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A potentially scalable method for the harvesting of hMSCs from microcarriers

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ABSTRACT

The use of hMSCs for allogeneic therapies requiring lot sizes of billions of cells will necessitate large-scale culture techniques such as the expansion of cells on microcarriers in bioreactors. Whilst much research investigating hMSC culture on microcarriers has focused on growth, much less involves their harvesting for passaging or as a step towards cryopreservation and storage. A successful new harvesting method has recently been outlined for cells grown on SoloHill microcarriers in a 5 L bioreactor [1]. Here, this new method is set out in detail, harvesting being defined as a two-step process involving cell 'detachment' from the microcarriers' surface followed by the 'separation' of the two entities. The new detachment method is based on theoretical concepts originally developed for secondary nucleation due to agitation. Based on this theory, it is suggested that a short period (here 7 min) of intense agitation in the presence of a suitable enzyme should detach the cells from the relatively large microcarriers. In addition, once detached, the cells should not be damaged because they are smaller than the Kolmogorov microscale. Detachment was then successfully achieved for hMSCs from two different donors using microcarrier/cell suspensions up to 100 mL in a spinner flask. In both cases, harvesting was completed by separating cells from microcarriers using a Steriflip® vacuum filter. The overall harvesting efficiency was >95% and after harvesting, the cells maintained all the attributes expected of hMSC cells. The underlying theoretical concepts suggest that the method is scalable and this aspect is discussed too.

1. Introduction

Currently there is significant emphasis on improving human mesenchymal stem cell (hMSC) expansion techniques due to the interest in these cells for treating a number of diseases/disorders [2]. Studies have taken place investigating the culture of hMSCs on a range of commercially available microcarriers including Cytodex-1 [3–5], Cytodex-3 [6–8], Cultispher-S [9–11], Cultispher-G [12], SoloHill Plastic P102-L [13] and Collagen [14].

The choice of microcarrier depends on several factors, including cell attachment and proliferation, and whether they are xeno-free. However, in the majority of cultivation studies listed above, there has been little focus on the harvesting procedure, or consideration of the ability to effectively harvest should the process increase in scale [15]. Yet, detachment or dissociation of cells from the microcarrier surface and subsequent retention of cell quality is equally as important as cell attachment and proliferation given that the product of interest for cell therapies is the cell itself. It is also important to recognise that, independent of whether the cells are to be reused for further expansion following a passage or stored prior to use for therapeutic or other purposes, harvesting involves two steps. Firstly, the cells are detached from microcarriers to produce a cell–microcarrier suspension; and secondly, a further separation step leaving the cells in suspension without the microcarriers present. Therefore we have employed the term 'harvesting' to denote this two step process of recovering the hMSCs free from microcarriers. The term 'detachment' or 'dissociation' is used just for the first step of removing the cells from the microcarrier surface; and the second step is designated 'separation' and involves the use of techniques such as filtration or centrifugation. These issues will only be exacerbated as expansion scale increases and therefore it is crucial to consider cell harvesting strategies from the outset so as to ensure a viable, complete bioprocess.

Typically, cell detachment from microcarriers is by enzymatic digestion [16]. Weber *et al.* (2007) [17] studied detachment using different enzymes and a range of microcarriers (Biosilon, Cytodex 1, Cytodex 3, RapidCell and P102-L). They expanded hMSC-TERT

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Nomenclature

$d_{microcarrier}$	diameter of microcarrier
d_E	size of a small entity in suspension
D	agitator diameter
$D_{M\text{-}I}$	detachment parameter due to microcarrier–impeller impacts
$D_{M\text{-}M}$	detachment parameter due to microcarrier–microcarrier impacts
$h_{efficiency}$	harvesting efficiency
h_{actual}	number of viable cells obtained after harvesting
$h_{expected}$	number of viable cells in culture
M	mass of media
N	agitator speed
P	power
Po	power number
Re	Reynolds number
T	bioreactor diameter
X	microcarrier concentration.

Greek letters

ε_T	local specific energy dissipation rate
$\bar{\varepsilon}_T$	mean specific energy dissipation rate
Φ	$\Phi = \varepsilon_{T\,max}/\bar{\varepsilon}_T$
λ_K	Kolmogorov turbulence microscale
μ	viscosity
ν	kinematic viscosity
ρ_L	liquid density

Subscripts

js	just suspended
max	maximum

cells in spinner flasks and then detached the cells exposing them to either trypsin, accutase, collagenase or a trypsin-accutase mixture generally for 6 min. The cells cultivated on Biosilon, RapidCell and P102-L were detached successfully, especially with the trypsin, and trypsin-accutase mixture achieving high cell yield and viabilities near 100%. The results using collagenase were generally poor for Cytodex 1 and Cytodex 3 with any of the enzyme solutions where even after exposure for up to 10 min, significant detachment of the cells was not obtained. After another spinner flask expansion and harvest, the cells were successfully differentiated to adipocytes. Though a very interesting paper, the scale at which the harvesting was undertaken was only 5 mL, very small compared to current and predicted future need.

Others have suggested that a non-enzymatic harvesting procedure would be preferable so as to avoid potential cell damage or change in the immunophenotype of the cell [18,19]. Work by Yang et al. [8] has highlighted the possibility of harvesting hMSCs without the need for enzymatic digestion by using a modified Cytodex-3 microcarrier coated with a thermo responsive polymer. The hMSCs were expanded on these microcarriers, after which the culture temperature was lowered to 32 °C where it was suggested that hMSCs were able to detach from the microcarriers. However, this method involves an extra process step (coating of the microcarrier with the polymer), which in effect makes the innate surface properties of a microcarrier redundant and may prove problematic if a process has been designed to take advantage of its original surface properties. It may also prove difficult to obtain an even coating of the thermo responsive polymer across the entire surface of the microcarrier especially on scale-up.

Enzyme-free dissociation buffers also exist but are rarely reported to be used for routine passaging of cells and some formulations may not be suitable for use with hMSCs. Indeed, one study using a Gibco-brand enzyme-free dissociation buffer to detach hMSCs grown on tissue culture plastic found that cell viability following detachment was significantly reduced compared to trypsin controls (~70% compared to >90%, respectively) and that cells also failed to reattach to tissue culture plastic [20].

In a recently-published paper [1], we have reported the successful cultivation of hMSCs on Plastic 102-L microcarriers (SoloHill, USA) in a 5 L bioreactor (2.5 L working volume) agitated at an impeller speed, N_{JS} (rev/s), just sufficient to keep all the microcarriers in suspension. This microcarrier was selected from a range of commercially available and in-house produced microcarriers, based initially on an assessment of the efficiency of cell proliferation in microtitre plates [21]. It was found that the Plastic P102-L without any surface pre-treatment gave a level of attachment as good as any other and better than Cytodex-1. A complimentary study in spinner flasks also showed similar relative levels of attachment to the different microcarriers (data not shown) [21]. Given the desire to choose a xeno-free microcarrier and not having special thermo-responsive microcarriers available, it was decided to choose Plastic P102-L for this work. Successful cell harvesting (detachment plus further cell–microcarrier separation) to allow for the recovery of cells was reported [1]. However, as the main thrust of that paper was to report in detail how the successful cultivation was undertaken, the harvesting aspects of the work were not described in detail.

Here, we concentrate mainly on the development of the detachment step associated with the earlier study. Based on the above analysis of the literature, there did not seem to be any reason to choose as a starting point any detachment method other than that recommended by the manufacturers, SoloHill, of the Plastic P102-L [22]. This protocol involved exposure to trypsin-EDTA for 15 min; but as set out below, it did not prove at all successful for primary hMSC lines from two different donors. However, having cultured the cells on Plastic P102-L beads, moving to surface-modified beads [8] did not seem advisable and in addition, that approach did not seem suitable for scale-up. Therefore, we decided to try to develop a new detachment method based on certain theoretical concepts that would, unlike the technique proposed by SoloHill, be scalable and reproducible. Here we discuss how these concepts applied at this relatively small scale should form a sound basis for scaling up to commercial levels and describe the experiments and results obtained, mentioned briefly previously [1]. However, in order not to repeat the precise details already reported concerning cultivation conditions in the spinner flasks and the 5 L bioreactor from which the microcarriers and attached cells were obtained for harvesting, the reader is referred to the previous paper [1]. Here, the emphasis is entirely on the two cell harvesting steps applied to two hMSC donor lines giving a technique which should be suitable for scaling up to a full hMSC production process.

2. Theoretical basis for the new detachment protocol

Dos Santos et al. [13] used TrypLE Express aided by intense agitation (650 rpm) for 5–7 min to speed up the separation of cells from a microcarrier–cell suspension in a 0.5 mL sample taken from a spinner flask culture in order to assess the number of live cells that had been grown. It was decided to develop a larger scale protocol based on using agitation to speed up the process. This approach is not an obvious one as generally there is a concern that excessive agitation damages cells, particularly animal cells because of the lack of a cell wall [23]. However, it is also recognised that excessive exposure to the types of enzymes used in detachment can cause processing problems even when the cells are not themselves the product [24]. The amount of damage depends on the type and concentration of

the enzyme and the time of exposure [8,19]. On the other hand, there is increasing evidence that animal cells are less sensitive to mechanical stress than originally thought both with respect to cell viability and density [25,26] and protein quality [25]. It was therefore decided to use trypsin-EDTA for detachment but to enhance it by increased mechanical stress from agitation for a shorter time than that suggested in the manufacturer's protocol to minimise damage to the cells whether from stress or enzymatic action.

2.1. Stresses on cells and microcarriers from agitation

There are a number of mechanisms that can give rise to mechanical stress on microcarriers and the cells on them [23,27]. When growing cells, there are two issues, both of which can lead to a deterioration in performance in the bioreactor; one aspect relates to cell growth and/or viability and the other to cell quality. These issues are that the stress will detach the cells from the microcarriers and subsequently performance will be compromised; or the stress will damage the cell whilst still on the microcarrrier. However, when detachment is desired, then providing a high stress for a short time to ensure the cells are detached but not directly damaged may represent an appropriate strategy.

2.1.1. The impact of turbulent flow

The most commonly addressed stress associated with agitation is that arising from turbulence. In agitated bioreactors, provided the Reynolds number, Re $(=\rho ND^2/\mu)$ is $\geq 2 \times 10^4$, the flow is turbulent. Under these conditions, Kolmogorov's theory can be applied to the flow to establish the potential for damage to cells in suspension [28]. The theory has also been applied to cells on microcarriers. However, most published work in which mechanical stress issues with microcarriers have been addressed were carried out in spinner flasks or similar sized bioreactors with gentle agitation, just sufficient to maintain microcarriers in suspension, N_{JS} which gave Re values just $<2 \times 10^4$, i.e. just in the transition regime [6,27,29,30]. Nevertheless, for the want of any other approach, with microcarriers, it has always been assumed that Kolmogorov's theory is still applicable under these transitional flow conditions.

The application of Kolmogorov's theory to fluid dynamic stress due to turbulence on freely suspended cells is discussed in detail elsewhere [23,31]. This theory suggests that provided the size of the biological entity, d_E, that is suspended in the flow is less than the Kolmogorov scale, λ_K, then the entity should not be damaged where

$$\lambda_K = \left(\frac{\nu^3}{\varepsilon_{T\max}}\right)^{1/4} \quad (1)$$

where $\varepsilon_{T\max}$ is the maximum local specific energy dissipation rate close to an impeller and ν is the kinematic viscosity. The mean specific energy dissipation rate, $\bar{\varepsilon}_T$, is numerically equal to the specific power, P/M where

$$\bar{\varepsilon}_T = \frac{P}{M} = \frac{Po\rho_L N^3 D^5}{M} \quad (2)$$

where Po is the impeller power number, D is the impeller diameter and M is the mass of medium and microcarriers in the vessel. In addition,

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

where Φ depends on the impeller type and impeller diameter/vessel diameter ratio, D/T. It is also difficult to determine experimentally and depends significantly on the model used to convert raw turbulent flow measurement whether laser-based [32] or using hot-film anemometry [33] into $\varepsilon_{T\max}$. Values of Φ from about 10 to greater than 100 have been reported with a trend to smaller values with large D/T ratios and vice versa [34]. A value used for studies of a range of biological entities has been about 30 [35]. Alternatively, if Φ is unknown, $\varepsilon_{T\max}$ can be estimated by the long-standing recommendation [36], which is still considered appropriate [37], that all the energy dissipation occurs in the impeller zone, of volume equal to the impeller swept volume. This approach also implies that Φ increases with smaller D/T ratios. Thus, provided the impeller power number is known, it is possible to estimate the Kolmogorov scale.

This theory has been successfully applied to bacteria [38], yeast [39] and animal cells [23]. However, for cells on microcarriers, perhaps because the structure of the entity (cells on microcarriers) is different or perhaps because of the Re number indicating flow in the transitional regime, it has been found that cell growth is not compromised if $\lambda_K \geq \sim 0.6 d_{microcarrier}$ [30]. Similar results were found by Hewitt et al. [6] using the swept volume concept to estimate $\varepsilon_{T\max}$.

2.1.2. The effect of microcarrier impacts

Little work has been reported on the 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 (the removal of tiny nuclei, a process known as secondary nucleation, from the surface of relatively large crystals, typical of the size of microcarriers) from such mechanisms [40]. Theories for the two cases of particle–particle impacts [41] and particle–impeller impacts [42] for secondary nucleation are similar to those developed later for those two mechanisms for microcarrier culture by Cherry and Papoutsakis [27]. The details of these models are beyond the scope of this article but in essence they suggest that 'damage' (whether destroying cells or just removing them from microcarriers) is very sensitive to mean specific energy dissipation rate.

For example, for microcarrier–impeller impacts [42] based on a model related to the frequency of circulation of microcarriers through the impeller zone, the kinetic energy of impact should impact occur, and the probability of impact, the potential for damage, $D_{M\text{-}I}$ is related to the agitation parameters by the functionality,

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

and is proportional to particle concentration, X.

For microcarrier–microcarrier impact on the other hand, the model [41] is based on an energy cascade, starting with that put in by the impeller causing both fluid and particle motion. Of the latter, part goes to overcoming fluid drag, part to elastic deformation following impact and part to damage, $D_{M\text{-}M}$. The latter is related to agitation and other parameters by;

$$D_{M\text{-}M} \propto d^6_{microcarrier}\bar{\varepsilon}_T^{1.5}X^2 \quad (5)$$

where $d_{microcarrrier}$ is the diameter of the microcarrier. The equations developed by Cherry and Papoutsakis [27] are very similar.

The precise formulation of these equations is not important. What is important is that the impaction mechanisms do not apply to free suspension cells because they are too small to cause either type of impact to occur. Secondly, the mechanism associated with the Kolmogorov scale is only weakly dependent on specific power (or relatively on agitator speed) since

$$\lambda_K \propto \bar{\varepsilon}_T^{-1/4} \propto N^{-3/4} \quad (6)$$

whilst the impact mechanisms are very sensitive to agitator speed. Thus;

$$D_{M-I} \propto N^4 \quad (7)$$

and

$$D_{M\text{-}M} \propto N^{4.5} \quad (8)$$

Thus, it should be possible to dramatically increase parameters which lead to cell detachment by increasing agitation intensity (speed) whilst at the same time keeping the Kolmogorov scale greater than the cell size once it has been removed. Those ideas are the fundamental ones behind this new method of cell detachment.

3. Material and methods

3.1. Assessing live cells in relation to detachment efficiency

The cells used in all studies were human bone-marrow derived MSCs which were obtained from Lonza (Lonza, Cologne AG) from two healthy donors after the patients provided informed consent. The local Ethical Committee approved the use of the sample for research. All studies were conducted with hMSCs at passage 3 for both primary cell lines, hereafter referred to as hMSC1 and hMSC2. For further 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. [1]. Of particular importance for this study is the bead and cell densities used in the stirred spinner flasks and bioreactor, which were 5000 beads/ml and 5 cells/bead respectively, to give 6000 cells/cm^2, approximately the same as in the T-flask. At confluence, the maximum cell density expressed as cells/cm^2 was very similar in all configurations.

A critical element in assessing detachment is the ability to determine accurately the number of live cells on the microcarriers before detachment is commenced; and then the numbers removed (or possibly still remaining on the microcarriers after detachment has been tried). Ideally, all cells should be removed and all cells before and after removal should be viable. Here, viable cell number was assessed using the automated NucleoCounter NC-100 (Chemometec, Denmark) which involved permeabilising the cell membrane by adding Buffer A100 (Chemometec, Denmark) in an equal volume to the cell sample. The sample was then mixed using a vortex mixer for 30 s and then left to settle for 30 s to ensure that all cells were exposed to the permeabilisation buffer. Buffer B (Chemometec, Denmark), a stabilisation buffer, was then added in equal volume to that of Buffer A and similarly was mixed for 30 s and left to stand for 30 s before running the sample. The NucleoCassette (Chemometec, Denmark) was then used to withdraw the permeabilised cell sample and run on the NucleoCounter NC-100. Nuclei released following cell permeabilisation are stained by propidium iodide contained within the NucleoCassette.

Initially, it was established that the number of live hMSCs attached to microcarriers could be accurately counted this way, based on the concept that since the cells would be permeabilised, they would release their nuclei into the cell suspension and could then be subsequently analysed. This ability to count cells whilst still attached to microcarriers was investigated by taking samples of cell–microcarrier cultures in spinner flasks. After 4 days in culture in two separate 100 mL spinner flasks as described in detail in our previous paper [1], four 2 mL samples were collected from each flask. For two of the samples, the number of cells still attached to the microcarriers was estimated as set out above (i.e. assuming that permeablisation had released the nuclei); and two samples after cell detachment from the Plastic P102-L microcarriers following the method described by Dos Santos et al. [13]. In summary, they took a small (0.5 mL) microcarrier–cell suspension sample from a spinner flask and washed it with PBS and TrypLE Express. The suspension was then held at 37 °C for 5–7 min whilst agitating at 650 rpm using a temperature controlled sample tube mixer, ThermoMixer comfort (Eppendorf, Germany). Subsequently, medium was added to stop enzymatic activity and the cells were filtered from the microcarriers suspension, the cell number and viability being determined using the Trypan Blue exclusion method. Essentially, the same technique was used here except detachment was achieved using trypsin-EDTA.

3.2. Detachment using the manufacturer's protocol

The hMSC1 and hMSC2 cells were grown in duplicate in 100 mL dimple-bottomed spinner flasks at a working volume of 100 mL (vessel height = 135 mm, vessel diameter, T = 60 mm) (BellCo, USA) with a magnetic horizontal stir bar (length = 40 mm) and a vertical paddle (paddle diameter, D = 50 mm). The horizontal stir bar was set to 5 mm above the dimple bottom of the spinner flask and agitation was achieved using a Bell-Ennium^{TM} Compact 5 position magnetic stirrer platform (BellCo, USA). With a 100 mL working volume, the height of the liquid in the vessel was 50 mm and agitation was set at 30 rpm (N_{JS}), the speed assessed visually as being necessary to just maintain all of the microcarriers fully in suspension. The spinner flask was installed in a 37 °C, humidified incubator supplemented with air containing 5% CO_2.

The cells were then cultured for a period of 6 days, with a 50% medium exchange after 72 h as described in detail in [1]. Microcarrier samples were then collected at day 6 and the number of cells attached to the microcarrier was determined as described above using the automated NucleoCounter NC-100.

A harvesting procedure suggested by the microcarrier manufacturer (SoloHill) was then applied. This method involved stopping agitation and allowing the culture to settle. The medium was then removed, ensuring that the settled microcarriers were not disturbed. The microcarriers were then washed twice by adding 100 mL of Ca^{2+} and Mg^{2+} free PBS and agitating the culture at 40 rpm for 15 min at 37 °C in the presence of 5% CO_2. 30 mL of 0.25% (v/v) trypsin and 0.02% (w/v) EDTA was then added to the spinner flask and allowed to incubate without agitation for 15 min again at 37 °C with 5% CO_2. Though not directed by the manufacturer, it was considered important, as when detaching cells from T-flasks, that after the 15 min incubation, the microcarrier–cell suspension containing trypsin and EDTA was diluted with 45 mL of growth medium to minimise the impact of trypsin on the quality of the cells. Finally, the cell suspension was gently pipetted up and down to dislodge the cells into a single cell suspension as directed by the manufacturer; presumably to expose the cells to elongational shear stresses [43]. This suspension of cells was then centrifuged for 5 min at 220 rpm and resuspended in 20 mL of growth medium and the number of cells present was again assessed.

3.3. The new harvesting protocol for detachment followed by separation into a cell-only suspension

The new protocol was developed for harvesting from a spinner flask based on the theory set out in Section 2.2. Here the detachment principle as applied to the current flasks is first quantified and then the precise experimental procedure is given.

As reported in [1], hMSC1 cells were successfully cultivated by agitation at N_{JS} (30 rpm) in the spinner flask identified in Section 3.2. An estimation of the maximum specific energy dissipation rate was obtained from [6] where the same spinner giving the same N_{JS} as here was used, gives $\varepsilon_{T\max} = \sim 9 \times 10^{-4}$ W/kg. Thus, from Eq. (1), λ_K is ~180 μm, a value as expected, well above the eddy size (suggested from the work of Croughan et al. [29,30]) at which damage should occur. For detachment, it was then decided to significantly increase the speed for a short time to enhance detachment but keep it less than that at which bubbles would be dragged into the suspension through the upper surface as damage due to stresses associated with bubble disengagement through that surface can be extremely damaging for animal cells [23]. Thus, a speed of 150 rpm and a time of 7 min were selected.

Under these conditions, the five fold increase in impeller speed increased the detachment mechanisms, $D_{M\text{-}I}$ and $D_{M\text{-}M}$ by factors of ~625 and ~1400 respectively. However, once detachment was complete, whilst the value of $\varepsilon_{T\max}$ increased to ~0.11 W/kg, the Kolmogorov scale was only reduced to 55 μm, well above the size of the detached cells. Therefore, once detached, damage to the cells should not occur. It was decided to use this approach to try to improve the technique proposed by SoloHill [22].

The actual experimental programme enabled both the new protocol and the SoloHill one to be directly compared. The hMSC1 and hMSC2 cells were grown in four 100 mL spinner flasks at a working volume of 100 mL as set out in detail in [1] and as described in Section 3.2. For detachment, initially samples were taken on day 6 to ascertain the viable cell number in each spinner flask prior to harvesting. Then, the technique also set out above was used to detach the cells using in two of the flasks the method proposed by SoloHill and in the other two using the new agitation strategy after the trypsin-EDTA had been added. Thus, the microcarrier suspension was agitated for 7 min at 150 rpm. In addition, because in monolayer culture, the tissue culture flask is usually manually tapped to aid detachment after removal from the incubator, it was decided to increase the speed to 200 rpm for the final 5 s. After agitation, the cells were quenched with 70 mL growth medium.

With SoloHill's detachment protocol, there was no effective separation step to generate a cell suspension free of microcarriers. Therefore, a new separation method needed to be developed too. Given that the size of the cells and the microcarriers are so different, it was felt that it would be appropriate to use vacuum filtration. For this step, therefore, the separation was achieved by using a Steriflip® 60 μm filtration unit (Millipore, UK) so that the cells and suspending fluid passed through and the microcarriers were filtered out. Even though aggregation of cells has been reported [5], none were observed here and filtration occurred in approximately 5 min. Moreover, though there were concerns that an excessive filter cake would form thereby restricting flow, this problem did not occur. The cell suspension was then centrifuged at 220 g for 5 min and resuspended in growth medium. Cell number and viability were then determined as described above. In addition to these experiments in which cells were both grown and detached in spinner flasks, as reported earlier [1], 60 mL of the 5 L bioreactor culture (hMSC1 cells) was transferred to spinner flasks and the same harvesting protocol was used.

Finally, another, 2.5 L culture as described in [1] was successfully undertaken in the 5 L bioreactor and a sample from the bioreactor was analysed using the Nucleocounter. Then, 500 mL were transferred into 5 fresh spinner flasks to perform the harvest, i.e. 100 mL of the culture was placed into each spinner flask. These 5 spinner flasks were then harvested independently using the new harvesting protocol; and after harvesting the 100 mL suspensions from the 5 spinner flasks were pooled.

Whilst the harvesting has been performed in spinner flasks, in principle, as discussed later, the cell detachment step can be carried out in situ in the bioreactor without the need to transfer to spinner flasks. However, it is still envisaged that the final separation of the cells from the microcarriers post-detachment to complete harvesting would be undertaken outside of the bioreactor.

3.4. Cell characterisation

The hMSC1 and hMSC2 cells were characterised in accordance with the International Society for Cellular Therapy (ISCT) criteria which states that greater than 95% of the cell population should be positive for CD73, CD90 and CD105 and less than 2% positive for CD14, CD19, CD34, CD45 HLA-DR. Moreover, the cells should demonstrate the ability to adhere and grow as a monolayer on tissue culture plastic and retain the ability to differentiate down the adipogenic, osteogenic and chondrogenic lineages [44]. To show these effects, the hMSCs post-harvest were analysed by flow cytometry for the aforementioned immunphenotypic markers as explained in Rafiq et al. [1]. Cells were also cultured in the respective differentiation media (all obtained from Life Technologies, UK) for 21 days with differentiation medium exchanges every 72 h.

For cells undergoing adipogenic and osteogenic differentiation, hMSCs were seeded onto microwell plates at a density of 5000 cells/cm^2 and placed in a humidified incubator at 37 °C, 5% CO_2. For cells undergoing chondrogenic differentiation, a micromass of hMSCs was generated by seeding 5 μL of hMSC suspension at a density of 1.6×10^7 cells/mL into a microwell plate. The microwell plate was placed into the incubator for 2 h, after which 1 mL chondrogenic differentiation medium was added; the cells were then returned to the incubator.

Staining of the hMSCs differentiated towards the chrondrogenic lineage involved fixing the cells with 2% (v/v) paraformaldehyde (PFA) for 30 min at room temperature, cells were washed three times with Ca^{2+} and Mg^{2+} free PBS. After this, a 1% (w/v) Alcian Blue (chondrogenic) solution (Sigma Aldrich, UK) was added to the cells which were then incubated at room temperature for 60 min. The samples were washed with PBS three times and 1 mL distilled water was added. Upon final washing, the samples were observed under a Nikon TS-100 light microscope (Nikon, UK).

Staining of the hMSCs differentiated towards the osteogenic lineage involved fixing the cells with 10% cold neutral-buffered formalin for 20 min at room temperature. The cells were washed with Ca^{2+} and Mg^{2+} free PBS and left in distilled water for 15 min at room temperature before incubating with 4% (v/v) Napthol AS-MX phosphate alkaline solution (Sigma–Aldrich, UK) in the dark at room temperature for 45 min. The cells were washed three times with distilled water, after which the samples were incubated with 2.5% (v/v) silver nitrate solution (Sigma–Aldrich, UK) for 30 min at room temperature. The samples were then washed with distilled water three times before observing under the Nikon light microscope mentioned previously.

Cells for differentiation controls were cultured and stained under the same conditions and subject to the same medium exchange routine, with the exception that the cells were cultured with growth medium.

The assessment was performed on cells prior to expansion on the microcarriers and on the cells after harvesting. In addition, cells obtained after harvesting were seeded into T-25 flasks to determine their ability to attach to tissue culture plastic as well as to qualitatively identify any morphological changes to the cells post-harvest using a Nikon TS-100 light microscope (Nikon, UK).

4. Results

4.1. Assessment of cell viability before and after detachment

To determine whether we could effectively ascertain cell viability whilst the cells were still attached to the microcarrier, a study was conducted where samples of cell–microcarrier suspensions were detached according to the Dos Santos et al. [13] protocol and counted. These cell count values were then compared with values obtained when cells were not removed from the microcarrier using the Nucleocounter (as described in Section 3.1). In spinner flask 1, the cell density obtained was 1.48×10^5 cells/mL after detaching the cells (again using the Dos Santos et al. method [13]), whilst the value was 1.52×10^5 cells/mL after counting with the cells still attached to the microcarrier (the Nucleocounter method). The values for spinner flask 2 were 1.36×10^5 cells/mL and 1.28×10^5 cells/mL respectively. The difference between the viable cell densities from these two counting methods were not

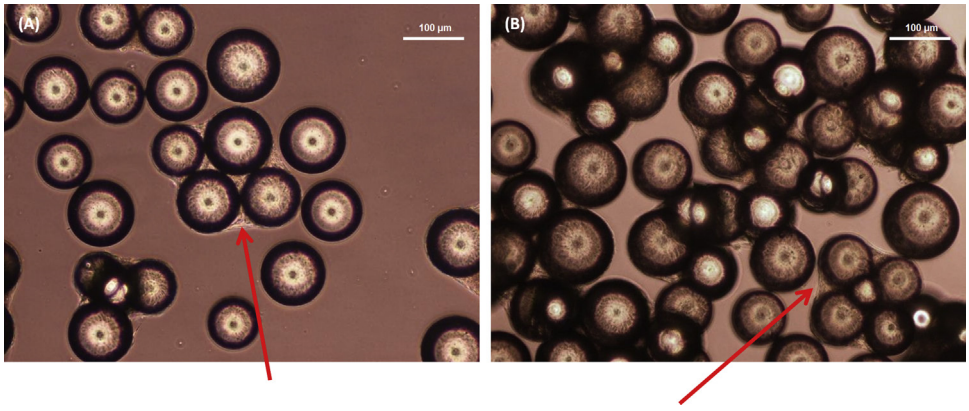

Fig. 1. Phase-contrast image of microcarrier and cell suspension after dissociation during the manufacturer's harvesting procedure for (A) hMSC1 and (B) hMSC2. Magnification 10×.
Arrow: hMSC still attached to the microcarrier

statistically significant as determined by the Bland–Altman analysis (where differences on the Bland–Altman plot were deemed insignificant based on 95% limits of agreement). As such, the two methods can be considered comparable. Any difference was probably due to sample variations used for the analysis. Clearly, the technique was able to assess the number of live cells initially attached and then detached.

4.2. Detachment efficiency using the manufacturer's protocol

Based on the cell density obtained on the microcarriers at the time of sampling from the spinner flask, there should have been ~14.6×10^6 viable cells present. However after harvesting, only 0.356×10^6 cells were obtained resulting in a harvesting efficiency of less than 2.5%, based on the definition

$$h_{efficiency}\,(\%) = \frac{h_{actual}}{h_{\exp ected}} \times 100 \qquad (9)$$

where $h_{efficiency}$ is the harvesting efficiency (%), h_{actual} is the number of viable cells obtained after the harvesting protocol and $h_{expected}$ is the number of viable cells expected in the culture based on the 2 mL sample analysis of cells on microcarriers outlined above. That efficiency is clearly unacceptable.

Two reasons for this low efficiency were considered possible. Firstly, cells may not have been removed from the microcarriers; and secondly, the separation technique based on centrifugation may have been inefficient. However, the latter worked well for the detachment of cells from T-flasks [21] and microscopic examination of the microcarriers after this treatment clearly showed that large numbers of cells were still on them for both the hMSC1 and hMSC2 cells (Fig. 1). To compare and contrast, Fig. 2 shows fresh microcarriers, i.e. the expected appearance of microcarriers when all the cells have been detached (Fig. 2A) and microcarriers during cultivation with many cells attached (Fig. 2B). Clearly, an improved detachment technique was required and the results from the one developed here are presented next.

4.3. 4.3 Detachment efficiency comparing the manufacturer's protocol and the new one

As in the initial study, the manufacturer's protocol resulted in a very low harvesting efficiency, this time 3.1% for hMSC1, after only 0.443×10^6 ($\pm 9.5 \times 10^4$) cells (mean value ± SD, $n = 4$) were successfully harvested from the microcarriers from an expected number of 14.3×10^6 ($\pm 8.5 \times 10^5$) cells (mean value ± SD, $n = 4$). The new approach, on the other hand, resulted in > 95% harvesting efficiency, with 14.5×10^6 ($\pm 3.5 \times 10^5$) cells (mean value ± SD, $n = 4$) successfully harvested from an expected number of 15.2×10^6 ($\pm 6.5 \times 10^5$) cells (mean value ± SD, $n = 4$). In both cases (harvest using the manufacturer's and the novel procedure), the cell viability of the harvested cells was greater than 98%.

Likewise for hMSC2, from an expected number of 15.4×10^6 ($\pm 6.6 \times 10^5$) cells (mean value ± SD, $n = 4$), only 0.752×10^6 ($\pm$ 5.9×10^4) cells (mean value ± SD, $n = 4$) were harvested using the manufacturer's protocol, a harvesting efficiency of 4.9%. In comparison, the new harvesting procedure again resulted in >95% harvesting efficiency where 14.2×10^6 ($\pm 6.2 \times 10^5$) cells (mean value ± SD, $n = 4$) were successfully harvested from 14.6×10^6

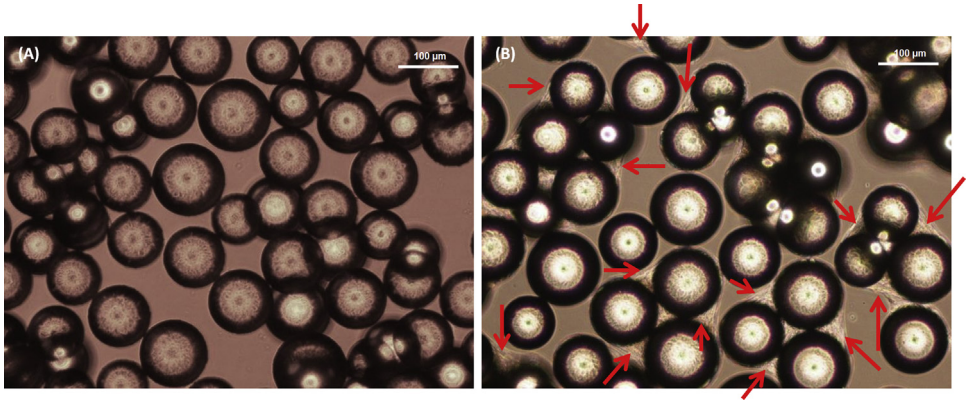

Fig. 2. Phase-contrast image of (A) fresh microcarriers with no cells attached and (B) microcarriers with many hMSCs attached as indicated by the arrows. Magnification 10x.

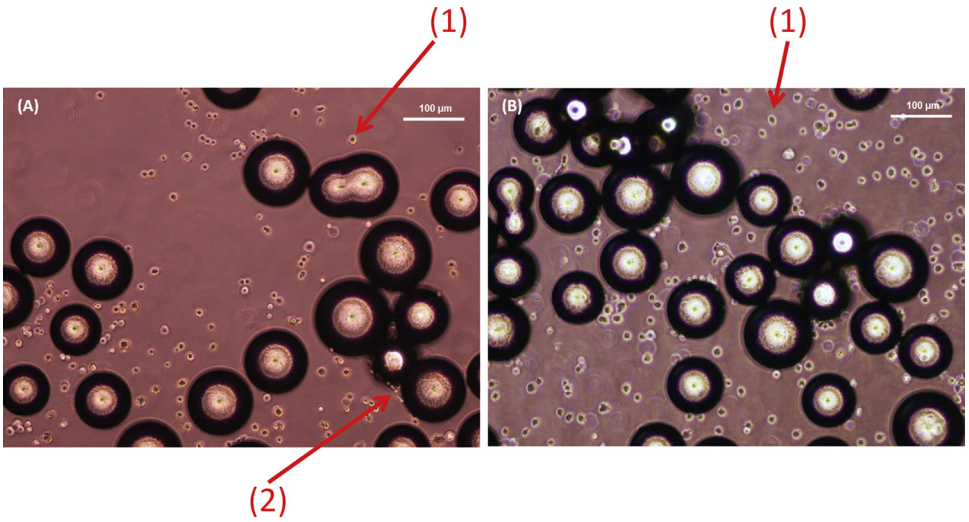

Fig. 3. Phase-contrast image of microcarrier and cell suspension after dissociation during the novel harvesting procedure for (A) hMSC1 and (B) hMSC2. Magnification 10x. Arrows: (1) Single hMSC successfully detached from microcarrier, (2) hMSCs still attached to microcarrier

($\pm 4.8 \times 10^5$) cells (mean value ± SD, $n = 4$). Clearly, the new harvesting procedure was significantly better than that based on the manufacturer's protocol, and could be considered a success. Similar results were obtained for the harvesting of samples taken from the bioreactor as described in Rafiq et al. [1].

The stark difference in the number of cells successfully harvested for both the hMSC1 and hMSC2 cells is clearly as a result of the agitation during trypsinisation, as illustrated in Fig. 3, which shows the microcarrier and cell suspension immediately after quenching with growth medium. Comparing this with Fig. 1 from the original study at the same point in the process, it is clear that the majority of the cells have been successfully harvested from the microcarriers with the new protocol whilst with the manufacturer's procedure, very few of the cells detached.

For the culture done in the bioreactor, the manufacturer's protocol was not applied. However, the analysis using the Nucleocounter indicated that the total viable cell number in the 2.5 L was $\sim 175 \times 10^6$ cells whilst in the 500 mL pooled from the 5 spinner flasks, the number of cells present was $\sim 35 \times 10^6$ cells, approximately one fifth of those present in the 2.5 L. Clearly, the whole procedure led to some small experimental error but essentially it showed that with the new technique ~ 100% of the cells were detached in each of the 5 spinner flasks.

4.4. Cell characterisation

After harvesting the hMSCs via both techniques, the intention was to characterise the cells in accordance with the ISCT guidelines to ascertain whether the harvesting procedure affected the quality of the cells. However as so few cells were obtained from manufacturer's harvesting procedure, it was not possible to perform all the characterisation techniques, and instead the hMSC1 and hMSC2 harvested cells were only characterised by inoculating a tissue culture flask with these cells to determine if they attached and formed a monolayer culture. Fig. 4A andB depicts the hMSC1 and hMSC2 cells respectively after they had been harvested via the manufacturer's protocol.

All characterisation analyses were performed on the cells obtained by the new procedure after filtration from the microcarriers whether they were grown initially in the spinner flasks or in the bioreactor. Table 1 demonstrates that the harvesting procedure had no effect on either the hMSC1 or hMSC2 immunophenotype based on the ISCT guidelines, with greater than 98% of the cells expressing CD73, CD90 and CD105, and less than 2% of the cells expressing CD14, CD19, CD34, CD45 and HLA-DR. Fig. 4C and D illustrates the monolayer culture of the hMSC1 and hMSC2 cells after harvesting with the novel procedure, thereby demonstrating the retention of the cells' ability to attach and proliferate on tissue culture plastic, whilst exhibiting the same fibroblastic, spindle-shaped morphology expected of hMSCs [45]. Furthermore, the cells stained positively for Alkaline Phosphatase and Von Kossa staining (Fig. 5A) demonstrating osteogenic differentiation, whereas the control did not stain at all (Fig. 5D). The cells formed visible lipid vacuoles in the cell cytoplasm typical of adipogenic differentiation (Fig. 5B), however these were not observed in the adipogenic control (Fig. 5E). With respect to the chondrogenic differentiation, the Alcian Blue staining illustrated in Fig. 5C stained the proteoglycans in the extracellular matrix. Micromass cultures were initiated, but during medium changes, the micromasses became dislodged. Nevertheless, the extracellular matrix clearly did still stain. In comparison, the chondrogenic control did not stain positive for Alcian Blue staining, even in the cells' extracellular matrix (Fig. 5F). These quantitative and qualitative assays fulfil the ISCT criteria, which include the expression/non-expression of cell surface markers, plastic adherence and the ability to differentiate toward the mesenchymal lineages. As such it can be stated that the novel harvesting procedure did not have any effect on hMSC quality.

Table 1
Immunophenotype of hMSC1 and hMSC2 after novel harvest procedure (mean value ± SD, $n = 4$).

Surface marker	hMSC1 Percentage expression	hMSC2 Percentage expression
CD73	98.3 ± 0.4	99.1 ± 0.6
CD90	99.2 ± 0.4	98.9 ± 0.3
CD105	98.5 ± 0.3	99.5 ± 0.4
CD14	0.19 ± 0.03	0.33 ± 0.2
CD19	0.89 ± 0.12	0.66 ± 0.15
CD34	1.1 ± 0.4	1.5 ± 0.3
CD45	1.4 ± 0.5	0.80 ± 0.32
HLA-DR	0.85 ± 0.08	1.77 ± 0.12

5. Discussion

The main advantage of the new detachment protocol is that there is a very high harvesting efficiency and the exposure time of

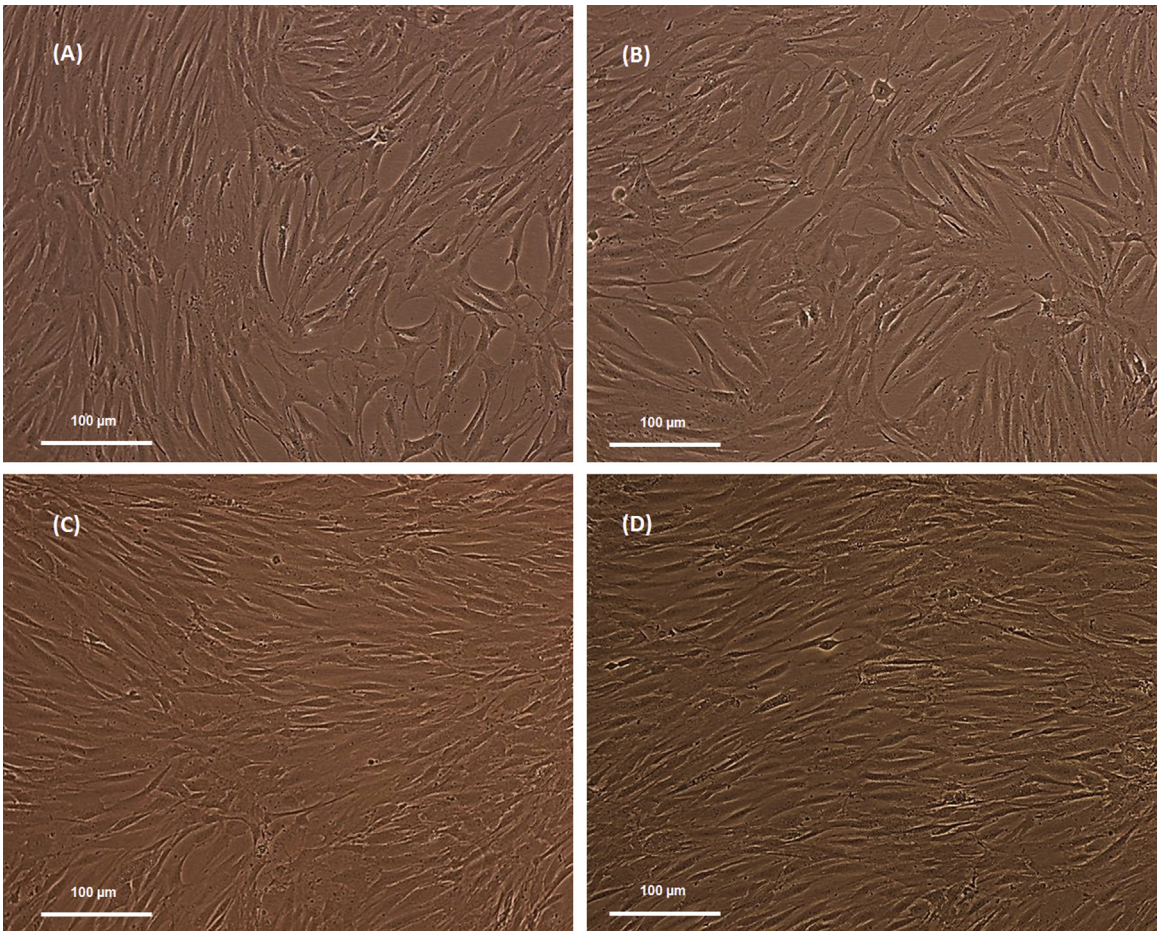

Fig. 4. Monolayer culture after harvesting for (A) hMSC1 using manufacturer's protocol, (B) hMSC1 using novel procedure, (C) hMSC2 using manufacturer's protocol and (D) hMSC2 using novel procedure. Magnification 10×.

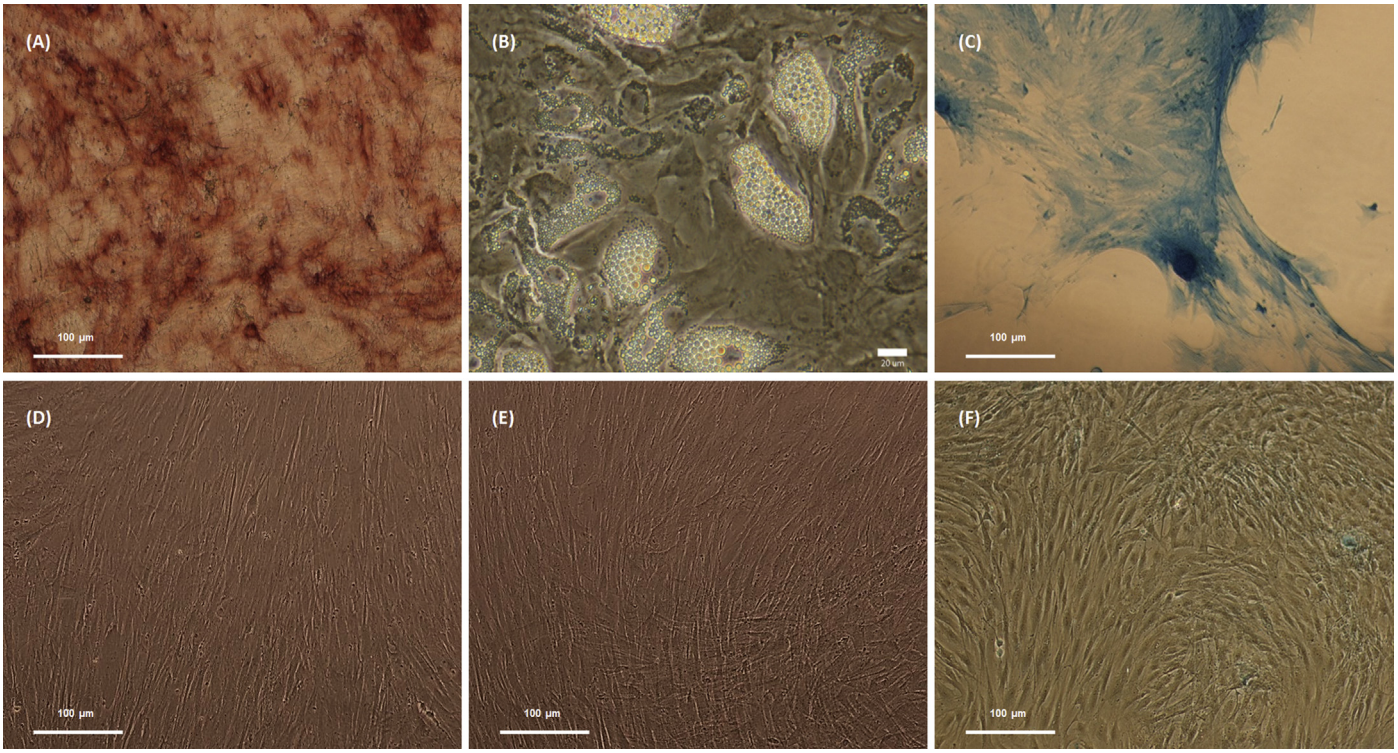

Fig. 5. Phase-contrast images of hMSCs harvested from the Plastic P102-L microcarriers using the novel harvesting procedure demonstrating differentiation potential down the (A) osteogenic, (B) adipogenic and (C) chondrogenic lineages. Control images for (D) osteogenic, (E) adipogenic and (F) chondrogenic differentiation. Magnification 10×.

the hMSCs to trypsin is greatly reduced. It is widely acknowledged that trypsin can cause cell damage, particularly if cells are exposed to trypsin for long periods [46], and with respect to hMSCs specifically, CD105 expression decreased with trypsin exposure time over a range of 5, 30 and 90 min [18]. On the other hand, Frauenschuh et al. [3] and Schop et al. [47] each developed a harvesting procedure, which required exposure to trypsin for over 1 h, and still reported that hMSCs maintained their immunophenotype and multipotency. Overall, the reduction to the precise time used in the new protocol may not be critical but keeping this time short is clearly advantageous.

On the other hand, exposure to the high agitation speeds involved might have been expected to damage cell quality attributes (cell immunophenotype or multipotency). That, however, is not the case. Here, it is proposed that the lack of damage is because once the cells are detached they are smaller than the Kolmogorov scale based on $\varepsilon_{T\max}$ estimated from the swept volume as explained above.

As pointed out above and emphasised in the recent review by Chen et al. [15], there has been very little focus on harvesting from those studies investigating the expansion of hMSCs on microcarriers. In many cases, it is difficult to ascertain whether the cultures were completely harvested, or whether the detachment of cells occurred only for small samples (usually in the range of a few mL). Yang et al. [8] appear to be the first to focus primarily on detachment and harvesting, with the focus on shifting away from trypsin to a thermoresponsive microcarrier which allows for the detachment of cells with a change in temperature. In principle, such a concept is promising. However, there are number of issues to consider. Firstly, the need to coat the microcarrier with the thermoresponsive substrate may affect the ease with which cells attach. Also, if a specific microcarrier was chosen for its coating or surface properties with respect to growth, the need to coat the microcarrier with another substrate would render the original properties unusable and therefore ineffective. Finally, the process of coating the microcarriers may itself be difficult to scale-up.

5.1. Scale-up considerations and sensitivity analysis of detachment

For scale-up of detachment, it seems quite feasible to build on the current approach. For both of the impaction mechanisms (Eqs. (4) and (5)), the mean specific energy dissipation rate, $\bar{\varepsilon}_T$, is a major factor. Based on this criterion, a similar performance should be expected on scale-up if it is held constant. For example, for treating the 2.5 L cell–microcarrier suspension in the 5 L stirred bioreactor, ideally the same $\bar{\varepsilon}_T$ should be used as in the spinner flask, namely 0.079 W/kg. Therefore, with its down pumping 3-blade 45°-pitch deep-blade impeller of diameter, 70 mm ($D/T = \sim 0.44$) to give the same $\bar{\varepsilon}_T$ (assuming a power number of 1.5 [48,49], a speed of 120 rpm would be required. Since the speed is little lower than in the spinner flask, if the dominant detachment mechanism is impeller impact, the time would be 1–2 min longer which is still short compared to other traditional methods. If the dominant mechanism is microcarrier–microcarrier impact, then the time should not change.

It is also useful to confirm that the Kolmogorov scale at this intensity of agitation in the 5 L bioreactor should not be a cause for concern. Zhou and Kresta [34] give a Φ value of 15–20 for both 'high' and 'low' shear impellers of $D/T = 0.5$, so a value of 30 seems a safe assumption for a D/T ratio of 0.44. In this case, $\varepsilon_{T\max}$ would be 2.34 W/kg and the Kolmogorov scale would be 26 μm, still above the size of the cells. This D/T ratio is a sensible one to use on scale-up as it minimizes the mean specific energy dissipation rate required to suspend microcarriers [50] and therefore the chance of damage during culture.

The microcarrier concentration used in this study was based on a preliminary assessment of its impact on the culture of these cells in spinner flasks [21] and our earlier work [6] with other MSCs. There, it was found that both higher and lower microcarrier concentrations gave lower MSC cell densities. With this microcarrier concentration, the cells grew to confluence, which limited the cell density achieved. Clearly, if the microcarrier concentration can be increased to give higher the cell densities in the bioreactors, that would be beneficial. Therefore an assessment of the impact of such a change on this detachment technique is valuable. Eq. (4) for microcarrier–impeller impacts indicates that mechanism increases with microcarrier concentration; whilst. Eq. (5) for microcarrier–microcarrier impacts shows that mechanism increases with the square of the concentration. Thus, the time required for detachment should remain the same or may actually decrease under these conditions.

5.2. Improvement and scale-up of separation

Though the harvesting efficiency using the new protocol was >95%, there is reason to believe that this efficiency can increase further especially for the separation step. It was noticed that whilst filtering the cells from the microcarriers using the Steriflip® vacuum filtration device, the cake (the growing deposit of microcarriers) started to act as another filter and entrapped cells on it and within it too. The use of a larger surface area of filter could reduce this tendency. For a further increase in scale, larger filters would be required and there may be a limitation to the volume of suspension that can be handled that way. Certainly, the filtering time will increase which may be an issue. Alternatively, a volume reduction step could be employed following cell detachment; indeed large-scale devices are now emerging on the market which appear to be suitable for this function, for example the kSep® centrifuge provides a scalable device for the concentration, washing and harvest clarification of up to 1000 L of culture broth [51].

6. Conclusions

There is little focus on harvesting in those studies reported in the literature investigating the expansion of hMSCs on microcarriers. In many cases, it is difficult to ascertain whether the cultures were completely harvested, or whether the detachment of cells occurred only for small samples (usually in the range of a few mL). Here, the protocol outlined has enabled MSCs from two different donors to be detached from SoloHill plastic microcarriers by using a short exposure to trypsin whilst providing an intense level of agitation. On the other hand, the protocol suggested by SoloHill [21] was unsatisfactory and both it and the use of thermoresponsive microcarriers [8] would be difficult to scale-up.

The approach seems well suited to scale-up with the mean specific energy dissipation rate required being very easily achieved at any scale; and because of the proposed mechanisms of detachment, it should be achieved in a short time. Also, once detached, Kolmogorov's theory suggests that, independent of scale, the cells would not be damaged as they are smaller than the Kolmogorov microscale. Once detached from the microcarriers, cell separation by simple vacuum filtration is also effective and again offers distinct scale-up potential. As applied here, the harvesting efficiency was >95% and cells after harvesting showed all the attributes expected of hMSC cells. The overall protocol is also sufficiently flexible that it should be capable of being adapted to meet the problem of cell line specificity. It might also meet the problem of cell/microcarrier specificity too, adjusting the agitation and the time for its application as well as the enzyme and its concentration to accommodate how tightly different cells bind to different microcarriers.

Indeed, this work suggests that for studies related to scale-up for allogeneic therapeutic purposes, it is very important that cells and microcarriers are considered not only with respect to attachment and growth but also with detachment in mind; and the potential for scale-up of the whole process because good attachment and growth without efficient detachment is not a viable large scale process.

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