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Reprint of "Multiphase mixing characteristics in a microcarrier-based stirred tank bioreactor suitable for human mesenchymal stem cell expansion"☆

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ABSTRACT

Large-scale human mesenchymal stem cell expansion calls for a bioreaction system, that provides a sufficient growth surface. An alternative to static cultivations systems like cell factories are disposable stirred tank reactors. Here, microcarriers provide the required growth surface, but these make it difficult to achieve a complete homogenization in the bioreactor, while avoiding shear stress. To gain insight into this process, we investigated the impact of different power inputs (0.02–2.6 W m^{-3}) on the mixing time (t_m). Whereas t_m was inversely proportional to agitation in a one-phase-system, aeration resulted in a constant mixing time at 30–70 rpm. A high microcarrier concentration (30 g L^{-1}) and low stirrer speed (30 rpm) in the liquid-solid system caused a 50-fold increase in t_m and the formation of a discrete non-mixed upper zone. The effect of the microcarrier concentration on t_m became negligible at higher stirrer speeds. In the three-phase system, microcarrier settling was prevented by aeration and a minimal specific power input of 0.6 W m^{-3} was sufficient for complete homogenization. We confirmed that a low power input during stem cell expansion leads to inhomogeneity, which has not been investigated in the three-phase system up to date.

1. Introduction

Human mesenchymal stem cells (hMSCs) are suitable for several applications in regenerative medicine, particularly in the field of cell therapy [1] due to their ability to support self-renewal and multi-linage differentiation, as well as their anti-inflammatory properties [2]. The limitation of hMSCs to anchorage-depend growth means that cell expansion is a challenging process. The expansion of hMSC is typically carried out in static cultivation systems such as tissue culture flasks or cell factories, but the growth surface is limited and elaborate harvesting processes are necessary [3–5]. Medical treatment with hMSCs requires processes that can expand a small number of isolated hMSCs up to an industrial scale, but the bioreactor system must provide gentle cultivation and harvest conditions because hMSCs are shear sensitive [6,7].

Rather than static systems, hMSCs can also be cultivated on macrocarriers in fixed-bed systems [8,9] or on suspended microcarriers in a stirred-tank reactor (STR). The advantage of the latter strategy is the high growth surface to volume ratio and greater spatial yield. The expansion of hMSCs in dynamic STRs has been demonstrated with different hMSC types and reactor configurations [10–13]. Promising results were achieved in disposable bioreactors e.g. the Mobius® CellReady 3 L bioreactor in fed-batch cultivation mode [14]. The flow regime in a STR becomes particularly important when the cells are growing on microcarriers, because a balance must be achieved between

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Abbreviations: c_v [−], solid fraction of the carrier; $c_{v,max}$ [−], maximum fraction of solids in the closest spherical packing; C [−], constant; D [m], inner vessel diameter; d [m], stirrer diameter; dmt [−], dimensionless mixing time; dpi [−], dimensionless power input; F_G [vvm], fraction of gas; g [m s^{-2}], gravity constant; n [s^{-1}], agitation rate; n_c [s^{-1}], critical impeller speed for solid suspension; Ne [−], Newton/power number; N_m [−], mixing number; P [W], power input; P_a [W], power input for aerated broth; Re [−], Reynolds number for stirrer; Su [−], suspension number; V [m^3], volume; V_a [m^3 s^{-1}], volumetric air flow rate; t_m [s], mixing time, usually for 95% homogeneity; η_l [kg m^{-1} s^{-1}], dynamic viscosity of medium/liquid; η_m [kg m^{-1} s^{-1}], mean dynamic viscosity; ρ_m [kg m^{-3}], liquid density; ρ [kg m^{-3}], density; ρ_c [kg m^{-3}], mean carrier density; ε_T [s^3 m^{-2}], mean energy dissipation; υ [m^2 N^{-1} s^{-1}], kinematic viscosity; τ [dyn cm^{-2}], shear stress

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the power input required to keep the solid particles in suspension and the protection of the hMSCs from shear stress. The solid microcarriers must remain suspended in the medium to ensure sufficient homogeneity and mixing in the bioreactor, but the power input is limited by the maximum shear stress that can be tolerated by the hMSCs without loss of viability or differentiation capacity [15].

The expansion of hMSCs involves a spatial and temporal mixing procedure based on the distribution of two or more components in the bioreactor volume. These components differ in at least one property, such as concentration, temperature, density, viscosity, particle size or shape. Because mixing is a key process that determines bioreactor performance, and is in turn influenced by many constructive and operational parameters, a detailed quantitative analysis of these factors and their impact on mixing efficiency and distribution is required for process optimization.

One useful criterion for the determination of mixing efficiency is the mixing time (homogenization time, t_m), which is defined as the time required to achieve a certain degree of homogeneity among the components. It offers specific information concerning bulk mixing (macromixing), but does not allow the quantitation of meso-mixing and micromixing. It can indicate the optimum hydrodynamic regime or bioreactor setup (e.g. stirrer type, use of baffles) and can predict how mixing efficiency is affected when a process is scaled up. In addition, different types of cultivation systems can be compared by calculating the dimensionless mixing number (N_m) [16] which represents the number of stirrer rotations required to homogenize the liquid [17].

$$N_m = t_m \cdot n \quad (1)$$

Mixing time depends on several geometrical factors (e.g. dimensions of the mixing system and bioreactor) and technological factors (e.g. fermentation conditions, physical characteristics of the medium, power consumption and dissipated energy). This general correlation describes the dependence of t_m on several factors:

$$t_m = f\left(\frac{d}{D}, n, \eta, \rho, \frac{V_a}{V}, \frac{P}{P_a}, \varepsilon_T\right) \quad (2)$$

Several equations can be used to calculate the mixing time, taking into account the type of fermentation (e.g. aerobic, anaerobic), the rheological characteristics of the broth, and the fermentation conditions [18–21], so the accuracy of such estimates are limited to the special conditions included in the calculation. Moreover, most models can only predict t_m for a Reynolds number (Re) exceeding 10,000. Where $Re < 10{,}000$, these models often require correction factors [22]. In a mixing process, the initial heterogeneity of the components has a major impact on the power input, especially where the components differ in viscosity and density. During the expansion of hMSCs, the power input is determined by a combination of stirring and the aeration.

Three studies have been published concerning mixing times in the Mobius® CellReady 3 L bioreactor, each involving a one-phase pure liquid system [23–25]. As the actual hMSC expansion process involves multiple phases, we investigated the mixing behavior of the this bioreactor in two-phase and three-phase-systems. Therefore, the mixing time at increasing agitation rates was determined in aerated and non-aerated processes with different working volumes and microcarrier concentrations. The selected parameters reflect the real hMSC expansion process: aeration (0.1 L min^{-1}), working volume (1 and 2.4 L), microcarrier concentration (15 and 30 g L^{-1}), agitation rate (30–110 rpm) and power input (0.01–6 W m^{-3}). The shear stress experienced by the cells under these process conditions was also evaluated.

2. Materials and methods

2.1. Mixing time determination

All mixing time experiments were carried out at 37 °C using the phenolphthalein pH shift color change method [26] in a Mobius® CellReady 3 L bioreactor (Merck Millipore, USA) with a scoping marine impeller (d = 0.0762 m). The bioreactor was assembled for stem cell cultivation with pH and temperature probes (for measurement) and an EXcell 230 sensor for the inline monitoring of absorbance (Exner Process Equipment, Germany).

Table 1
Conditions used to determine the mixing time in water at 37 °C and ambient pressure.

Factor	Lower limit	Upper limit	Center Points
Working volume (L)	1.0	2.4	–
Aeration (L min^{-1})	0	0.1	–
Microcarrier concentration (g L^{-1})	0	30	15
Agitation rate (rpm)	30	110	50 and 70

For mixing time determination, the bioreactor was filled with preheated distilled water and supplemented with approximately 2 mL 0.1% phenolphthalein (Fagron, Germany) before adjusting the pH to ~10 with 5 M NaOH to ensure a complete color change to pink. Here, the mixing time is defined by the time span from addition of acid to fully decolorization of the solution in the vessel. For decolorization, 5 M HCl in 5% excess was added as a pulse through the remaining probe fitting in the head plate. The mixing time measurement was started immediately after the addition of HCl and stopped when the color changed (95% criterion).

According to the experimental design (Table 1), five parameters were varied: volume, aeration, microcarrier concentration, and stirrer rotation/agitation rate. For every setting, the mixing time was determined in triplicates. The influence of aeration on the mixing time was determined by passing air through the micro-sparger at 0.1 L min^{-1}. The effect of the microcarrier concentration was investigated in the presence and absence of two different concentrations (15 g $L^{-1}$and 30 g L^{-1}) which are typical for stem cell expansion [27,28]. Solohill® collagen-coated microcarriers (Pall Corp., USA) with a density of 1022–1030 kg m^{-3} and a diameter between 125–212 µm were used. The mixing time under all the test conditions was determined over a range of agitation rates (30, 50, 70 and 110 rpm). Each experiment was done three times. The data represent the mean value and the respective standard deviation.

2.2. Cell expansion in the bioreactor

As a prove of concept, we have carried out a bioreactor cultivation with bone-marrow derived hMSCs (passage 3–7; kindly provided by EMD Millipore (USA)). The cells were cultivated at 37 °C in DMEM supplemented with 10% FCS and 2 mM L-glutamine (all Biochrom, Germany) containing 15 g L^{-1} microcarriers (Solohill® collagen coated, 360 cm^2 g^{-1}). The aeration rate was maintained at 0.1 L min^{-1}. Expansion was carried out in fed-batch mode (initial concentration 3350 cells cm^{-2}) in a Mobius® CellReady 3 L bioreactor with an agitation rate of 50 rpm. Feeding protocol was done according to previous published work [13,14,29]. The bioreactor was initially filled with 1 L of fresh medium, on day 8 the working volume was increased to 2 L with fresh medium and sufficient microcarriers to maintain the concentration at 15 g L^{-1}, and the agitation rate was increased to 80 rpm. To avoid substrate limitations, 0.4 L fresh medium and microcarriers were added on day 12 and the agitation rate was increased to 90 rpm. Cell dissociation prior to the addition of the fresh microcarriers has not been carried out in the bioreactor expansion.

Adherent cells on microcarriers were counted by membrane lysis and subsequent staining of cell nuclei with crystal violet. The microcarriers were settled in 1 mL homogeneous carrier suspension, the supernatant (900 µL) was removed and replaced with 900 µL 0.1% crystal violet in 0.1 M citric acid. The suspension was mixed and incubated at 37 °C for 45 min on a rocking shaker. The nuclei were counted with a