

LATEST ADVANCES IN
BIOPROCESSING: OVERCOMING
BOTTLENECKS

SPOTLIGHT

EXPERT INSIGHT

Next-generation stem cell expansion technologies

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Cell therapies, where living cells are used as therapeutic agents, either to regenerate tissues affected by disease or to act as delivery agents for secreted factors, are commonly based on the action of stem cells or their differentiated progeny. These cells also have great potential for drug discovery, providing access to inaccessible patient-specific cell types. However, stem cells, which are the most important 'raw material' for these applications, have to be expanded *in vitro* to generate the high quantities of cells that are expected to be required. Decades of research and application of bioreactor engineering concepts have provided a solid foundation for use of advanced culture technologies for stem cell manufacturing. The particular challenges of producing high quantities of functional cells are discussed here as well as innovative approaches and technologies that may revolutionize the field.

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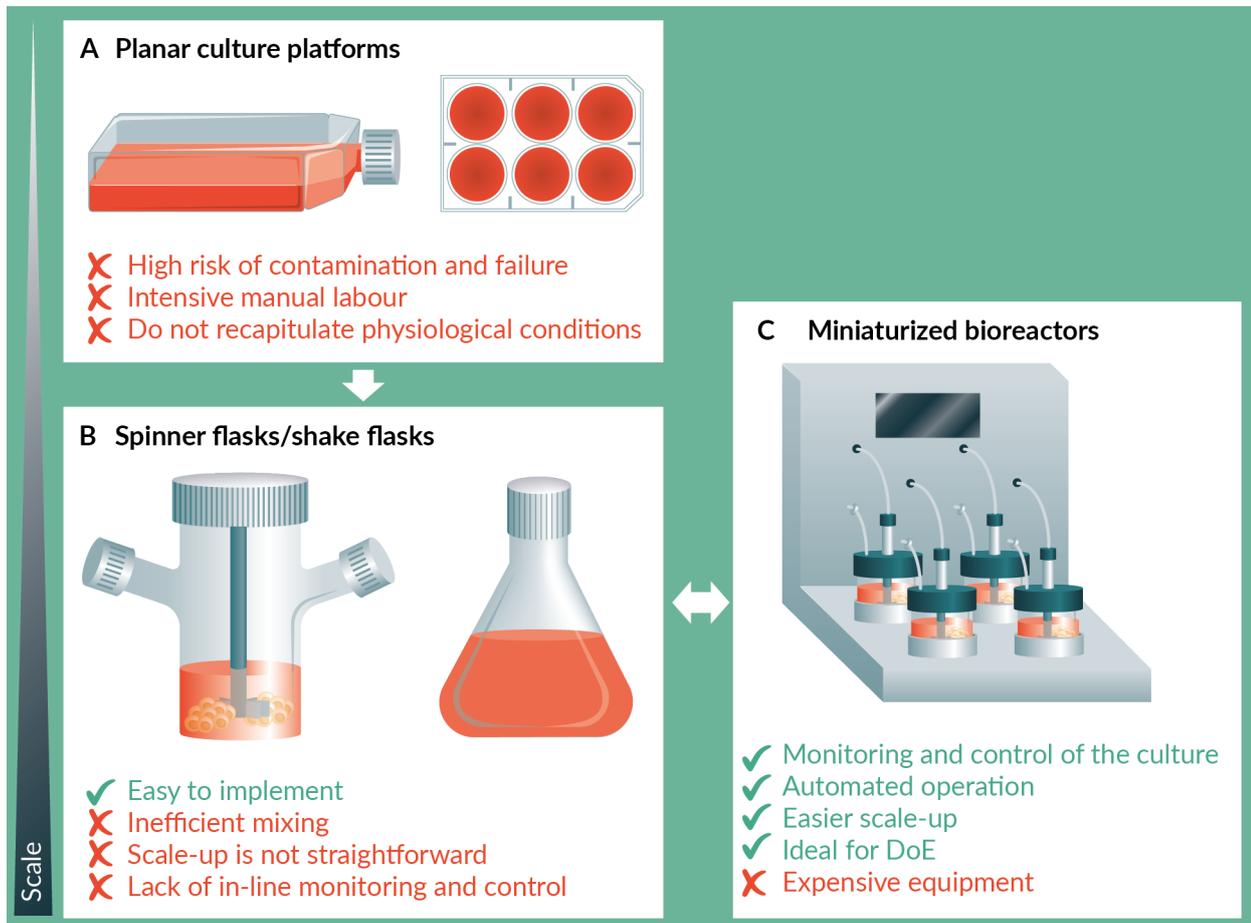
The generation of large numbers of stem cells and derivatives is a prerequisite for the successful use of these cells for cell therapies, disease modeling and drug discovery [1-3]. As stem cell-based therapies start to enter clinical trials, the need for robust methods and consistent processes capable of providing the necessary quantities of cells in a

predictable way, while ensuring the safety and functionality of the product, becomes clear [4,5]. Currently, stem cell culture is performed mostly using planar culture platforms, such as tissue culture plates and T-flasks (Figure 1A), where cells are cultured without agitation and are routinely passaged whenever they have no more available area to grow.

This culture setup is relatively easy to implement and works well in a small scale. The generation of larger cell quantities requires scaling-out, by increasing the number of plates, which quickly becomes impracticable in terms of manual labor and incubator space, besides presenting high risks of contamination or failure [6]. Multi-tray flasks, which are

► **FIGURE 1**

Initial steps of stem cell manufacturing process development.



The typical workflow is represented from static culture plates to small-scale vessels and alternative miniaturized bioreactors are also depicted. The advantages and drawbacks of these systems are highlighted.

essentially T-flasks with multiple culture layers, can be convenient for this scale-out approach but may lead to limitations in heat and oxygen transfer to the middle layers, possibly resulting in a heterogeneous final product [7]. The use of automated platforms for cell culture, which can hold and manipulate large quantities of flasks with minimal user input, is seen as a strategy to overcome the limitations associated with scaling-out planar culture platforms and work in this direction was performed using platforms such as the CompacT SelecT™ system (Sartorius Stedim) [8]. However,

even with automation, 2D culture has the problem of not correctly reproducing the *in vivo* environment [9], ultimately affecting cell fate as well as the growth rate or viability. The transition to a 3D culture paradigm has been performed using mostly two different approaches: microcarriers and cell aggregates [2,10-12]. Bioreactors constitute the ideal platform for 3D culture of stem cells and can be used with both these culture formats [13]. Besides allowing to culture the cells in an agitated environment, these vessels are commonly equipped with probes to monitor and control the

culture environment, for instance, in terms of temperature, pH and dissolved oxygen [13]. As a result of extensive research and practice with microorganism and mammalian cell cultivation for the production of biopharmaceuticals, there is a solid know-how in the area of bioreactor engineering which is now being applied to the manufacturing of stem cell-based products [14,15]. However, the particular characteristics of these bioprocesses are leading to development of new technological solutions that will be discussed here.

INNOVATIVE BIOREACTOR CONFIGURATIONS

Stirred-tank bioreactors are the most common bioreactor type used in the biotechnology industry. These bioreactors are typically made of glass or stainless steel and are equipped with a mechanical impeller to provide agitation and to maintain cells in suspension. The culture parameters (e.g., temperature, pH, dissolved oxygen) are constantly monitored by probes installed in the vessel, and can be maintained within a working range through the action of an automated controller. Working volumes can go from milliliters, in bench-top bioreactors, to thousands of liters in large bioreactors. There are multiple examples of the application of stirred bioreactors for stem cell culture, either culturing cells on microcarriers or as aggregates [16–20].

The development of a stem cell production process starts with the optimization of culture conditions, envisaging the generation of a high cell concentration in a short time. This optimization can be achieved by using different combinations of,

among others, culture media, agitation speeds, seeding densities or feeding strategies. However, a significant constraint for this optimization is the prohibitive cost of culture medium, due to the presence of expensive growth factors or cytokines, associated with a high frequency of culture medium renewal – in some cases close to daily 100% medium renewal – which limits the number of conditions to test. The mixing mechanism in stirred tank bioreactors is also not ideal for stem cells, which are more sensitive to shear stress than bacteria and, particularly in the case of cell manufacturing processes, cell integrity is fundamental for the final product quality. We will next describe innovative solutions that address these issues and that may re-invent stem cell culture in bioreactors.

Miniaturized bioreactors

A common strategy to overcome the constraints of the initial steps of process development described above consists in the use of small-scale vessels, like spinner flasks or shake flasks (Figure 1B), to minimize culture medium requirements. However, these vessels are typically not equipped with probes for culture monitoring or control and are agitated using magnetic bars, or orbital shakers, which do not mimic the impellers of higher scale bioreactors. More sophisticated, fully instrumented small-scale bioreactors have been introduced, such as the DASbox (Eppendorf) [21] or ambr 250 (Sartorius Stedim) [22] which closely mimic larger scale bioreactors, in terms of impeller geometry and control of culture conditions, allowing them to work in a 100–250 mL scale. A smaller scale system, the ambr 15 (Sartorius Stedim) [23], is

also available, as a high-throughput automated workstation which allows simultaneous culture of up to 48 individually controlled vessels with working volumes as low as 10–15 mL. The possibility of testing multiple conditions in parallel in a cost-effective way (using reduced working volumes), with minimal operator-associated variability is therefore a valuable tool for process development. In fact, a process for human Mesenchymal Stem Cell (hMSC) expansion, optimized using the ambr 15, was shown to have identical performance in a higher scale [23], which confirms the validity of this approach. These systems are also particularly interesting envisaging the implementation of a Quality by Design (QbD) strategy [24], a paradigm that is changing the industrial production of biopharmaceutical products [25].

The first steps to the design of a process under QbD guidelines requires the identification of critical quality attributes (CQA) and the critical process parameters that influence them. In the case of stem cell manufacturing, it is important to identify the most important process parameters (e.g., agitation speed, seeding density, etc.) and to understand their impact on the final product, establishing the range where it is possible to operate without affecting the CQA. The use of tools like Design of Experiments (DoE) allow to run multivariate experiments, where different process parameters are varied simultaneously, elucidating the interactions between those parameters and establishing the conditions for optimal culture outcomes [26,27]. To provide statistically significant results, a high number of experimental conditions have to be tested with an appropriate number

of replicates. Miniaturized bioreactor systems, like those described above, can run multiple vessels in parallel, varying and controlling the culture conditions individually, to generate data that allows to define response surfaces correlating parameter variation with quality outcomes [14]. As the process understanding increases, as well as the general stem cell biology knowledge, this rather empirical approach can be replaced with mechanistic models [24] that also correlate information about gene expression or metabolism with the CQA. Given the inherent variability associated with cellular systems, the use of automated small-scale bioreactors for process optimization allows to standardize operations performed during culture that are sources of experimental error (e.g., manual sampling or cell counting), improving the reproducibility and speeding up process development (Figure 1C).

Alternative mixing mechanisms

Stirred-tank bioreactors (Figure 2A) have been the most common choice for the dynamic culture of stem cells but, as there are concerns regarding the high and heterogeneous levels of shear stress in these vessels, alternative bioreactor configurations, with different agitation concepts, may be beneficial for the field [6,28].

A new bioreactor type, the Vertical-Wheel bioreactor (PBS Biotech) [29], has been recently introduced, with an innovative agitation mechanism designed to promote efficient mixing with minimum power input, which can be advantageous for shear-sensitive cells (Figure 2B) [29]. This bioreactor has a U-shaped bottom and is equipped with a large vertical impeller, resulting in

homogenous and gentle particle suspension, as well as a high mass transfer rate, under low agitation speeds. The Vertical-Wheel bioreactors were developed as single-use vessels, envisaging compliance with current Good Manufacturing Practices (cGMP), and are available in different volumetric scales. Miniaturized versions of these reactors (100–500 mL of working volume) can be used, as described above, for process development – although these vessels are not instrumented. Above the 3 L model, and up to 80 L, the bioreactors allow automated control of the culture and consistent mixing performance across the scales. However, since the Vertical-Wheel bioreactor technology is relatively new, more detailed engineering studies regarding fluid dynamics or mass transfer are still necessary for the complete characterization of the system. Nevertheless, successful use of this bioreactor for hMSC [30] and hiPSC [31] expansion using microcarriers has been reported and thus this should be also a promising technology for stem cell culture using other formats or cell types.

Stirred-tank bioreactors and Vertical-Wheel bioreactors generate fluid motion by the action of an impeller that, through its rotation, provides the dynamic environment that enhances culture medium mixing and particle suspension. However, other bioreactor geometries are available which generate mixing without a mechanical impeller. Hollow-fiber bioreactors [32], where anchorage-dependent cells are cultured attached to the fibers while culture medium is perfused continuously, in a way that resembles the human circulatory system, are a low-shear stress alternative to

FIGURE 2

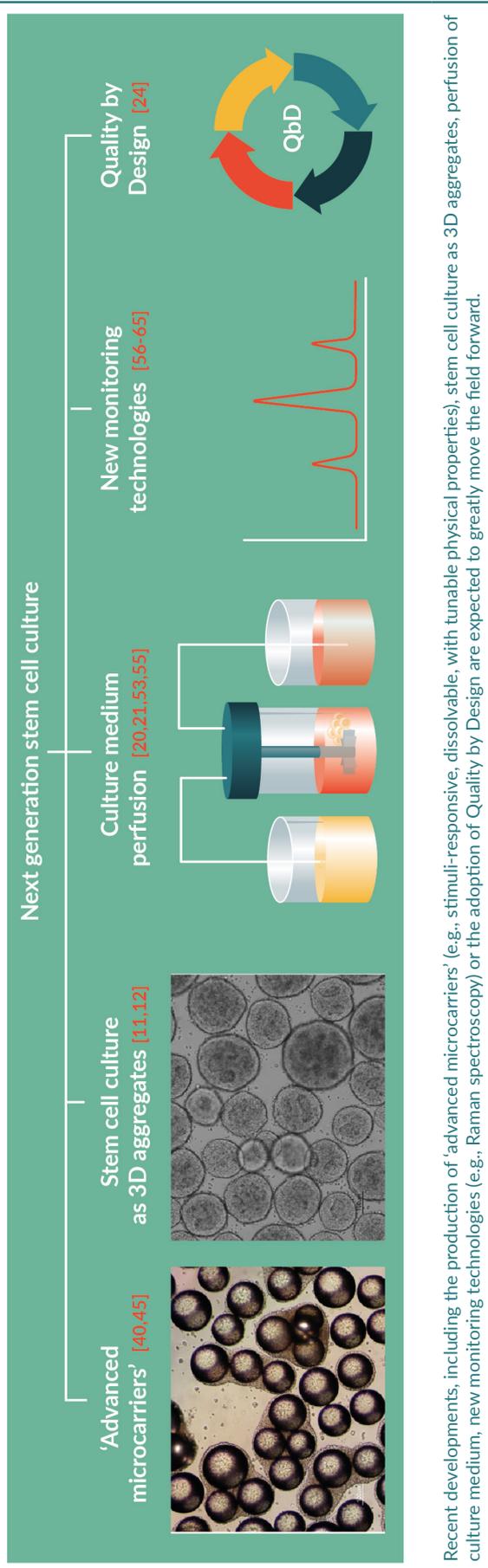
Common bioreactor systems for the large-scale culture of animal cells.



Stirred tank bioreactors (A) are the most common large-scale culture system for animal cells, including stem cells. Alternative bioreactor systems are shown including: (B) Vertical-Wheel bioreactors, (C) hollow-fiber bioreactors and (D) prototype bioreactors. The advantages and drawbacks of these systems are highlighted.

FIGURE 3

Towards the next generation of stem cell expansion technologies.



stirred bioreactors (Figure 2C). The Quantum Cell Expansion System (Terumo BCT) is a closed-system, automated hollow-fiber bioreactor that has been recently used for the expansion of hMSC [33,34] as well as neural stem cells (NSC) [35]. These studies present promising results but aspects such as monitoring of cell morphology or growth are still not straightforward with hollow-fiber bioreactors and can be the object of future research.

An interesting alternative geometry, conceived specifically to provide a laminar flow regime, while avoiding the presence of impellers/rotating components, was described by Massai and colleagues [36]. The bioreactor consists of a stainless steel base, a culture chamber and a lid. The continuous and closed-loop circulation of medium in the culture chamber leads to the generation of buoyant vortices for particle (e.g., microcarriers or cell aggregates) suspension under low-to-moderate shear stress, depending on the flow rate. The shape of the culture chamber was conceived and further improved using *in silico* models, in order to achieve optimized performance. Although the culture of stem cells was not reported, the authors show proof-of-concept of the application of this bioreactor with cancer cell spheroids. This study illustrates the design of innovative prototype bioreactor configurations for mammalian cell culture (Figure 2D), through computational modeling, prototyping and experimental validation, which may be an interesting solution, in particular for stem cell differentiation bioprocesses that may require tunable levels of shear to maintain cell viability but also, in some cases, to stimulate differentiation [37].

NEW STRATEGIES & TECHNOLOGIES FOR STEM CELL CULTURE

The bioreactor configurations described above have the potential to take stem cell manufacturing to a next level. Nevertheless, new approaches and strategies for improved cell culture performance combined with cutting-edge technologies for monitoring of the culture parameters and also for cell recovery/downstream processing are equally important. In this section, we will discuss the benefits and bottlenecks of different culture formats (microcarriers vs cell aggregates) and review how recent scientific and technological advances may lead to the development of 'advanced microcarriers', to alternative culture medium feeding strategies (perfusion) and also to new devices for culture monitoring that may impact the field of stem cell bioprocessing (Figure 3).

Development of 'advanced microcarriers'

The transition of stem cell culture from static 2D plates to a 3D format is often performed using microcarriers, which are particles (100–300 µm of diameter) to which cells adhere and grow, usually coated with extracellular matrix substrates [38]. The surface-to-volume ratio of microcarriers is much higher than in planar platforms and the available surface area for the cells to grow can easily be increased by adding more microcarriers. Keeping the microcarriers in suspension in stirred bioreactors requires high agitation speeds, which may damage the cells not only directly by the shear forces but also due to the bead-to-bead collisions. Also, the cells have to be harvested from the microcarriers at the end of

the culture. Indeed, the most commonly used microcarriers are polystyrene microspheres and, to meet the regulatory and safety demands for the presence of particulates, the microcarriers have to be efficiently removed from the final product [39]. The subsequent use of the cells expanded using this method therefore requires two downstream operations: detaching the cells from the microcarriers and a separation step to remove the microcarriers from the cell suspension. Innovative approaches have been reported to address these challenging stages. Similarly to what happens in culture plates, proteolytic enzymes are frequently used to detach cells from microcarriers. However, the action of these enzymes may be detrimental for cell viability. The use of microcarriers made, or coated, with stimuli-responsive biomaterials is a promising alternative strategy [40]. Stimuli-responsive materials change their chemical or physical properties in response to external environmental changes. The coating of microcarriers with poly N-isopropylacrylamide (pNIPAAm), a thermoresponsive polymer [41], allows cell detachment by decreasing the temperature under 32 °C, allowing to harvest the cells without proteases which may damage cell membrane proteins [42,43]. This approach has been successfully reported in the literature with Chinese Hamster Ovary (CHO) cells [44] and also with hMSC [45]. Polymers sensitive to other stimuli like pH, light, ultrasounds and electric or magnetic fields are also available with interesting properties for microcarrier production [40] but their use with stem cells has not been reported. However, although more efficient cell detachment can be obtained using these polymers, it remains necessary

to remove the microcarriers from the final product. This separation process can be performed by filtration using cell strainers, in small-scale processes, or using, for instance, tangential flow filtration systems, when moving to larger scales [39]. These operations may result in cell losses/decreased viability and increase the complexity of the process. The production of microcarriers made of biomaterials, such as hydrogels that can be dissolved enzymatically or by other means, with minimal harm to the cells, may also bring many advantages to the downstream processing stage and a commercial product with these characteristics has recently been introduced by Corning. Besides these bioprocessing aspects, the manipulation of the physical properties of the microcarriers is also interesting to modulate stem cell fate. Many studies describe the impact of mechanical cues on stem cell proliferation and differentiation (reviewed in [46]) and the use of microcarriers made of materials with different properties in terms of elasticity or topography may be a promising strategy for stem cell manufacturing [47]. Moreover, the microcarriers can be used as agents for controlled delivery of growth factors or small molecules, enhancing, for instance, differentiation efficiency [48].

Stem cell culture as 3D aggregates

A viable alternative to the use of microcarriers is growing the cells as self-organized spherical aggregates [11,12]. In contrast to microcarrier-based culture there is no need for external particles and/or matrices and, importantly, at the end of culture, the cells can be harvested directly, without the need to be separated from the particles, simplifying

the process and avoiding cell losses. Stem cell aggregates can also be used to generate organoids, which are cellular structures that replicate, in a simplified way, the structural and functional features of a specific organ [49].

Despite these advantages, aggregate culture may present limitations in terms of diffusion to the centre of the aggregates when they grow above 200–300 μm [50] and heterogeneity in aggregate size may lead to inconsistent results. These problems can be avoided if aggregate size is controlled either through frequent passaging (every 3–4 days), by adjusting the stirring rate or by chemical-based methods, as described in a recent publication [51]. Although aggregate culture is sometimes difficult to implement, in particular using bioreactors, encouraging advances have been recently reported either with hMSC [52] or hiPSC [21,53] and, in the future, interesting developments are expected regarding organoid culture in bioreactors [49,54].

Culture medium perfusion

In most of reported cases of stem cell culture in bioreactors, culture medium feeding is performed, similarly to what happens in static culture, using the so-called ‘repeated batch’ scheme [6], which consists of replacing a fraction or the whole volume of culture medium in regular time intervals. This methodology, however, may lead to significant oscillations in culture medium composition (e.g., nutrients, toxic metabolites, cell-secreted factors) and also in terms of pH or dissolved oxygen. Recent studies demonstrate the benefit of using perfusion of culture medium as a way to improve stem cell expansion [20,21,53,55].

Perfusion consists in the continuous addition of fresh medium and removal of spent medium while the cells are retained inside the bioreactor, leading to a more stable culture environment. However, while some bioreactor configurations depend on perfusion to operate (e.g., hollow-fiber bioreactors), the implementation of this feeding strategy with other bioreactors may be technically demanding, requiring cell retention devices and the availability of feeding and harvesting ports, which can be particularly complex when using simple devices, such as spinner flasks. However, the culture of hiPSC in instrumented small-scale stirred bioreactors using perfusion was implemented and reported to result in a 47% higher cell yield when compared with repeated batch [21], highlighting the advantage of

adopting this more physiological feeding strategy, which may be an additional way to better mimic the *in vivo* stem cell microenvironment.

Monitoring technology

As mentioned above, bioreactors are equipped with probes that allow monitoring of the culture environment. However, the probes used are mostly able to measure conventional physicochemical factors (e.g., temperature, pH, dissolved oxygen) and therefore, the ability to measure other more specific culture parameters (concentration of glucose or lactate and cell density) or even to characterize the cell population (e.g., undifferentiated stem cells vs differentiated cells) in real time are not frequently used – or not available at all – and may be important to further improve the culture

TABLE 1 Summary of maximum cell quantities obtained with different bioreactors and stem cell types.

Bioreactor	Cell type	Maximum cell density (cells/mL)	Total cells harvested	Ref.
DASbox (w/ perfusion)	hiPSC	$2.85 \pm 0.34 \times 10^6$	$3.56 \pm 0.43 \times 10^8$	[21]
ambr 15	hMSC	$1.51 \pm 0.06 \times 10^5$	$2.27 \pm 0.09 \times 10^6$	[23]
Vertical-Wheel (PBS 3)	hMSC	3×10^5	6.6×10^8	[30]
Vertical-Wheel (PBS 0.1)	hiPSC	$1.21 \pm 0.02 \times 10^6$	$9.68 \pm 0.16 \times 10^7$	[31]
Quantum CES	Human periosteum-derived stem cells	N/A	3.71×10^8	[33]
Quantum CES	hMSC	N/A	2.40×10^8	[34]
Quantum CES	hNSC	N/A	$2-3 \times 10^9$	[35]
Spinner flask (w/ pNIPAAm microcarriers)	hMSC	$\sim 5 \times 10^5$	$\sim 1.5 \times 10^6$	[45]
Stirred bioreactor (w/ perfusion)	hiPSC	$3.9 \pm 0.2 \times 10^6$	$7.8 \pm 0.4 \times 10^8$	[53]
Vertical-Wheel (PBS 0.1 w/ pNIPAAm microcarriers)	hMSC	$\sim 1.1 \times 10^5$	$\sim 6.6 \times 10^6$	[67]

N/A: Not applicable.

performance and reduce batch-to-batch variability.

The concentration of nutrients (e.g., glucose) and metabolites (e.g., lactate) during bioreactor culture is often measured off-line and the results are not used for automated feedback control of the culture. Methods for measurement of glucose or lactate concentrations, using spectroscopic analysis [56] are available and, as an example, mid-infrared (MIR) spectroscopy has been used to analyze the supernatants of hMSC cultures on microcarriers [57] and could be in the future used for real-time optimization of medium feeding to bioreactors. Other spectroscopic methods such as Raman or 2D-fluorescence were also used for the same purposes with mammalian cell lines and could, in principle, be also used with stem cells [58].

The possibility to have online measurement of other stem cell specific parameters would greatly increase the knowledge about the process and potentially facilitate the adoption of QbD [24]. A method for tracking stem cell growth on microcarriers, using an optical system, has been developed for automated and operator-independent measurement of cell viability, confluence and cell distribution [59]. Dielectric spectroscopy has also been used for viable cell quantification on microcarriers with encouraging results [60]. The characterization of the cells generated in the process, however, involves assays that often require sacrificing cell samples and lengthy protocols, such as transcriptional/proteomic profiling or functional assays (e.g., differentiation potential, *in vivo* transplantation), which are not suitable for real-time monitoring of bioprocesses. Alternative assays that can give indications of cell identity/

quality in real time are therefore necessary. Raman spectroscopy has been used to non-invasively measure molecular properties of stem cells during differentiation *in vitro* [61] as this label-free technique allows to monitor time-dependent molecular changes in live cells, providing information about cell phenotype, without affecting cell viability. Examples of the use of Raman spectroscopy include the ability to identify and distinguish NSC and NSC-derived glial cells [62], monitoring cardiac differentiation of human embryonic stem cells [63] and, more recently, for the discrimination of atrial and ventricular cardiomyocytes derived from pluripotent stem cells [64]. The possibility to integrate these techniques in bioreactor systems will be a huge step forward towards the manufacturing of high-quality stem cell-based products. Although online monitoring of stem cell/differentiated progeny properties is not yet possible, the use of Raman spectroscopy for evaluation of the maturity of cardiomyocytes derived from hiPSC in bioreactors has been described [65] and in forthcoming years we may see the emergence of groundbreaking devices for stem cell bioprocess monitoring.

TRANSLATIONAL INSIGHT

The success of stem cell-based therapies or, in a shorter-term, the use of stem cells in drug discovery and/or toxicity evaluation, will greatly depend on the availability of technologies for efficient and reliable cell manufacturing. Recent advances are opening new doors that will be crucial to overcome many challenges that still exist in the field. Currently, many steps of process optimization

are performed in small-scale culture vessels, which are difficult to monitor and control. Miniaturized bioreactors, as discussed above, allow the performance of high-throughput experiments, in order to establish the optimal ranges of culture parameters, in a vessel geometry that can be translated to larger scale bioreactors. The use of these bioreactors also may lead to more reproducible experimental outcomes and the detailed process data collected can be used to implement QbD strategies [24]. The availability of online monitoring technologies, for important parameters for stem cell culture, is expected to lead, as well, to tighter control of the bioreactor culture performance and to improved outcomes. Agitation/mixing paradigms are also evolving to take into account the requirements of stem cell cultivation. Traditional stirred bioreactors have been used for stem cell culture but there are concerns about the possibility of shear-induced cell death or differentiation [66]. Different strategies have been used to design bioreactors with low-shear environments and it is predicted that new bioreactor geometries (as illustrated in [36]) can be introduced in the future using computational fluid dynamics modeling and the current availability of 3D printing equipment may facilitate even more the construction of new prototypes that can after be validated experimentally.

The inventive combination of some of the different bioprocessing approaches here described may also be a way of targeting culture challenges, as exemplified in a recent work where hMSC aggregates were obtained by combining pre-expansion on pNIPAAm-coated microcarriers, followed by temperature-induced cell detachment and

aggregate formation using Vertical-Wheel bioreactors [67]. Most of the current bioreactor technology has been developed envisaging the production of high concentrations of proteins and not high concentrations of functional, living cells. The methodologies here described, including the use of new bioreactors, different culture modes ('advanced microcarriers', aggregates), feeding strategies (perfusion) or monitoring devices, were already able to generate relatively high cell densities (Table 1). Further developments of these technologies, together with new fundamental biology knowledge, will allow the design of new stem cell-oriented bioprocesses, which will take the field into the next generation of stem cell culture.

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