100 mL working volume), adopting a minimum mean specific energy dissipation rate, $\bar{\epsilon}_T$, strategy to reduce the possibility of damage to the cells [2]. In order to ensure adequate mass transfer to the cells of key nutrients (including oxygen) from, and unwanted metabolites to the culture medium, it was felt essential that the microcarriers were just fully suspended in it. The agitation speed at this condition is defined as N_{JS}, and this choice of speed set the minimum $(\bar{\epsilon}_T)_{JS}$. This strategy was successful, producing up to 3.8×10^5 cells/mL. Subsequently using the same strategy, we cultivated hMSCs in a 5 L (2.5 L working volume) bioreactor, which at the time was the largest scale reported in the peer reviewed literature and the first time hMSCs had been cultured on microcarriers in a stirred-tank bioreactor at the litre scale [3]. This study also showed that the

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Nomenclature

hMSC	human mesenchymal stem cell
ISCT	International Society for Cellular Therapies
DMEM	Dulbecco's modified eagle medium
FBS	fetal bovine serum
SFM	serum free medium
HPL	human platelet lysate
d_C	cell diameter, m
d_{entity}	diameter of biological entity, m
$d_{microcarrier}$	size of microcarrier, m
D	impeller diameter, m
M	fluid mass, kg
N	agitator speed, rev/s
P	power input, W
Po	power number, dimensionless
Re	Reynolds number (ND^2/ν), dimensionless
S	suspension parameter, dimensionless
T	bioreactor diameter, m
X	mass of microcarriers/mass of media × 100, dimensionless
ϵ_T	local specific energy dissipation rate, W/kg or m^2/s^3
$\bar{\epsilon}_T$	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	$(\epsilon_T)_{max}/\bar{\epsilon}_T$, dimensionless
ρ_L	fluid density, kg/m^3
$\Delta\rho$	density difference between microcarriers and medium, kg/m^3

Subscripts

D	during detachment
JS	at the just suspended condition
max	maximum

specific oxygen uptake rate was extremely small compared to cells in free suspension and could easily be met by surface aeration at N_{JS}.

On trying to harvest the cells using the microcarrier manufacturer's protocol, we found it very unsuccessful. Since, in addition, there was a lack of literature on methods of harvesting all the cells efficiently at such a scale, we developed a method and briefly reported it [3]. In it, enzymatic detachment was aided by a short period of intense agitation (7 min at 5 times N_{JS}) of the suspension of cells attached to microcarriers enabled detachment to be achieved in a 100 mL spinner flask agitated by a magnetic spinner bar with spinner blades. After detachment, the enzymatic action was quenched by the addition of growth medium. The essential idea behind the method was the need to minimize the time cells were exposed to stress both from the enzymatic detachment solution and from fluid dynamic processes. The microcarriers were subsequently separated from the cells by vacuum filtration. We also reported that the cells after harvest met all the quality attributes as defined by the ISCT [3,4].

Subsequently, we published further details on this harvest method which included a theoretical justification for the detachment method as carried out in spinner flasks for hMSCs from two different donors [5]. In essence, we showed that the increase in agitation speed led to a one to two orders of magnitude increase in the mechanisms likely to remove cells from the ~200 μm microcarriers, namely fluid generated stresses or impacts between microcarriers and impellers or between themselves. What we also showed was that once the cells had been removed at this higher speed, even at the maximum specific energy dissipation rate close to the impeller, $(\epsilon_T)_{Dmax}$, the cells were smaller than the Kolmogorov microscale of turbulence, $(\lambda_K)_D$, and therefore should not be damaged [6]. We also suggested that because the technique was based on established agitation principles, it should be well enough understood to offer flexibility and thus be scalable, either up or down.

In one of our recent papers in 2015 [7], we used the same basic culture and detachment protocols as described above, undertaking both in the same spinner flask but with greater than three times the number of hMSCs per mL and in serum-free medium. In addition, the cells once harvested were exposed to a downstream holding time of four hours and a serum-free cryopreservation process. We then recovered the cells from cryopreservation and showed that they were able to successfully attach to and proliferate on tissue culture plastic and demonstrated the ability to form colonies as well as meet the ISCT minimum criteria.

We are now using this same N_{JS} criteria for culture followed by the detachment technique in-situ on a regular basis in stirred bioreactors from the 15 mL scale using the ambr™ (TAP Biosystems) [8] through spinner flasks to the 250 mL DASGIP bioreactor (Eppendorf). We have also used it with cells from a number of donors with a wide range of microcarriers (some with surface coatings), and with different media (containing animal or human serum, or serum-free) and detachment enzymes. Our general approach has been, in each case, based on the detachment concepts presented previously [5]. However, because the full protocol for detachment is yet to be established, and each bioreactor geometry presents different challenges, the precise high agitation intensity has been varied to meet each particular case as explained below.

It should also be noted that the experimental studies here were not aimed at optimizing or proving the versatility of the detachment technique. In each case, the main objective was to investigate some other aspect of hMSC culture, for example the impact of different donors on culture performance or the use of serum-based versus serum-free media. During each run, the culture was conducted at N_{JS} and on the basis of our earlier work [3,5], it was assumed that an appropriate detachment protocol could be found; and that the detachment achieved would enable an in-depth analysis of the main aim of the investigation to be undertaken more effectively since all the cells would be available for analysis. The detachment technique has proved to be as versatile as expected, and since there is so little in the literature on detachment of the whole suspension culture in stirred vessels, we thought it important to make the results available to the community. However, the paper does not give any other significant details on the culture conditions or the outcome of the other, albeit, main aims of these experimental studies.

2. Theoretical background to detachment and cell damage

This aspect has been discussed in detail earlier [5]. However, the broad brush principles are reiterated here. During culture at N_{JS}, the mean specific energy dissipation rate, $(\bar{\epsilon}_T)_{JS}$ which is numerically equal to the specific power, $(P/M)_{JS}$, imparted to the medium is given by:

$$\left(\bar{\epsilon}_T\right)_{JS} = \left(\frac{P}{M}\right)_{JS} = \frac{\mathrm{Po}\rho_L N^3{}_{JS} D^5}{M_{JS}} \quad (1)$$

where Po 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, as discussed in our earlier papers [2,5], because the Reynolds numbers, Re, are in the transitional regime ($\sim 10^3 < \mathrm{Re} < \sim 10^4$), it is the standard approach for considering the impact of fluid dynamic stress on

organisms. 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, then the entity should not be damaged where

$$(\lambda_K)_{JS} = \left(\frac{\nu^3}{(\epsilon_T)_{JSmax}}\right)^{1/4} \quad (2)$$

where ϵ_{Tmax} is the maximum local specific energy dissipation rate close to an impeller and ν is the kinematic viscosity. In addition,

$$\epsilon_{Tmax} = \Phi\bar{\epsilon}_T \quad (3)$$

where Φ depends rather weakly on the impeller type (it is very similar with both so-called 'high shear' Rushton turbines and 'low shear' hydrofoil impellers [9]) and more on impeller diameter/vessel diameter ratio, D/T [9]. A value of Φ used for studies of a range of biological entities at $D/T = 0.33$–0.4 has been about 30 [10]. For cells on microcarriers, d_E has usually been considered as the size of the microcarrier, $d_{microcarrier}$, typically ~200 μm, and earlier work in 1987 also conducted in spinner flasks and using arguments based on Kolmogorov's theory showed that cell growth is not compromised provided $\lambda_K \geq \sim 0.6 d_{microcarrier}$ [11].

The same theory can be applied when a high agitation speed, N_D is used for detachment so that

$$(\lambda_K)_D = \left(\frac{\nu^3}{(\epsilon_T)_{Dmax}}\right)^{1/4} \propto N^{-3/4} \quad (4)$$

thus, $(\epsilon_T)_{Dmax}(\propto N^3)$ is much greater than $(\epsilon_T)_{JSmax}$ and $(\lambda_K)_D << d_{microcarrier}$, so the cells are liable to be detached. However, once detached, the cells are in free suspension with a diameter d_C of about 13–18 μm, so that $(\lambda_K)_D > d_C$ and hence the cells should not be damaged. Other detachment mechanisms such as impeller-microcarrier impacts and microcarrier–microcarrier impacts are increased even more with agitation speed, $\propto N^4$ and $N^{4.5}$ respectively [5]. Which of these three mechanisms is the cause of detachment is difficult to determine but clearly, there is potentially much scope for increasing them by modest increases in speed. This potential is exemplified in the detachment results presented here.

Table 1
Summary of BM-hMSC line nomenclature and donor information.

hMSC line nomenclature	Donor age (years)	Donor gender	Donor ethnicity
BM-hMSC 1	20	Male	Black
BM-hMSC 2	19	Female	Black
BM-hMSC 3	24	Male	Caucasian
BM-hMSC 4	25	Male	Hispanic

3. Material and methods

For specific details of the T-flask monolayer, spinner flask and bioreactor culture conditions used for growing the cells, the reader is directed to Rafiq et al. [3]. The cells used in all studies were human bone-marrow derived MSCs purchased from Lonza (USA). These MSCs were isolated from healthy donors after the patients provided informed consent and the local Ethical Committee approved their use for research. The details of the donors and cells are given in Table 1 and the types of stirred bioreactors employed are listed in Table 2. Their internal surfaces were coated with sterile Sigmacote (Sigma–Aldrich, UK) to siliconise them, thereby preventing attachment of the cells and microcarriers to the vessel surface and such treatment proved critical for the 15 mL ambr™. Table 2 also gives the microcarriers employed (coated and uncoated), the different culture media (with and without serum) and the different enzymes and reagents used to aid detachment. The microcarrier and cell densities used were ~6000 microcarriers/mL and 5 cells/microcarriers respectively, to give 6000 cells/cm^2.

In all cases, the agitator speed used for culture of the cells was the minimum speed required to just completely suspend the particles, N_{JS}. It can often be calculated from the equation

$$N_{JS} = \frac{S d_p^{0.2} \left(\frac{g\Delta\rho}{\rho_L}\right)^{0.45} \nu^{0.1} X^{0.13}}{D^{0.85}} \quad (5)$$

where $\Delta\rho$ is the density difference between the solid particles (microcarriers) and the surrounding fluid (medium) and S is a geometric parameter depending particularly on the impeller type in baffled vessels. However, because of their configuration, S values are not available for the spinner flask or the ambr™. Also, though the unbaffled DASGIP bioreactor has a pitched blade turbine for which S values are available with normal baffling, because of the

Table 2
Combination of bioreactor, microcarrier (coated and uncoated), hMSC cells and media used along with the detachment enzyme (In all cases, >95% of cultured cells were recovered after harvest with viability >95%; and the ISCT quality criteria [4] were always met (see text in Section 4.1)).

Number	Vessel type	Microcarrier type	Surface coating	Cell line	Dissociation reagent	Culture medium
1 [5]	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 1	Trypsin/EDTA	DMEM (10% FBS)
2 [5]	100 mL Spinner Flask	Solohill plastic	None	BM-hMSC 2	Trypsin/EDTA	DMEM (10% FBS)
3	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 3	Trypsin/EDTA	DMEM (10% FBS)
4	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 4	Trypsin/EDTA	DMEM (10% FBS)
5	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 2	TrypLE express	DMEM (10% HPL)
6	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 3	TrypLE express	DMEM (10% HPL)
7	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 1	TrypLE express	DMEM (10% FBS)
8	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 1	Accutase	DMEM (10% FBS)
9	100 mL Spinner flask	Solohill plastic	None	BM-hMSC 1	Trypsin/EDTA + accutase	DMEM (10% FBS)
10	100 mL Spinner flask	Solohill plastic	Fibronectin (irvine scientific)	BM-hMSC 2	Trypsin/EDTA	DMEM (10% FBS)
11 [7]	100 mL Spinner flask	Solohill plastic	Fibronectin (Irvine Scientific)	BM-hMSC 2	TrypLE express	SFM (irvine scientific
12	100 mL Spinner flask	Solohill plastic	Fibronectin (irvine scientific)	BM-hMSC 1	TrypLE express	SFM (irvine scientific
13	100 mL Spinner flask	SoloHill plastic	LN-521 (BioLamina)	BM-hMSC1	Trypsin	DMEM (10% FBS)
14	100 mL Spinner flask	Solohill plastic	LN-521 (BioLamina)	BM-hMSC 1	TrypLE express	SFM (irvine scientific
15	100 mL Spinner flask	Solohill collagen	None	BM-hMSC 1	Trypsin/EDTA	DMEM (10% FBS)
16	100 mL Spinner flask	Solohill Collagen	None	BM-hMSC 2	Trypsin/EDTA	DMEM (10% FBS)
17	100 mL DASGIP bioreactor	Solohill plastic	None	BM-hMSC 2	Trypsin/EDTA	DMEM (10% FBS)
18	15 mL ambr™ bioreactor	Solohill plastic	None	BM-hMSC 1	Trypsin/EDTA	DMEM (10% FBS)
19	15 mL ambr™ bioreactor	Solohill plastic	None	BM-hMSC 1	TrypLE express	SFM (irvine scientific
20	15 mL ambr™ bioreactor	Solohill plastic	Fibronectin (irvine scientific)	BM-hMSC 1	Trypsin/EDTA	DMEM (10% FBS)
21	15 mL ambr™ bioreactor	Solohill plastic	Fibronectin (irvine scientific)	BM-hMSC 1	TrypLE express	SFM (irvine scientific)
22 [3,5]	5L Bioreactor (harvest in 100 mL spinner flask)	Solohill plastic	None	BM-hMSC 1	Trypsin/EDTA	DMEM (10% FBS)

Table 3
Agitation parameters during culture and detachment.

Culture platform	Culture volume/detachment volume	Power no., Po/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	6.3×10^{-3}	112	2.5	1.31	30
250 mL DASGIPbioreactor	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 [8].
(2)From reference [9].
(3)From reference [2].
(4)From reference [5] since agitators in the DASGIP and Sartorius bioreactors are similar 3 blade, pitched turbines.
(5)From reference [5].

sensitivity to the precise geometry [12], these S values are not appropriate. Finally, though S valuesare available for the configuration of the Sartorius bioreactor, Ibrahim and Nienow [13], who studied microcarrier suspension, found that the correlation (Eq. (5)) overpredicted N_{JS} by 50%. They suggested that it was because the studies that had established S were undertaken at much higher $\Delta\rho$ values. Thus, here N_{JS} was assessed experimentally by visual assessment, as has been the usual method [12,13]. The measured values of N_{JS} are shown in Table 3 for each bioreactor (diameter, T) along with the volume of medium used, the impeller diameter, D, and its power number, Po.

After culture, the detachment protocol described in detail before [3,5] was employed whether for passaging or for harvest. In summary, this procedure involved stopping agitation and allowing the culture to settle. After settling, as much as possible of the medium was removed (where it could then be used to assess nutrient uptake and metabolite production), ensuring that the settled microcarriers were not disturbed. The microcarriers were then washed twice with Ca^{2+}- and Mg^{2+}- free PBS whilst agitating in the presence of 5% CO_2. An appropriate amount of dissociation reagent was then added to the bioreactor (the volume used being given in Table 3 which also sets the value of M_D required to calculate $\left(\bar{\epsilon}_T\right)_D$ and then agitated at the speed, N_D for 7 min in each of the cases listed in Table 2. After filtration, the suspension of cells was then centrifuged for 5 min at 220 rpm and finally the separated cells were resuspended in growth medium. The reasons for the speeds chosen for detachment are given in the discussion of the results.

As in our previous work [3,5,7], after both culture and harvesting in order to identify whether there had been any change in the cells as a result of their treatment, they were analysed: (1) according to the ISCT panel of markers [4] to ascertain their immunophenotypic expression; (2) by tissue-culture adherence and morphology; and (3) for their multilineage differentiation potential. As before, all were appropriate showing that the cells maintained their desired quality attributes. In addition, the post-harvest viability (via acridine orange uptake and DAPI exclusion) as determined by the Nucleocounter NC-3000 (Chemometec, Denmark) was always >95%.

4. Results and discussion

4.1. Overview

In Table 2, Cases 1–4 were part of a study on the impact of different donors on culture variability as were Cases 5–9 whilst also investigating the impact of various media for growth and enzymes for detachment. Cases 10–14 were especially aimed at investigating different microcarrier coatings whilst in Cases 15 and 16, the microcarriers were changed. In all those cases, spinner flasks were used for culture followed by detachment in the same vessel whilst in Case 17, it was established that the DASGIP bioreactor could be used for both steps. Finally, in Cases 18–21, it was shown that the ambr™, which is so effective for clone selection with animal cells [8], was able to cultivate and detach stem cells at the 15 mL scale under a wide variety of conditions.

Table 2 also indicates that in all Cases, the cell recovery after harvesting was >95%. This value was assessed as set out in detail elsewhere [3] and was used here for Cases 1, 2 and 22 as already published [3,5] and in addition for Case 18. In summary, harvest is considered a two stage process [3]; detachment of cells from microcarriers followed by the separation of cells in suspension from microcarriers, in this work by filtration. The assessment of harvest efficiency was based firstly on a count of the cells on the microcarriers after culture followed by visual observation of the suspension after detachment by intense agitation, which indicated that the microcarriers no longer had cells attached to them and that they were present in suspension as single cells. After filtration to separate cells and microcarriers, counting of the cells was again undertaken after centrifugation and resuspension. This method was applied on each occasion for all the runs undertaken in those 4 cases and in every experiment, the harvesting technique recovered >95% [3,5]. In the other cases in Table 2, again observation after agitation showed that all cells had been removed as single cells and since the same filtration technique was used in every case, it can be assumed that the same overall harvesting efficiency would be obtained. As already noted, after harvesting, the cells in all cases had a viability >95%.

For each Case set out in Table 2, typically approximately 10 or more runs were undertaken and the cell density with each combination varied as a result of the impact of the different parameters being assessed; and in most cases, except as set out below, values of between 1 and 5×10^5 cells/mL were obtained. With BM-hMSC 3 and 4 which were less able to proliferate, the values were generally less than 1×10^5 cells/mL. With Cases 5, 6, 8, 9 and 22, only 4 runs were undertaken just in order to assess reproducibility. Typically, the reproducibility for any given case was ±8% or less of the mean value for that Case.

It is planned to publish the detailed outcome of these studies separately in the future so they will not be discussed in further detail here. However, we believe it is important to show how the agitation strategy outlined earlier for culturing and harvesting cells has now been applied successfully to so many cases. Therefore, the remainder of the paper concentrates on that strategy. Importantly however, the work also showed that the strategy adopted did not distort the main aims of the experiments. This conclusion could be reached because the relative performance of the cell lines from the different donors reported for growth in T-flasks [14] was the same during experiments with the same cells on microcarriers (data not shown).

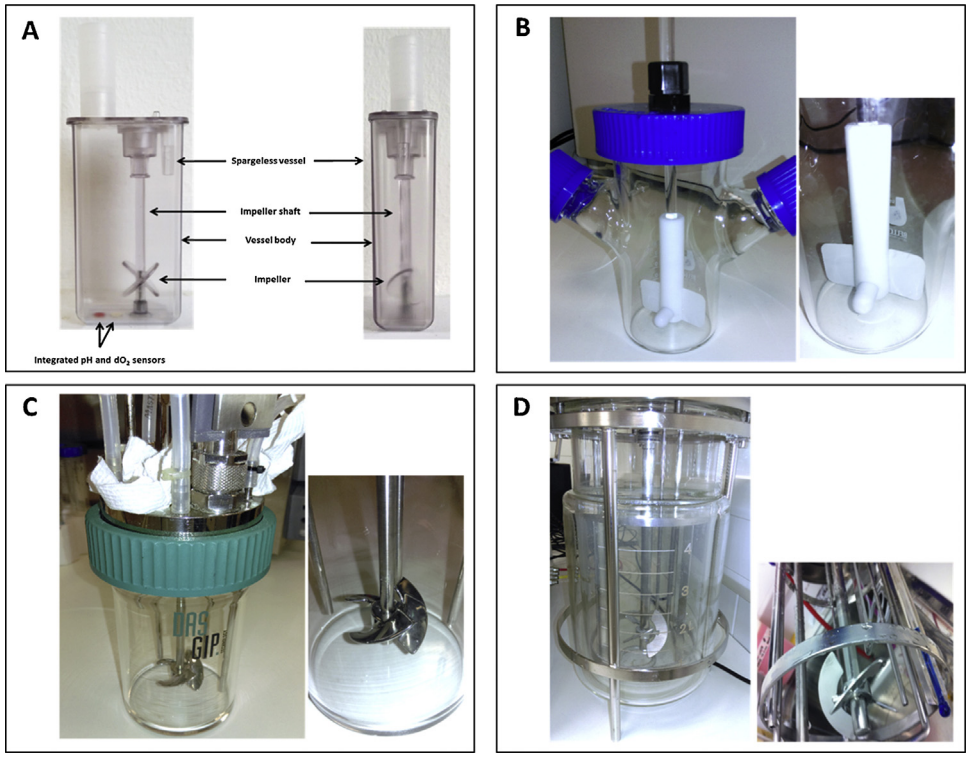

Fig. 1. The bioreactors used: (A) the 15 mL operating volume ambr^{TM} (vessel body, 63 mm high × 31 mm wide × 18 mm deep); (B) 125 mL spinner flask; (C) 250 mL DASGIP; (D) 5 L Sartorius Stedim Univessel.

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

Perhaps because of the very low density difference between the microcarriers and the medium, which is the most important physical property determining N_{JS} as indicated by Eq. (5) [12,13], in each case, the choice of microcarrier had a negligible effect on it. Hence, only one speed for N_{JS} is given in Table 3 for each bioreactor.

Taking each bioreactor in the order of Table 3, the shape of the 15 mL ambr^{TM} (Fig. 1A) together with the flow patterns generated by the impeller [8] 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 aggregates which increase as the culture progresses. Hence, the need for very careful coating of the inside of the vessel with sterile Sigmacote. The small scale leads to a high agitation speed [8] but the shape of the vessel base and position of the impeller in the ambr^{TM} enhance it further, leading to a high mean specific energy dissipation rate. Estimating Φ as 18 (Table 3) from the work of Zhou and Kresta [9] based on the equivalent diameter of the ambr^{TM} vessel [8] (giving $D/T = 0.48$) gives $(\epsilon_T)_{JSmax} = 0.14$W/kgand the Kolmogorov scale, $(\lambda_K)_{JS}$ as 52 μm. Thus, $(\lambda_K)_{JS}$ is ~0.25 of the size of the microcarrier, much smaller than has generally been considered acceptable for satisfactory culture on microcarriers [2,3,5,11]. Yet the cells proliferated to confluence as can be seen from the cell density achieved (Table 2) and maintained their quality attributes.

Dealing next with the unbaffled spinner flasks (Fig. 1B), the mean specific energy dissipation rate is low [2], probably because of the large D/T (~0.7) radial flow impeller [12]; 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. In order to use the same criterion for all the geometries studied in this paper for assessing $(\epsilon_T)_{JSmax}$, for this large D/T ratio, Φ has been estimated to be 10 [9]. Thus, $(\epsilon_T)_{JSmax}$ is 6.3×10^{-3} W/kg giving $(\lambda_K)_{JS} = 112$ μm. This value is much closer to the size suggested in the earlier work that λ_K should be >~0.6 times the size of the microcarrier for satisfactory culture [11], possibly because both studies were done in spinner flasks.

The DASGIP (Fig. 1C) is also unbaffled but has many probes in it, which produce some effective baffling and reduce the swirling motion found in bioreactors without them. The axial flow impeller is an efficient impeller for suspension at $D/T = 0.48$; and $N_{JS} = 1.92$ rev/s [12,13]. Estimating Φ as 18 [9] for this D/T ratio gives $(\epsilon_T)_{JSmax} = 0.046$W/kgand$(\lambda_K)_{JS} = 68\mu$m, somewhat bigger than with the ambr^{TM} but still smaller relative to the size of the microcarrier (~1/3) than generally expected for good growth. Finally, for the 5 L Sartorius bioreactor [3] (Fig. 1D) with $D/T = 0.43$ giving $\Phi = 25$ [9], $(\epsilon_T)_{JSmax} = 0.049$W/kgand$(\lambda_K)_{JS} = 67\mu$m. Thus, though for these two fairly similar geometry bioreactors, N_{JS} is higher for the smaller scale as expected [12], the agitation parameters that determine the potential for damage, namely $(\epsilon_T)_{JSmax}$ and $(\lambda_K)_{JS}$ are very similar. Again, as with the spinner flasks, cell culture was satisfactory and cells had the desired hMSC quality attributes.

4.3. The Kolmogorov scale during detachment in each bioreactor

As can be seen in Table 3, for the 15 mL ambr^{TM}, N_{JS} and $(\epsilon_T)_{JSmax}$ were very high. Initially it was intended to use the protocol established for detachment with the spinner flask, namely $N_D = 5N_{JS}$ [3,5] However, this speed was beyond that available in the ambr^{TM}, and so a detachment speed close to the maximum was chosen, $N_D = 2N_{JS}$ (13.3 rev/s) which still gave $(\epsilon_T)_{Dmax}$ more than twice as high as in the spinner flask. At this value of $(\epsilon_T)_{Dmax}$ $\left(= 2.8\text{W/kg}\right)$, $(\lambda_K)_D = 24\mu$m, still greater than the size of the detached cells. Nevertheless, as in all other cases, the cells maintained the hMSC quality attributes with a viability >95%. Subsequently, the speed was reduced to 10.8 rev/s as indicated in Table 3 in order to give $(\epsilon_T)_{Dmax}$ approximately equal to that parameter in the spinner flask and increasing $(\lambda_K)_D$ to 29 μm. Detachment was equally successful in the ambr^{TM} under each of the conditions given in Table 3.

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Detachment in the spinner flasks was originally undertaken successfully with microcarrier–cell suspensions from the 5 L bioreactor [3]. The same spinner flask conditions were used here with the same value of N_{JS} and with $N_D = 5N_{JS}$ to give $(\epsilon_T)_{Dmax} = 1.31$W/kgand$(\lambda_K)_D = 30\mu$m for all the combinations in Table 2. Thus, in all cases, $(\lambda_K)_D$ was greater than the size of the detached cell and detachment was successfully achieved.

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 reduce to $\sim 3.25N_{JS}$ to avoid this phenomenon, to give $(\epsilon_T)_{Dmax} = 2.23$W/kgand$(\lambda_K)_D = 26\mu$m . Both these parameters indicate more severe detachment conditions than in the spinner flasks but $(\lambda_K)_D$ is still bigger than the cell size; and, again, as with the ambr^{TM}, successful detachment was achieved.

5. Conclusions

Here we report 22 combinations of conditions in which hMSCs have been cultivated on microcarriers in four different types and sizes of bioreactor; and 21 combinations of detachment in-situ in the three smallest. The detachment technique was developed when the microcarriers manufacturer's protocol failed to work when cultivating cells in the 5 L Sartorius bioreactor; and this development was done at the 100 mL spinner flask scale [3,5] as indicated in Table 2. Currently, we do not have the facilities available for downstream processing of the volume involved at the 5L scale to enable successful harvest without compromising the process, so to date, culture at this scale has not been repeated.

It is shown here that the agitation speed required to just suspend the cells, N_{JS}, and the associated maximum specific energy dissipation rate, $(\epsilon_T)_{Dmax}$, is very different in the four stirred bioreactor configurations as is usually the case for suspending particles in stirred systems [12]. Indeed, in the 15 mL ambr^{TM} bioreactor, $(\epsilon_T)_{JSmax}$ is so high that $(\lambda_K)_{JS}$ is approximately equal to 25% of the size of the microcarrier, much smaller than has generally been considered acceptable for satisfactory culture on microcarriers [2,3,5,11]. In that respect, the situation is very similar to the use of the ambr^{TM} for cells in free suspension where $\bar{\epsilon}_T$ values up to $\sim$0.4 W/kg have been used successfully during clone selection [8] whilst a typical value at larger scale is of the order of 0.025 W/kg or less [6]. In other cases, $(\lambda_K)_{JS}$ varied from about 30% (250 mL DASGIP and 5 L sartorius) to 60% (spinner flask) compared to the microcarrier size. Regardless of these differences, in all cases, with different media (with serum and serum free), microcarriers (coated and uncoated) and cell lines, hMSCs proliferated successfully and met the desired critical quality attributes. Indeed, though the different combinations of other factors, which was the main aim of the investigations collected here, led to different cultivation performances (which will be reported later), there was no indication that the difference was due to the use of N_{JS} for cultivation or that it was not an appropriate agitation strategy.

Subsequently, all the hMSCs cultivated under these different conditions were detached by using different enzymes and a high agitation intensity strategy to give $(\lambda_K)_D < \sim 0.17$ times the size of the microcarrier but $> \sim 1.6$ times the cell size. In each case, this strategy led to successful detachment recovering >95% of the cells with >95% viability and meeting the usual desired quality attributes. It is suggested that these two agitation strategies offer a very useful approach to the scale-up of stem cell culture.

However, as higher cell densities are achieved, the mean specific energy dissipation rate required to give adequate mass transfer of oxygen may not be sufficient at N_{JS}. Clearly, the ability of the cells to proliferate at $(\lambda_K)_{JS} = \sim 25\%$ of the microcarrier size encourages one to think that possibly, as with other animal cells in free suspension [15], hMSCs are more robust than has generally been perceived. Nevertheless, more work is required to establish at what value of $(\lambda_K)_{JS}$, cell damage (or detachment) compromises cell proliferation; and also to optimise cell detachment by the use of a short period of high intensity agitation (enzyme concentration, time, agitator speed/specific power).

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