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Advances in human mesenchymal stromal cell-based therapies – Towards an integrated biological and engineering approach

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ABSTRACT

Recent advances of stem cell-based therapies in clinical trials have raised the need for large-scale manufacturing platforms that can supply clinically relevant doses to meet an increasing demand. Promising results have been reported using stirred-tank bioreactors, where human Mesenchymal Stromal Cells (hMSCs) were cultured in suspension on microcarriers (MCs), although the formation of microcarrier-cell-aggregates might still limit mass transfer and determine a heterogeneous distribution of hMSCs. A variety of MCs, bioreactor-impeller configurations, and agitation conditions have been established in an attempt to overcome the trade-off of ensuring good suspension while keeping the stresses to a minimum. While understanding and controlling the fluid flow environment of bioreactors has been initially under-appreciated, it has recently gained in popularity in the mission of providing ideal culture environments across different scales. This review article aims to provide a comprehensive overview of how rigorous engineering characterisation studies improved the outcome of biological process development and scale-up efforts. Reconciling these two disciplines is crucial to propose tailored bioprocessing solutions that can provide improved growth environments across a range of scales for the allogeneic cell therapies of the future.

1. Human mesenchymal stromal cell expansion platforms and current limitations

1.1. Mesenchymal stromal cells for cell therapy

Human Mesenchymal Stromal Cells (hMSCs) are a heterogeneous group of cells with the ability to secrete bioactive factors that can modulate the immune system and promote tissue repair. Due to these features, hMSCs are being globally explored as a treatment to a wide array of clinical indications. Various clinical trials have shown MSC therapies to improve the patients' health conditions, indicating that they could soon become a realistic practice in the clinic. As a consequence, the generation of clinically relevant amounts of cells is crucial to the successful and sustainable commercialisation of off-the-shelf cell products (Viswanathan et al., 2019).

Originally, hMSCs were cultured in static 2D culture dishes, but dynamic 3D culture systems represent widely used alternatives in which higher cell yields can be obtained. Suspension culture systems typically reduce manual operations and offer greater process reproducibility and higher product quality as critical process parameters (e.g. dissolved oxygen, pH) can be fully controlled. Since hMSCs require a surface to attach and grow on, the transition to suspension cultures includes the introduction of MCs (Nienow et al., 2016a). Stirred-tank reactors (STRs) and spinner flasks are the most well-known and well-characterised suspension expansion platforms in which hMSCs are grown on MCs.

Research groups also explored a number of conceptually different bioreactor designs for hMSC manufacturing. Examples include the vertical wheel bioreactor (Sousa et al., 2015), hollow fibre bioreactor (Cheatham et al., 2019), roller bottle (Tozetti et al., 2017), packed bed bioreactor (Tsai et al., 2016), Tide motion bioreactor (Chiew et al., 2019), or rocked (wave) bioreactors (de Sá da Silva et al., 2019).

1.2. Scale-down models of STRs

Despite the wide range of bioreactor designs, large scale hMSC expansions have been mainly carried out in STRs. The optimisation and development of tailored cell culture mediums, MCs materials, and other improved cell culture protocols concerning the cell adhesion, feeding procedures and cell recovery from MCs have been mostly performed in spinner flasks and have greatly increased cell expansion (Chen et al., 2015; Lam et al., 2017; Tan et al., 2016). Indeed, most studies to date

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Table 1
Overview of STR models which have been used for MC-based hMSC expansion.

Scale	Employed Working Volume	Reactor Type	Sources
Spinner flasks	0.025–0.3 L	Various manufacturers	(Carmelo et al., 2015; Caruso et al., 2014; Chen et al., 2015; Cunha et al., 2017; de Soure et al., 2017; dos Santos et al., 2014, 2011; Eibes et al., 2010; Elseberg et al., 2012; Ferrari et al., 2012; Frauenschuh et al., 2007; Goh et al., 2013; Gupta et al., 2019; Heathman et al., 2016, 2015; Hervy et al., 2014; Hewitt et al., 2011; Hupfeld et al., 2014; Jossen et al., 2018, 2016, 2014; Kaiser et al., 2013; Leber et al., 2017; Loubière et al., 2019b; Mizukami et al., 2016; Nienow et al., 2016a,b,c, 2014; Petry et al., 2016; Rafiq et al., 2018, 2016, 2013; Schirmaier et al., 2014; Schop et al., 2010; Shekaran et al., 2015; Sion et al., 2020; Sun et al., 2010; Takahashi et al., 2017; Tozetti et al., 2017; Yuan et al., 2018, 2014; Zhao et al., 2015)
Laboratory-scale bioreactors	0.015–0.5 L	Sartorius Ambr	(Nienow et al., 2016a,b,c; Rafiq et al., 2017)
		Sartorius Biostat Qplus	(Cunha et al., 2015; Sousa et al., 2015)
		Eppendorf DASGIP/DASBox	(Heathman et al., 2018, 2019; Nienow et al., 2016a,b,c; Teixeira et al., 2017)
	> 0.5–2 L	Sartorius UniVessel Glass	(Chen et al., 2015; Goh et al., 2013; Hupfeld et al., 2014)
		Sartorius UniVessel SU	(Cunha et al., 2017; Schirmaier et al., 2014)
		Eppendorf CelliGen 310	(Mizukami et al., 2018, 2016; Tozetti et al., 2017)
		Eppendorf Bioflo 110	(dos Santos et al., 2014)
		Applikon Biotechnology Glass	(Elseberg et al., 2012; Justice et al., 2011)
	> 2–5 L	Merck Millipore Mobius CellReady	(Grein et al., 2016; Kehoe et al., 2013)
		Sartorius UniVessel Glass	(Rafiq et al., 2013)
Pilot-scale bioreactors	> 5–50 L	Merck Millipore Mobius SU	(Lawson et al., 2017)
		Sartorius CultiBag STR	(Schirmaier et al., 2014)
		Pall Allegro STR	(Bayne et al., 2019)

used spinner flask cultures, as they are easily available scale-down model reactors (see Table 1). However, with the development of miniature bioreactor models (e.g. DASbox, Eppendorf, Germany) or automated high-throughput microbioreactors like the ambr®-system (Sartorius, Germany), there are now alternatives available that may replace spinner flasks in future scale-down studies. Despite offering greater reproducibility through automation, additional process development efforts and standardised protocols are needed to make of them a viable and widespread alternative (Rafiq et al., 2017).

1.3. STRs as expansion platform

The optimisation of many biological culture parameters has led to notable advances using STRs that are equipped with axial and/or radial flow impellers. Cell viabilities of over 90% as well as cell densities of 10^5–10^6 cells mL^{-1} culture medium are typically reported by dos Santos et al. (2014), Goh et al. (2013), Kehoe et al. (2013), Rafiq et al. (2013), and Tan et al. (2016). In few cases hMSC expansion on MCs was achieved in STRs with a capacity up to 50 L (Jossen et al., 2014a; Lawson et al., 2017; Schirmaier et al., 2014). In addition to academic groups, some companies have also reported the production of hMSCs at 50 L scales, including but not limited to Pall (Bayne et al., 2019), Lonza (Gupta, 2016), and GenCure (GenCure Web Announcement, 2019). Lonza reportedly manufactured up to 2×10^6 viable cells mL^{-1} within 10 days in 50 L reactors. GenCure announced the successful culture of a 50 L batch of 1.2×10^9 bone-derived hMSCs, which were expanded into 20 billion cells within 10 days (final cell concentration 4×10^5 cells mL^{-1}). The increasing number of similar reports indicate the determination of major industry players to push STR solutions forward in order to address the critical need for a large amount of consistent and clinical grade hMSCs to supply both clinical trials and a global demand post-market authorisation. A summary of studies using STRs for hMSC expansion at different reactor scales is provided in Table 1.

1.4. Current upstream limitations in STRs

Even though stirred-tank reactor systems are the most widely spread hMSC suspension culture platform, they are still subject to some limitations. These can be encountered during the attachment, expansion, and harvesting phase of the process. For example, harvesting cells from MCs typically involves the use of enzymes, which could negatively affect the quality of the cell product. The use of biodegradable microcarriers, however, could overcome this obstacle (Lam et al., 2017). During cell expansion, agitation of the culture medium will promote mixing, which is necessary to generate sufficient oxygen and mass transfer within the reactor, but may also induce stress levels that have been correlated with the upregulation of gene and protein expression towards a certain lineage (Delaine-Smith and Reilly, 2012). In view of the fact that hMSCs are intended to be administered to the patient, morphological changes to the cells and subsequent decrease of their therapeutic potency must be avoided within every large-scale expansion device (Heathman et al., 2015). Microcarrier clumping, which is more pronounced at low agitation rates, is another aspect that has to be carefully considered. MC aggregation may be further stimulated by the increase in cell density with prolonged culture duration and can result in detrimental hypoxia and nutrient deprivation (Ferrari et al., 2012; Mizukami et al., 2016). As suggested in recent studies, microcarrier aggregation could potentially be overcome by the addition of fresh microcarriers into the cell culture (Rafiq et al., 2018; Sion et al., 2020). Due to a phenomenon called bead-to-bead transfer, which describes cell migration from confluent microcarriers to empty ones, cell confluence and thus aggregation can be delayed. Another reported obstacle is low attachment efficiencies on MCs during inoculation, which can result in a prolonged lag-phase at the beginning of the expansion process. This in turn would increase production time and the hereto connected manufacturing costs (Heathman et al., 2018).

2. Addressing manufacturing limitations through engineering characterisation of the bioreactor

2.1. Engineering characterisation for bioprocess development

A critical amount of process development efforts should be invested in acquiring a better understanding of the fluid dynamics environment within the expansion chamber, irrespective of the bioreactor selected. Amongst others, flow and mixing dynamics studies in bioreactors are essential to fully understand their impact on cell attachment to the MCs, hence potentially improving cell-inoculation protocols. In addition, the

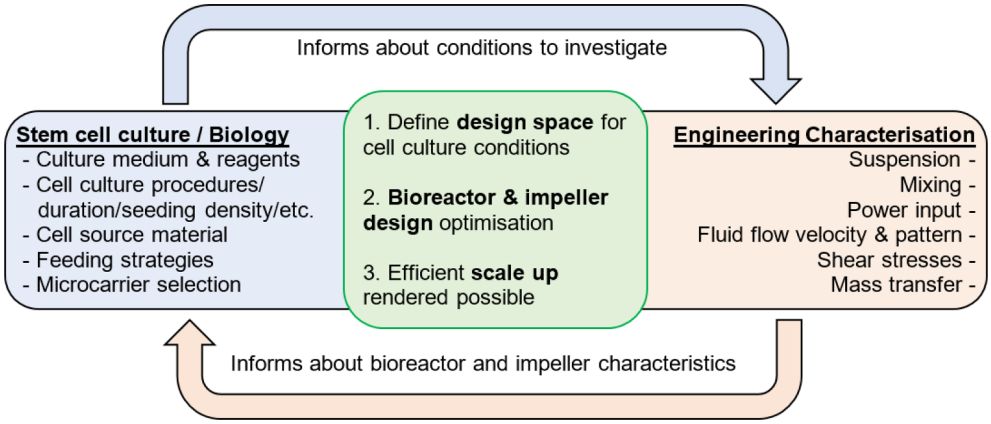

Fig. 1. Feedback loop of engineering characterisations that support biological cell culture experiments. An integrated approach leads to culture condition optimisation, bioreactor design improvement, and further development of scale-up protocols.

effects of different flow regimes on solid suspension during the expansion stage may inform operators on the optimal agitation strategy (Kinney et al., 2011; Samaras et al., 2019). Computational fluid dynamics (CFD) approaches have been used to construct a digital twin of the bioreactor set-up and could represent a viable tool to improve stem cell culture protocols (Liovic et al., 2012). Combined with experimental works, simulations are necessary to gain full insight into the 3D bioreactor performance and to overcome cell culture and scale-up challenges by means of improving bioreactor design or enhancing cross-comparability between different types of bioreactors (Kaiser et al., 2011). The characterisation of critical engineering parameters in stirred-tank bioreactor systems is therefore vital for the successful establishment of a design space in which the process can be performed across scales (Fig. 1).

For example, to inform agitation strategies and improve operational protocols in spinner flasks cultures, Berry et al. (2016) employed CFD models that were validated against Particle Image Velocimetry data. Lagrangian particle tracking enabled the group to characterise stresses "experienced" by suspended microcarriers (rather than the fluid within the bioreactor as a whole). The authors identified levels of maximum stresses (≈ 0.1–0.5 Pa at 50–70 rpm) and were therefore able to recognise whether certain agitation speeds would lead to stress levels that have been associated with stem cell differentiation. Furthermore, they found that intermittent agitation exposed microcarriers to elevated levels of shear that have been reported to induce morphological changes in MSCs, and subsequently suggested to avoid multiple restarts of the impeller, e.g. during the initial cell attachment phase (Berry et al., 2016). A comprehensive summary of reported shear stress levels inducing change in MSCs (of human and other animal origin) is given in Table 2, with values in the order of 0.25–2 Pa.

The level of stress encountered in STR cultures are usually estimated from equations 1 and 2, where the mean energy dissipation rate, $\bar{\varepsilon}$, is related to the impeller power number:

$$\bar{\varepsilon} = \frac{P}{M} = \frac{N_P \rho_L N^3 D^5}{M} \quad (1)$$

and N_P is the impeller power number, ρ_L is the density of the fluid, N is the impeller speed, D is the impeller diameter, and M is the mass of the liquid. This, however, provides only a coarse estimate across the bioreactor volume, and recent studies employing high spatial resolution measurement techniques with direct estimates of all energy dissipation terms (12 in total) have shown that phase-resolved values of energy dissipation rate are at least 50 times greater than $\bar{\varepsilon}$ (Ducci and Yianneskis, 2005; Micheletti et al., 2004), with instantaneous peaks of energy dissipation in proximity of the impeller being 80 $\bar{\varepsilon}$ (Huchet et al., 2009). Based on the maximum dissipation rate, ε_{max}, a reference maximum stress value, τ_{max}, can be obtained from the following equation:

$$\tau_{max} = \sqrt{\frac{\varepsilon_{max}}{\nu}}\eta \quad (2)$$

where ν is the kinematic viscosity of the liquid, η is the dynamic viscosity of the liquid, and ε_{max} = 50–80 $\bar{\varepsilon}$. It should be noted that the energy dissipation comprises both normal and shear viscous stresses and therefore τ_{max} is representative of both. Following these steps, expected reference stress values in two commonly used single-use STRs have been outlined in Table 3.

The Sartorius UniVessel SU is equipped with two down-pumping 3-Blade Segment impellers (blade angle = 30°, D = 0.054 m, N_P = 1.9) and has typically a working volume of 2 L. The Möbius CellReady 3L reactor (Merck Millipore) has a maximum working volume of 2.4 L and is equipped with a scoping Marine impeller (D = 0.0762 m, N_P = 0.22) (van Eikenhorst et al., 2014). Both the Möbius CellReady (τ_{max}≈ 0.66–0.84 Pa at 200 rpm) and the Sartorius UniVessel SU (τ_{max}≈ 0.64–0.80 Pa at 200 rpm) reach maximum shear stresses that are below the widely reported threshold of 1–2 Pa, but exceed thresholds of 0.25–0.5 Pa which were also shown to affect MSCs in some studies (Table 2). While these speeds are well above the agitation requirements for light microcarriers with a specific density of ≈1.03 (as N_{JS} ≈ 40–50 rpm), they might be employed for heavier microcarriers (e.g. specific densities > 1.2).

2.2. Scale-up of hMSC expansion in STRs

The most commonly used scale-up criterion in hMSC culture is based on microcarrier suspension (Cunha et al., 2017; Jossen et al., 2014a; Rafiq et al., 2013). Other scaling parameters are the power input per unit volume (P/V), volumetric oxygen transfer coefficient (k_La), impeller tip speed, mixing time, or shear-related factors. These engineering parameters must be determined experimentally and can be complemented by CFD simulations (Jossen et al., 2016; Kaiser et al., 2011). Schirmaier et al. (2014) reported the successful expansion of hMSC in 35 L culture volume (CultiBag STR, Sartorius). This study provided the basis for Jossen et al. (2014a), who described the subsequent scale-up of the hMSC production process to 50 L working volume. Both studies consisted of a combination of CFD simulations, PIV, and MC suspension measurements to predict the fluid flow characteristics. They ultimately performed the scale-up using the impeller speed that kept none of the MCs at rest (with MCs moving along the vessel bottom), reaching viable cell densities of up to 7.2 × 10^5 cells mL^{-1} (Jossen et al., 2014a). In one of the largest-scale hMSC cultures to date, the systematic identification of critical MC suspension parameters were used to expand hMSCs in the Möbius 50 L bioreactor (Merck Millipore) at the maximum working volume of 50 L (Lawson et al., 2017). A cell density of 2.56 × 10^5 hMSCs mL^{-1} with > 95% viability within

Table 2
Overview of shear stress effect on MSC gene and protein expressions.

Source	Cell type	Methodology	Main observation	Applied shear stress
(Hu et al., 2017)	Human MSCs (bone marrow)	• Parallel plate flow chamber • Flow duration: 72 h	• Upregulated mRNA expression of Osx (**osteogenic differentiation marker**), while adiponectin decreased • ALP staining increased • Late osteogenic marker Ocn unchanged • Increase in intracellular Ca^{2+}	1.2 Pa
(Kim et al., 2016)	Human MSCs (bone marrow)	• Tubular scaffold • Flow duration: o 4 days (continuous) o 4 days (continuous) followed by 3 or 4 days of 3% or 5% circumferential stretch	• Increased mRNA and protein levels of **endothelial cell markers** (vWF, CD31, VE-cadherin, E-selectin) after one day	0.25 Pa
(Liu et al., 2012)	Human MSCs (bone marrow)	• Perfusion culture system for cells seeding in scaffolds • Flow duration: o Intermittent: 1 h–11 h–1 h (high–low–high shear) o Continuous: 13 h (high shear)	• Increased ALP activity under intermittent fluid shear stress in comparison to continuous shear stress • Intermittent shear stress upregulated activity of ERK1/2 and FAK	Low shear: 0.034 Pa High shear: 0.42 Pa
(Kim et al., 2011)	Human MSCs (bone marrow)	• Tubular construct • Flow duration: 24 h, starting 24 h after seeding	• Expression of vWF and calponin • CD31 (PECAM-1) significantly expressed • Higher expression of myocardin, myosin heavy chain, SM-22a (**smooth muscle cell markers**)	0.25–1 Pa
(Yourek et al., 2010)	Human MSCs (bone marrow)	• Parallel plate flow chamber • Wall shear stress on cell monolayer	• Shear stress stimulated towards **osteoblastic phenotype** in the absence of chemical induction • Increased alkaline phosphatase activity in osteogenic medium • mRNA expression of BMP-2 and osteoponin significantly higher at day 4 and 8 • Shear stress with no effect in alignment and shape of hMSCs	0.9–2.2 Pa
(Bassaneze et al., 2010)	Human MSCs (Adipose tissue)	• Shear stress by cone plate viscometer • Flow duration: 24, 48, or 96 h	• **Increased VEGF** production (mediated by nitric oxide) • No change in MSC surface markers compared to static control • No induction of endothelial markers	1 Pa
(Grellier et al., 2009)	Human MSCs (bone marrow)	• Parallel plate flow chamber (steady flow) • Flow duration: 30 and 90 min	• Increase in ALP gene expression	1.2 Pa
(Stavenschi et al., 2017)	Murine MSC cell line	• Oscillatory fluid flow in flow chamber • 2 Hz for 2 h • 2 Hz for 2 h, 2x/day for 4 days, followed by 2 weeks static culture	• **Osteogenic gene expression** upregulation (Cox2, Runx2, Opn) • Osteogenic lineage commitment (increased collagen and calcium deposition)	2 Pa
(Arnsdorf et al., 2009)	Murine MSC cell line	• Oscillatory fluid flow in parallel plate flow chamber • 1 h, 1 Hz (sinusoidal)	• Upregulated gene expression of Runx2, Sox9, PPARg • **Osteogenic differentiation** mechanism	1 Pa
(Bai et al., 2010)	Rat MSCs (bone marrow)	• Parallel plate flow chamber (steady flow + VEGF) • Flow duration: 12, 24, or 48 h	• **Endothelial differentiation** for shear stress $<$ 1.5 Pa (24 h, but not for 48 h). No differentiation for shear stress $>$ 2 Pa • Elongation and alignment of MSCs to the direction of flow • VEGF treatment enhanced EC differentiation, reduced apoptosis and resulted in less cells washed off the plates	1–2.5 Pa
(Huang et al., 2010)	Rat MSCs (bone marrow)	• Parallel plate-type device • Flow duration: 24 h continuous, then static culture and differentiation	• Increased mRNA expression of GATA-4, NKx2.5, MEF2c, b-MHC at day 7 of differentiation (no expression in untreated cells) • **Endothelial marker expression**	0.5–2 Pa 1.5 Pa
(Kreke et al., 2008)	Rat MSCs (bone marrow)	• Parallel plate flow chamber • Intermittent flow (5 min on/5 min off) or • Continuous flow for 24 h • Differentiation incl. osteogenic medium, at day 6 of differentiation start of fluid flow	• Elevated expression of Col-1a1, OPN, BSP, OCN (**osteogenic marker expression**) under continuous and intermittent flow compared to static controls (at day 13 of differentiation)	0.23 Pa
(Dong et al., 2009)	Canine MSCs (bone marrow)	• Cells in scaffold • Flow duration: 4 days (2 days at 0.1–1.5 Pa, 2 days at 1.5 Pa)	• **Increased endothelial cell markers** at mRNA and protein level (PECAM-1, VE-cadherin, CD34) • Reduced protein levels of a-SMA and calponin (downregulated smooth muscle-related markers)	0.1–1.5 Pa
(Knippenberg et al., 2005)	Goat MSCs (adipose tissue)	• Pulsating fluid flow through parallel plate flow chamber + osteogenic differentiation • 5 Hz pulse (pulse amplitude: 0.3 Pa)	• Undifferentiated MSCs acquired **bone cell-like phenotype** • NO production increased, COX-2 upregulation	0.6 Pa (mean) 8.4 Pa (max.)

Table 3
Expected stress in two model STRs at various impeller speeds and maximum working volume. Water at room temperature (T = 20 °C) was used as an exemplary cell culture medium ($\nu = 10^{-6}$ m^2 s^{-1};η = 0.001 Pa s; ρ_L = 998.2 kg m^{-3}).

Impeller Speed [rpm/rps]	Impeller power number[1] [-]	$\bar{\varepsilon}$ [W kg^{-1}]	ε_{max} [W kg^{-1}]	τ_{max} [Pa]
Möbius CellReady 3L				
50 / 0.83	0.89	0.0006	0.0276–0.0441	0.17–0.21
100 / 1.67	0.44	0.0022	0.1090–0.1744	0.33–0.42
150 / 2.50	0.25	0.0042	0.2091–0.3345	0.46–0.58
200 / 3.33	0.22	0.0087	0.4361–0.6978	0.66–0.84
Sartorius UniVessel SU 2L[2]				
50 / 0.83	3.0	0.0002	0.0100–0.0159	0.10–0.13
100 / 1.67	2.5	0.0013	0.0664–0.1063	0.26–0.33
150 / 2.50	2.1	0.0038	0.1883–0.3013	0.43–0.55
200 / 3.33	1.9	0.0081	0.4039–0.6462	0.64–0.80

[1] van Eikenhorst et al., 2014.
[2] The power number of the Sartorius UniVessel SU 2L is a total of both impellers, however the energy dissipation rates and stress values correspond to a single impeller.

11 days of culture was achieved. Despite the difference in geometry from conventional stirred-tank bioreactors, the authors underlined the importance of an engineering characterisation for a successful scale-up.

2.3. Informing cell culture processes in STRs through engineering characterisation techniques

In addition to facilitating the efforts to overcome scale-up challenges, the determination of critical engineering parameters in bioreactors can also be beneficial in improving hMSC cultures by reducing the number of experiments needed to find the optimal operating conditions. Several studies show that rigorous quantitative analyses are crucial to understand the relationship between the hydrodynamic environment and the biological outcomes.

Characterisation studies for a range of commercially available bioreactors that are widely used in the field were conducted by van Eikenhorst et al. (2014). The authors compared the Möbius CellReady 3 L, the Sartorius UniVessel SU 2.5 L, the Sartorius UniVessel 3 L, and the CelliGen BLU 5 L (New Brunswick) with respect to their volumetric mass transfer coefficient, mixing efficiencies, and suspension characteristics at a range of power input per unit volumes (P/V = 0–400 W m^{-3}; depending on impeller type). Similarly, Grein et al. (2016) also investigated mixing in the Möbius CellReady 3 L and determined mean intensity of the shear stresses for one-, two- and three-phase systems at different power inputs. Detailed flow characterisations in the Möbius CellReady 3 L were also published by Odeleye et al. (2014), who combined PIV measurements with a biological study to elucidate the impact of the fluid dynamic characteristics on the culture performance and productivity of a GS-CHO cell line.

Engineering characterisation studies provided the basis to develop a novel harvesting method that is now widely applied in the field (Nienow et al., 2016c). Kaiser et al. (2013) improved the cell expansion by analysing the impact of fluid flow on proliferation of hMSCs in spinner flasks. Jossen et al. (2018) performed numerical and experimental investigations and established a growth model with 76–96% accuracy in predicting the growth of immortalised human adipose tissue-derived stromal cells (hTERT-hASCs) on MCs in spinner flasks. To inform the culture of induced pluripotent stem cells (iPSCs), others have investigated the flow in small-scale bioreactors (Ismadi et al., 2014). Samaras et al. (2018) elucidated the impact of intermittent agitation in DASGIP bioreactors on iPSC-derived cardiomyocyte differentiation and was able to derive specific process recommendations.

3. Addressing manufacturing limitations through engineering characterisation of the impeller

3.1. Agitation strategies in STRs

Identifying and maintaining an optimal agitation strategy can be a delicate task as the right balance may change over time with varying cell density or culture characteristics (e.g. medium viscosity). While slow mixing could lead to gradients and to cells settling at the bottom of the reactor (potentially reducing viability and potency), fast mixing could lead to cells experiencing hydrodynamic forces which could inhibit growth or impair cell integrity. Main causes of damage could be the interaction between cells on MCs and turbulent eddies, or shear forces through the rise and bursting of bubbles in bulk flow and at the liquid surface. In the boundary layers near solid objects, especially the impeller, shear forces can also be amplified. Given the wide-spread emphasis on reducing shear stress, the starting point for a cell culture protocol is associated to the minimum speed at which a cell culture is suspended. This is often denoted as the "just suspended speed" (N_{JS}). N_{JS} as a culture and scaling criterion was first used in the context of stem cell expansion by Hewitt et al. (2011) after it had been formulated over 60 years ago by Zwietering (1958) in chemical engineering applications.

Due to potential changes in agitation rate throughout the culture, it becomes critical to find not only the lower but also the upper agitation limit for an hMSC-bioreactor culturing system. To ensure that all microcarriers are properly in suspension, the lower agitation limit should be N_{JS}, which is visually determined or calculated on the basis of the Zwietering correlation (Zwietering, 1958). From a nutritional and mixing point of view it makes sense to assume N_{JS} to be the ideal culture condition as all MCs are suspended and have access to cell culture medium. Additionally, oxygen requirements of MSCs are reported to be low enough for N_{JS} and headspace sparging to sufficiently meet the uptake rate of the MSCs at the benchtop scale. However, to increase microcarrier homogeneity or to decrease potential aggregate formation, exploring higher agitation rates could turn out to be beneficial and may support higher overall cell densities. It is common practice to select the upper agitation limit by comparing the size of the microcarriers to the local Kolmogorov length scale (λ_K), which is directly related to the local rate of viscous dissipation of turbulent kinetic energy, ε (fully turbulent flow regime, $Re > 1 \times 10^4$). In brief, this approach suggests that MSCs attached to microcarriers could be damaged in suspension by turbulent eddies only when their size is equal or larger than the smallest eddy size found in a flow (i.e. λ_K), while eddies larger than MCs would only determine microcarrier transport across the reactor. When determining the smallest eddy length λ_K in a flow, the maximum energy dissipation rate, ε_{max}, should be used.

$$\lambda_K = \left(\frac{\nu^3}{\varepsilon_{max}}\right)^{0.25} \quad (3)$$

One could therefore define the upper agitation limit as the speed at which the Kolmogorov eddy length becomes smaller than the MC, or $N_{\lambda K}$. However, recent bioreactor cultures showed that even for eddy lengths that are 30% of the microcarrier diameter, no changes in hMSC quality attributes were observed (Nienow et al., 2016b) so that cases where N or $N_{JS} > N_{\lambda K}$ can nevertheless be explored. The various agitation strategies applied at the respective culture stages are summarised in Fig. 2.

Cell attachment strategies range from static (1.5) (e.g. Hupfeld et al. (2014)) to intermittent stirring (1.3 and 1.4) (e.g. Caruso et al. (2014), dos Santos et al. (2014), Elseberg et al. (2012)) to continuous agitation (1.1 and 1.2) (e.g. Goh et al. (2013)). There is no consensus in the literature with respect to what cell attachment strategy is most ideal as all strategies have shown to work to a certain degree. From the perspective of reducing shear stresses, it may be reasonable to avoid intermittent agitation, as sudden acceleration/deceleration produce higher levels of shear. However, it may also have a beneficial impact on

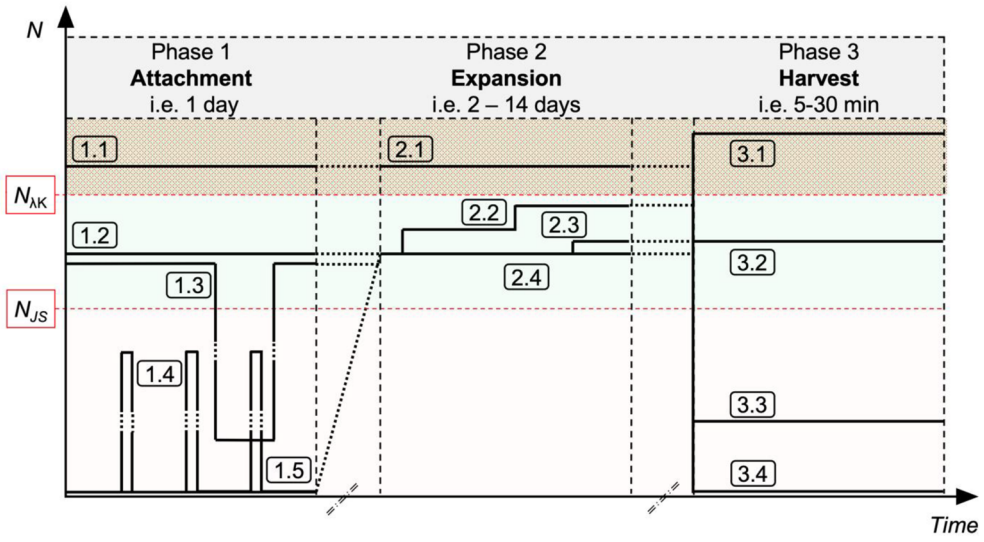

Fig. 2. Qualitative representation of the three agitation phases during hMSC expansion and the most common agitation strategies found in the literature. It has to be stressed that the agitation strategies at the respective phases are independent from each other. For example, line 1.3 could be followed by any of the strategies shown in phase 2 or 3.

the cell attachment efficiency as it prevents that only one layer of MCs are being seeded (i.e. during static attachment the side of the MCs that face the culture medium) through the intermittent resuspension of the MCs. The subsequent cell expansion phase is typically 7–14 days long. Constant agitation (2.1 and 2.4) equals often, but not always, N_{JS} (e.g. Rafiq et al. (2013)). (2.3) shows an increase in agitation speed to avoid bead aggregation and ensure efficient MC suspension (e.g. Chen et al. (2015)). Multiple increases in agitation speed (2.2) over the course of the expansion phase have also been performed (e.g. Lawson et al. (2017), Mizukami et al. (2016)). Lastly, four main strategies have been reported for cell harvesting. 3.1 and 3.2 strategy reproduces a short but strong agitation (e.g. above or within Kolmogorov limit scale) (e.g. Heathman et al. (2018); (3.3) strategy corresponds cell harvesting that involves some sort of oscillatory shaking (dos Santos et al., 2011)); (3.4) strategy represents cell harvesting with no agitation (e.g. Shekaran et al. (2015)).

3.2. Engineering characterisation for impeller design optimisation

The selection of an impeller should be based on a number of parameters that characterise the impellers' ability to minimise potential cell damage and to increase the mixing and microcarrier suspension efficiencies (or at least keep those constant). Some of these parameters include the mean fluid velocity within the vessel, the energy dissipation rate and resulting Kolmogorov length scale, the turbulent kinetic energy, the size of trailing vortices, the oxygen and mass transfer rate, the power number, N_p, the pumping number, N_q, and the just suspended speed, N_{JS} (Nienow, 2010).

Many of the impeller designs currently used for bioprocessing applications originated from other fields such as the chemical engineering industry. The performance of various impellers and impeller/tank configurations are therefore very well documented in the literature from groups across these disciplines (de Lamotte et al., 2017; Delafosse et al., 2018; Ducci et al., 2008). Collignon et al. (2016) studied three conventional impeller geometries and concluded that an up-pumping marine propeller would be more suitable for hMSC culture in small-scale bioreactors (250 mL) than an 'elephant ear' impeller. Through studying the hydrodynamics via CFD simulations, the up-pumping configuration was found to less damage the suspended microcarriers by reducing the frequency and magnitude of exposure to high level of energy dissipation. This is in agreement with the review by (Wang et al., 2016) who argued that upward pumping impellers are advantageous as they prevent the cells from being forced down into the base of the vessel and indeed the commercial Möbius CellReady 3L single-use STR operates a Marine impeller in up-pumping direction, too. Having said this, however, it is interesting to point out that the majority of the literature studied axial flow impellers in down-pumping mode due to their lower specific power required to suspend microcarriers (Ibrahim and Nienow, 2004)

Depending on the desired process outcome, new impeller designs or modifications of existing ones can significantly improve flow characteristic features. Martínez-Delgadillo et al. (2019) recently attempted to improve the impeller performance by changing the blades of a 4-blade pitched turbine. Another recently published study dealt with the characterisation of multiple novel impeller geometries that are relevant for biochemical engineering processes where "low" shear stresses are desired (Bliatsiou et al., 2019). In the context of hMSC culture, only few studies have adjusted the existing design or introduced a novel impeller design. In a study from 2014, Jossen et al. (2014b) improved the culture conditions in a UniVessel SU 2 L by reducing shear stress through the increase of the impeller blade angles and reduction of the impeller clearance. Recently, Loubière et al. (2019a) proposed a CFD-based strategy to improve the design of an 'elephant ear' impeller by varying its size, blade slope angle, and position in the reactor. The presented method is an interesting tool to explore multiple impeller configurations within the bioreactor set-up of choice, for example, to minimise power input per unit volume. These studies highlight that even slight modifications of the original design can improve critical process attributes in favour of the hMSC culture.

4. Conclusions

Large-scale bioreactors are indispensable in the commercialisation and clinical translation of allogeneic cell-based therapies. While the design of novel impellers has seen little innovation in the past years, many existing agitator designs and bioreactor configurations have been characterised in detail in an attempt to facilitate the culture and large-scale expansion of hMSCs. The scale-up of microcarrier-based suspension cultures to industrial sizes may only be fully successful if novel bioprocessing and agitation solutions will be designed. These in turn must be implemented with the help of rigorous engineering characterisation studies and considerations of fluid dynamics characteristics. In this respect, this review article aimed to advocate and encourage a more widespread adoption of an interdisciplinary approach in hMSC bioprocess research and development.

CRediT authorship contribution statement

Tom A. Wyrobnik: Writing - original draft, Writing - review & editing, Investigation, Conceptualization, Visualization. **Andrea Ducci:** Writing - review & editing, Conceptualization, Visualization, Supervision. **Martina Micheletti:** Writing - review & editing, Conceptualization, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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