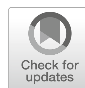
Process Design for Human Mesenchymal Stem Cell Products in Stirred-Tank Bioreactors



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Abbreviations

ad	Adipose tissue
APIs	Active pharmaceutical ingredients
ATMPs	Advanced therapy medicinal products
bm	Bone marrow
CBMP	Cell-based medicinal products
CD	Cluster of differentiation
CPP	Critical process parameter
CQA	Critical quality attribute
DO	Dissolved oxygen
ECM	Extracellular matrix
EMA	European medicines agency
EVs	Extracellular vesicles
FBS	Fetal bovine serum
FDA	Food and drug administration
GMP	Good manufacturing practice
hESC	Human embryonic stem cell
IL	Interleukin
INF	Interferon
ISCT	International society of cell therapy
MSCs	Mesenchymal stem cells
PAT	Process analytical technology
PX	Passage number X

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QbD	Quality by design
QTPP	Quality target product profile
R&D	Research and development
sCTMP	Somatic cell therapy medicinal products
STR	Stirred-tank bioreactor
TNF	tumor necrosis factor
uc	Umbilical cord

Nomenclature

N_c	Critical agitation rate
d_S	Diameter of the stirrer
d_T	Diameter of the tank
h_S	Height of the stirrer
k_La	Volumetric mass transfer coefficient
T	Temperature
p	Pressure

1 Introduction

Human mesenchymal stem cells (MSCs) for cell therapy are classed as advanced therapy medicinal products (ATMPs), which are defined as medicines for human use that are based on genes, cells, or tissue engineering, excluding vaccines. The global ATMP sector is growing rapidly, with over 900 companies worldwide, 1060 clinical trials involving ATMPs, and 14 products already approved for the market [1]. In a molecular context, ATMPs are highly complex products, even more so than biologicals such as antibodies or insulin. The active pharmaceutical ingredients (APIs) of ATMPs are complex entities such as viable cells and/or infectious viruses, which require elaborate and costly characterization. The regulation of ATMPs is not globally harmonized. The two major regulatory authorities, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), differ slightly in terms of ATMP subclasses. The FDA classification is relatively broad and covers two major groups: gene therapy products and cellular therapy products. However, the EMA differentiates between gene therapy medicinal products and cell-based medicinal products (CBMPs), and further divides CBMPs into somatic cell therapy medicinal products (sCTMPs) and tissue engineered products. The fourth class in the EMA classification is the combined ATMPs, featuring mixtures of other product types [2]. The largest class is the sCTMPs, representing 39% of all ATMPs (www.grandviewresearch.com data from 2019) and 18% of all sCTMPs contain MSCs as the API [3].

The therapeutic application of MSCs requires an average of 416 million cells per dose [4], far exceeding the number of cells that can be isolated by tissue aspiration. All MSC manufacturing processes must therefore include an *in vitro* expansion

step. One option for MSC expansion is the use of static culture vessels such as T-flasks, which are common consumables in many research and development (R&D) laboratories. However, assuming that 13.9 million MSCs can be produced per T-175 flask (175 cm^2 growth surface), 30 such flasks would be needed per dose per patient. A phase I clinical trial with 20 patients would therefore require 600 flasks, a phase II trial with 200 patients would require 6000, and a phase III trial with 2000 patients would require 60,000. Cultivation of the latter would require 450 standard CO_2 incubators (160 L) and 130 trained staff. This simple calculation shows the limitations of the so-called scale-out approach. Bioreactors are therefore preferable for the scale-up of MSC cultivation for clinical trials. For comparison, the MSCs required for a phase III trial involving 2000 patients can be produced using microcarriers in one stirred-tank bioreactor with a working volume of 1050 L. This is not only more economical but also allows the precise control the MSC microenvironment, which is necessary to ensure the functionality of the final MSC product.

2 MSC-Based Products Are Non-typical Stem Cell Products

MSCs have been studied for several decades, but a precise definition has been surprisingly challenging. In 2006, the International Society of Cell Therapy (ISCT) defined minimal criteria that must be met before cells can be defined as MSCs. Such cells must (i) show plastic adherence; (ii) express the cluster of differentiation (CD) surface markers CD73, CD90 and CD105, but not CD11b, CD14, CD19, CD34, CD45 or HLA-DR; and (iii) be able to differentiate into cartilage, bone, and fat cells *in vitro* [5]. To define MSCs as "stem cells" is misleading because MSCs *in vivo* show non-typical stem cell behavior. Stem cells are capable of both self-renewal and differentiation *in vivo*, whereas MSCs are only capable of self-renewal and do not differentiate *in vivo*. Instead, MSCs stimulate local stem cells to differentiate and to regenerate the destroyed or dysfunctional tissue. Therefore, the therapeutic benefit of MSCs reflects the properties of their secretome.

The MSC secretome comprises a pool of cytokines, chemokines, growth factors and extracellular vesicles (EVs) carrying proteins, lipids, and various RNAs, and differs widely among MSC isolates and subpopulations. MSCs can modulate immune cells, reduce inflammation, apoptosis, or fibrosis, and improve angiogenesis [6]. These modes of action are clinically relevant, as seen when surveying the clinical trials involving MSCs. There are currently 374 phase I, 314 phase II and 45 phase III trials with MSCs as the API (www.clinicaltrials.gov, search term *mesenchymal stem cells*, 2021). A quarter of these trials are in the field of immunology, using the immunomodulatory properties of MSCs to treat conditions such as Crohn's disease, graft-vs-host disease, or immunodeficiency. Another significant proportion of the trials exploit the anti-inflammatory effect of MSCs to treat rheumatic diseases such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis. Their anti-apoptotic potential is being used in clinical trials targeting stroke and cardiac defects. As well as inhibiting cardiomyocyte apoptosis, MSCs

are useful in cardiac therapy because they secrete growth factors such as VEGF and improve angiogenesis [7]. Since 2016, MSCs have also been defined as medicinal signaling cells, which properly reflects their therapeutic activity [8].

For the clinical use of MSCs, manufacturing (including *in vitro* expansion) must follow FDA/EMA guidance and must produce a sufficient quantity of viable cells. However, the most important goal is to ensure the MSCs are therapeutically functional, due to their role as the API. The definition of therapeutic functionality differs for each therapeutic approach, and the *in vitro* MSC expansion step must therefore be adapted for each MSC product. It is important to understand if and how the various process parameters affect the MSC product, allowing the critical process parameters to be tightly controlled, thus ensuring reproducibility, standardization, and economic efficiency.

3 MSC Functionality Is Determined by the Microenvironment

The successful manufacturing of functional MSCs is primarily dependent on the microenvironment *in vitro*. MSCs are found in various human tissues. They were initially isolated from bone marrow (bm-MSCs) based on their plastic adherence, but today they are usually isolated from adipose tissue (ad-MSCs) or umbilical cord blood (uc-MSCs), which are more accessible [9]. MSCs are also found in various other adult, fetal and perinatal tissues [10]. Regardless of their origin, isolated MSCs are heterogeneous and polyclonal cells, but even monoclonal MSCs become heterogeneous during *in vitro* expansion [11]. MSCs have different growth rates depending on their source, but even MSCs from the same source tissue but different donors show different growth performance [12]. For example, ad-MSCs proliferate more quickly than bm-MSCs [13], and juvenile uc-MSCs proliferate more quickly than adult MSCs [14]. Furthermore, potency is often dependent on origin. The immunomodulatory activity of bm-MSCs exceeds that of other MSCs [13], whereas ad-MSCs show stronger immunosuppressive effects than bm-MSCs [15], Wharton-jelly MSCs inhibit mitogen-induced T-cell responses to a greater extent [16], and uc-MSCs show the highest angiogenic capacity *in vitro* [17]. These differences reflect the microenvironment of the cells *in vivo*, which defines the functionality and properties of MSCs *in vitro*.

MSCs are influenced by several factors *in vivo*, including other MSCs, other cells (e.g., neighboring cells, immune and cancer cells and their EVs), the inflammatory regulators in the environment, the components, stiffness, elasticity, and topography of the surrounding extracellular matrix (ECM), nutrients (e.g., glucose, lipids, oxygen, and trace elements), waste products, and soluble factors such as chemokines, cytokines, and hormones (Fig. 1). These factors clearly differ between bone marrow, adipose tissue and the umbilical cord. MSCs are surrounded by other cells, with which they communicate via surface receptors, soluble factors,

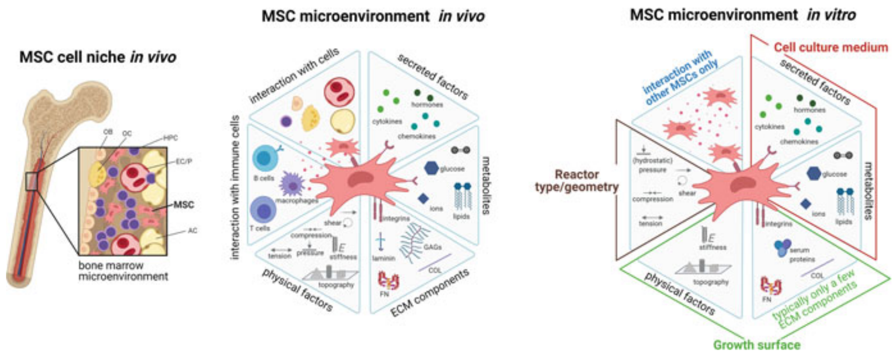

Fig. 1 The properties of MSCs are defined by their niche and microenvironment. Left panel: MSCs *in vivo* are affected by their tissue of origin (in this case, bone marrow) and the corresponding microenvironment. MSCs are influenced by surrounding cells such as osteoblasts (OB), osteoclasts (OC), hematopoietic stem cells (HPC), endothelial cells/pericytes (EC/P), adipocytes (AC) and immune cells such as T cells, B cells and macrophages. Middle panel: As well as interactions with cells, including other MSCs, the properties of MSCs are influenced by physical factors such as shear and pressure, extracellular matrix (ECM) components such as fibronectin (FN), glycosaminoglycans (GAGs) and collagen (COL), metabolites such as glucose, ions and lipids, and secreted factors such as hormones, chemokines and cytokines. Right panel: The *in vivo* microenvironment can be only partly imitated *in vitro*. The only cellular interactions are with other MSCs. Most physical factors (shear and pressure) are attributed to the reactor design, whereas the ECM components are restricted and mainly provided by the growth surface. The cell culture medium provides certain metabolites and secreted factors, and its composition is very flexible

and EVs. Accordingly, MSCs are often described as donor cells (providing EVs to other cells types) but they also act as recipients and their behavior is thereby modulated by their neighbors. Each cell produces a secretome that forms a microenvironment affecting surrounding cells, including MSCs [18]. For example, neural cells (and their EVs) facilitate MSC neuronal induction [19], whereas endothelial cells and their EVs influence MSC proliferation, migration, and the secretion of soluble factors such as matrix metalloproteases (MMP-1 and MMP-3), chemokine ligand 2 (CCL-2), and interleukin (IL)-6 [20]. Immune cells such as monocytes also communicate with MSCs. Lipopolysaccharide-activated monocytes secrete soluble factors and EVs that modulate the MSC phenotype [21]. MSCs also react to inflammation or cancer. An acute inflammatory environment induces the immunosuppressive effect of MSCs, whereas a chronic inflammatory environment causes pro-inflammatory behavior [22]. EVs from cancer cells stimulate MSCs to produce and secrete inflammatory cytokines such as IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 [23]. Cancer stem cells can also induce epigenetic changes in recipient cells. MSCs are attracted to the tumor environment and change their phenotype, becoming pro-tumorigenic. This correlates with the overexpression of genes involved in cell migration, ECM remodeling, angiogenesis and tumor growth [24].

As stated above, MSCs can remodel the ECM but the ECM also exerts a reciprocal influence. In their natural niche, MSCs form cell−cell connections via cadherins and connexins, and also interact with ECM components. MSCs are influenced by the biochemical constituents of the ECM, but also by its stiffness and topography [25]. The ECM surrounding ad-MSCs and bm-MSCs induces changes in MSC quantity, morphology, and function [26]. MSC proliferation is enhanced when the origin of the MSC matches the tissue origin of the cultured ECM [27]. Each tissue has a certain concentration of ECM components but also a certain stiffness and elasticity. MSCs usually originate from very soft tissues such as marrow, or soft tissues such as fat. MSCs adhere only weakly in these tissues, which is necessary to maintain their self-renewing capability. Such MSCs are characterized by only low levels of integrin-mediated signaling through focal adhesion kinase (FAK), retaining levels of extracellular signal-regulated kinase (ERK1/2) to support growth but not differentiation [28].

Given the above, it is clear that the interactions between MSCs and their microenvironment are very complex. Expanding MSCs beyond their natural niche induces massive changes in their microenvironment, which means that every step during *in vitro* cultivation has a non-neutral effect on MSC biological properties. An *in vitro* expansion process must therefore mimic the cellular niche to ensure that MSCs remain functional and therapeutically active. However, several aspects of the natural niche cannot be replicated *in vitro* (Fig. 1). Interactions with other cell types and the immune system are absent *in vitro*, so the only intercellular interactions are with other MSCs. Interactions involving cell–cell connections, cytoskeletal elements, the ECM, and overall tissue topography can have profound effects on multipotent MSCs. Harvesting MSCs from a bone marrow niche with its condensed cell-rich environment and culturing them *in vitro* removes the cell−cell cadherin and connexin connections and replaces them with cell−substrate and cell−matrix interactions as the cells produce more ECM [29].

A certain set of physical and ECM interactions can be replaced *in vitro* by the growth surface/matrix. Several growth surfaces are available, including the planar (often polystyrene) surfaces found in T-flasks, curved growth surfaces such as microcarriers, and 3D matrices such as porous microcarriers, hydrogels, and spheroids. The planar polystyrene surface does not imitate the natural niche very well because it is stiff, with an elasticity module ($E = 1$–10 GPa) much higher than that of the natural MSC matrix (bm-MSC $E < 0.3$ kPa; ad-MSC $E = 2$–6 kPa) [30]. MSCs grown on stiff, planar surfaces show limited expansion potential and lower differentiation capacity with increased passage number. Moreover, the stiffness of the growth surface/matrix can alter transcription [31] and thus modulate MSC behavior and differentiation [32]. This suggests that MSCs retain an environmental memory, meaning that transcription is still altered even if MSCs are transferred from T-flaks to a softer substrate later [33]. The whole MSC manufacturing process must therefore be considered in this context because the routine passaging of MSCs on planar polystyrene surfaces may inadvertently and permanently alter their phenotype and functionality.

On planar surfaces, the ECM and other proteins build up as a continuous layer which establishes an apical-basal polarity and a restricted adhesion to the *x*–*y* plane. The adhesion is often very strong and spaced over large distances (~5 μm), which promotes unwanted differentiation because MSCs undergoing osteogenesis require stronger adhesion [28]. In contrast, curved surfaces transduce cytoskeletal changes that influence MSC migration and differentiation [34]. The cell–cell interactions are less strong, with fewer adhesion points. The 3D materials are often softer than flasks, and the ECM forms discrete fibrils. MSCs can establish 3D networks without polarity, and spreading is sterically hindered. With topography and elasticity/stiffness, a certain set of physical factors are defined by the growth surface/matrix. Other physical factors, such as tension, pressure, compression and especially shear, are provided by the bioreactor system. Static culture vessels apply few if any of these factors, whereas bioreactors provide a more nurturing environment where the strength of the factors can be controlled by the reactor geometry, conditions, and equipment setup.

Soluble components such as cytokines, chemokines, hormones, nutrients, trace elements, ions, and lipids are provided *in vitro* by the cell culture medium. This requires a detailed understanding what MSCs really need and how the components influence MSC properties. In the absence of such knowledge, fetal bovine serum (FBS) was formerly used as an obligate component of MSC culture medium. Although its exact composition is undefined, FBS offers various adhesion factors, cytokines, EVs, hormones, and protease inhibitors that support cell growth [35]. As our understanding of cell requirements has improved, and given more recent ethical and regulatory concerns, FBS is no longer allowed for the manufacture of clinical MSC products and serum-free or chemically defined media are preferred.

The plasticity of MSCs (their ability to change due to the conditions in their microenvironment) can be exploited. If the effect of environmental parameters is known, the bioprocess can be designed to trigger the production of MSCs with a certain therapeutic function. Specific cultivation conditions should therefore be defined to prevent differentiation into unwanted cell linages and the total loss of therapeutic potential.

4 Bioreactor Systems Can Create the Appropriate Microenvironment for MSC Expansion *In Vitro*

The transition from laboratory-scale experiments to industrial biomanufacturing processes is hampered by the intricacy of MSCs and their interactions with the microenvironment. To ensure a consistent and standardized manufacturing process, the development is grounded in the quality-by-design (QbD) principle, which provides a rational framework and combines scientific knowledge from biological and engineering perspectives. This requires a clear definition of the quality target product profile (QTPP). The product attributes are known as critical

quality attributes (CQAs) and define the product in terms of physical, chemical, and biological properties. Therefore, the identity, purity, and potency of each MSC product is tightly controlled but varies for individual MSC products and their therapeutic indications, which cannot be transferred from one product to another. In short, the identity of MSCs is determined by morphological and phenotypic analysis, meaning the presence or absence of specific surface markers as described above. The potency of MSCs is generally dependent on therapeutic indications. Several potency assays are available but only specific assays are applicable for individual MSC products. For example, the assessment of a differentiation potency assay is only appropriate for MSCs that develop their therapeutic mechanism based on tissue formation. Sterility and purity, meaning the absence of contaminants such as unwanted cell types, particles, or pathogens, must be proven to ensure safety and efficacy [6]. Every process parameter that influences the CQAs of an MSC product is described as a critical process parameter (CPP). Ideally, CPPs are controlled throughout the manufacturing process, but some CPPs are difficult to access depending on the bioreactor design.

Static cultures in T-flasks or hyperflasks are not only difficult to scale up, they also lack process control. This led to the commercial development of large-scale planar bioreactor systems with an integrated stirrer and pH and dissolved oxygen (DO) control, providing surface areas of up to 12.24 m^2, equivalent to ~700 T-175 flasks (Table 1). Although these systems are suitable for MSC expansion, drawbacks include limited monitoring of cell growth, dissimilarity with *in vivo* conditions, and labor-intensive and time-consuming operations. Nevertheless, these systems can be used for the preparation of MSCs for phase I and II clinical studies involving only a small number of patients.

Table 1 Large-scale single-use bioreactors potentially suitable for the manufacture of MSC-sEVs. All bioreactor systems monitor pH, temperature, gas flow and stirring speed except the NANT XL, which is not aerated or stirred. Suspension bioreactors were prepared with 15 g/L microcarriers giving a specific surface area of 360 cm^2/g

Bioreactor system	Process mode	Monitored parameters	Growth surface [m^2]	Working volume [L]	Supplier
Planar bioreactors					
NANT XL	Batch	CO_2, confluency	0.318	–	VivaBioCell
Xpansion200	Batch	pH, DO, confluency	12.24	21.9	Pall
Hollow-Fiber bioreactors					
Quantum	Perfusion	T, DO	2.1	0.189	Terumo
Fixed bed bioreactors					
BioBLU 5p	Perfusion	T, pH, DO	18	3.75	Eppendorf
iCellis	Perfusion	T, pH, DO, biomass, p	500	25	Pall
Scale-X nitro	Perfusion	pH, T, DO, p	600	–	Univercells
Suspension bioreactors					
Mobius 2000 L	Batch	T, pH, DO, biomass	1080	2000	Merck
Xcellerex XDR 2000	Batch	T, pH, DO, biomass	1080	2000	Cytiva
Allegro STR 2000 L	Batch	T, pH, DO, biomass	1080	2000	Pall

In contrast to planar bioreactor systems, MSCs in hollow-fiber and fixed-bed or packed-bed bioreactors create a 3D microenvironment [36]. Fixed-bed and packed-bed bioreactors provide a large surface area for cell growth over a bed of macro carriers, which the cells use as a substrate. Metabolites are provided by the constant supply of fresh medium, and waste products are removed continually. In hollow-fiber bioreactors, MSCs grow in the interstitial spaces of a cartridge of hollow fibers that mimic blood capillaries and thus simultaneously deliver metabolites while removing waste products [37]. Both bioreactor systems can prevent the inhibition of cell growth by the buildup of toxic metabolites, and the process can therefore be extended for several months, increasing MSC yields and economic efficiency [38]. Both bioreactor systems achieve a high yield of cells per unit volume because the cells grow very densely, close to physiological conditions, helping to maintain their CQAs. However, these bioreactor systems must still overcome challenges hindering large-scale manufacturing, including (i) heterogeneous cell distribution; (ii) reduced metabolite availability and waste product removal due to high cell densities and insufficient diffusive mass transfer; (iii) lack of direct cell growth monitoring, relying instead on metabolism-derived approximations such as mass balance of oxygen levels; and most importantly (iv) low harvesting efficiency [39, 40]. Given the high cell densities, the enzymatic contact surface is restricted and long incubation times are required for detachment, which reduces cell viability [41, 42].

The importance of harvesting for MSC manufacturing has led to the introduction of suspension bioreactors such as stirred-tank reactors (STRs) for the large-scale expansion of MSCs. For example, in a 50-L STR with a working volume of 35 L, a 50-fold expansion was achieved with a final yield of 2.6×10^{10} cells [43]. For industrial-scale manufacturing, several disposable STRs are commercially available (Table 1). MSCs are anchorage-dependent cells, so the growth surface is generally increased by the use of mostly spherical microcarriers with cell-specific properties to encourage attachment, proliferation and harvesting. However, microcarriers that ensure proper attachment and proliferation are not necessarily suitable for biomanufacturing processes when the cell is the API. For example, MSCs attach strongly to Cytodex I microcarriers but the harvesting efficiency is only ~20% [44]. Therefore, a well-designed microcarrier screening process should include attachment, proliferation, harvesting kinetics and MSC functionality. Once suitable microcarriers are identified, the exponential growth phase can be extended by bead-to-bead transfer without enzymatic treatment, ensuring high cell yields, surface-to-volume ratios and economic efficiency over a range of scales [45]. Furthermore, STRs do not suffer from the disadvantage of heterogeneous cell distribution as seen in hollow-fiber and fixed-bed/packed-bed bioreactors. Convective mass transport prevails instead of diffusive processes, ensuring the sufficient availability of nutrients and oxygen. The homogeneous cell distribution in STRs also allows representative sampling if necessary. Most importantly, STRs are compatible with process analytical technology (PAT) to guarantee process control [46]. In addition to online controlled parameters such as pH, temperature and DO, and the offline measurement of glucose levels, cell growth, viability and size can be monitored online by impedance spectroscopy [47]. This online technology ensures process transparency and control during MSC biomanufacturing. STRs are therefore

the most suitable bioreactors for the manufacture of MSCs as products because of their process flexibility, economy and tight control of CPPs, allowing them to meet CQAs with low batch-to-batch variations. We therefore focus below on CPPs for MSC expansion in STRs.

5 CPPs for MSC Expansion in STRs

5.1 Cell-Related Parameters for MSCs

Regardless of the bioreactor system, the medium, growth surface and other cell-related parameters have a profound impact on the success of MSC expansion. As stated above, cell–cell interactions *in vitro* are restricted to MSCs because no other cells are present. The relevant CPPs include the MSC source, age and density. The source and donor of the MSCs should be fixed, because important MSC properties such as doubling time are strongly dependent on this parameter. For example, under the same cultivation conditions and medium, uc-MSCs had a significantly shorter doubling time (4 days) than adult MSC (7 days) [48].

MSC age is also important because aging (population doubling *in vitro*) causes MSCs to increase in volume [49], proliferate more slowly, begin to lose the expression of MSC markers, and become more fibroblast-like in morphology [50]. MSCs reach senescence *in vitro* after a source-dependent number of doublings, for example ~50 in the case of uc-MSCs [51] and ~ 70 in the case of ad-MSCs [52]. The MSC expansion process should therefore be started with a distinct population doubling and/or stopped before the population doubling limit is reached. This limitation can be overcome using an immortalized MSC line if the line displays the desired therapeutic functions.

For MSC expansion, the initial cell density and final cell density must be standardized in order to reach the same number of population doublings during one passage [53]. The final cell density is restricted by the growth surface area and the efficiency of harvesting, but is typically in the range 5×10^4–1×10^5 cells/cm^2. The initial cell density varies from 100 to 10,000 cells/cm^2. MSCs derived from initial high-density cultures feature a larger number of flat cells and the proliferation rate is lower. However, the initial density should not be too low, because cultures initially plated at a density of 10–100 cells/cm^2 do not expand effectively [54]. The initial cell density must be chosen carefully because it also affects cell age, given that cells with lower initial densities require additional rounds of doubling to reach the final cell density. In an expansion process requiring several passages, these cumulative age differences lead to different cell populations even though the passage number remains the same. Furthermore, even monoclonal MSCs become heterogeneous during expansion. If the initial seeding density is too low, the risk increases that certain MSC subgroups may overgrow the general MSC population. The fastest growing MSC subgroup is not necessarily the one

with the best therapeutic potential. Accordingly, cellular dynamics during the MSC expansion process must be monitored carefully. A fast growth rate is not sufficient alone and the therapeutic efficacy of the expanded MSCs must be considered as well.

MSCs *in vivo* are surrounded by several cells, so replicating this effect *in vitro* by providing the corresponding EVs may be beneficial. EVs from differentiated cells, immune cells and cancer cells can all modulate the properties of MSCs [18]. Other interactions, with living or inactivated bacteria, can increase the absolute number of MSCs, improve their immunomodulatory properties, and promote the expression of anti-inflammatory factors [55]. The easiest parameters to control *in vitro* are the physicochemical factors, which are mainly related to the culture medium and the growth surface.

5.2 Physicochemical Parameters for MSCs

MSCs respond to physical parameters such as hydrostatic pressure, tensile stress, compression, vibration, and ultrasound by modifying their transcriptional profiles (mechanotranscription). Many of these factors can promote MSC differentiation [56], but it is unclear whether they can also influence the fate of undifferentiated MSCs, or affect their proliferation or functionality. Given that mechanical stimuli are part of the natural MSC niche, such factors are likely to play a key role in the biological and structural responses of MSCs.

More is known about the impact of chemical/biochemical factors on MSCs. The availability of oxygen in the natural cell niche is low (2–7% pO_2) [57], whereas many bioreactors strive to achieve atmospheric oxygen conditions (21% pO_2). High oxygen levels promote the generation of reactive oxygen species (ROS) which damage MSCs and induce apoptosis [58]. Many studies have therefore highlighted the need to cultivate MSCs under hypoxic conditions from isolation until transplantation [59–65]. Hypoxia (typically 2–5% pO_2) is known to increase bm-MSC density, inhibit senescence and maintain the undifferentiated state [66–69]. Even the composition of MSC-derived EVs changes during hypoxia, reflecting the upregulation of hypoxia inducible factor 1 α (HIF-1α) and miR-126, improving the therapeutic efficacy of bone fracture healing [70].

After oxygen, the second most important requirement for MSCs is glucose. Although a low glucose concentration (5.5 mM) is maintained *in vivo* [71], many cell culture media contain high levels of glucose (22 mM). The effects of high glucose levels have been reported, with conflicting claims, but there is evidence for a limited impact on MSC proliferation and function [72–74]. Low glucose levels (5.5 mM) slightly increased the frequency of apoptosis in ad-MSCs [75] but weakly promoted the proliferation of bm-MSC [76]. High glucose levels may be a pathological trigger for MSCs, creating disease-specific microenvironments in conditions such as diabetes. Other physicochemical factors such as pH and osmolarity are also associated with diseases, and these factors must be kept within physiological ranges to ensure the health of MSCs cultivated *in vitro*. Even weak

acidity (pH 6.8) and hyperosmolarity (485 mOsm) can inhibit the proliferation of ad-MSCs [75] and bm-MSCs [77], and promote necrosis. Trace elements and metal ions are essential for MSCs, but some metal ions promote differentiation (e.g., Mg^{2+} promotes osteogenesis and Li^{+} promotes myogenesis [78]).

Cytokines are potent regulators of MSC behavior *in vivo* and *in vitro*. The priming of MSCs by cytokines *in vitro* has been described in detail. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α are the most prominent inducers of immunosuppressive MSC behavior, promoting survival and proliferation [79], but interleukins such as IL-1α, IL-1β and IL-2 also induce an immunosuppressive phenotype [22]. Stromal cell-derived factor 1 (CXCL12/SCDF-1) is a chemotactic for MSCs, promoting survival, proliferation, and paracrine functions [80]. The microenvironment *in vivo* combines several cytokines and each cytokine has a concentration-dependent effect [78]. Therefore, a design-of-experiments (DoE) approach may be useful to evaluate the impact of cytokines on the therapeutic function of MSCs, allowing the identification of concentration-dependent effects and also interactions between two or more growth factors.

5.3 Microcarriers Provide the Growth Surface for MSCs in a STR

Although part of the physicochemical parameters, we discuss the MSC growth surface/matrix separately because it is essential for MSC expansion. MSCs are strictly anchorage dependent and will undergo a form of programmed cell death known as anoikis if a substrate is unavailable. In a STR, the growth surface is often provided in form of microcarriers, which are small beads (100–300 μm in diameter) with a similar density to the medium, allowing homogenous distribution in the bioreactor by stirring. Microcarriers are considered as a form of 3D cultivation, but the cells nevertheless grow as a monolayer on the curved surfaces, so the term pseudo-3D is more appropriate. Microcarriers can be classified as porous or non-porous. Porous microcarriers mimic 3D cell–cell interactions more accurately than their non-porous counterparts, but the surface of the latter can be modified (e.g., by coating with ECM molecules) to enhance cell attachment, or by physical treatment to change the surface charge and wettability [81].

Most microcarriers recommended for human MSC expansion are commercial non-porous beads with a polystyrene core and various coatings or surface treatments. These are very stiff and the coatings, if present, are generally not thick enough to enable full control over the surface mechanical stiffness sensed by the cells. A coating must be 10–20 μm thick to mask the stiffness of the underlying substrate [82]. The influence of microcarrier stiffness on MSC properties has not been evaluated in detail, perhaps because the curvature effect on mechanical stress makes the results difficult to interpret. MSCs may therefore be less sensitive to the stiffness of microcarriers than planar surfaces [83]. The interaction between MSCs and microcarriers is responsible for cell attachment, proliferation, and detachment.

MSCs from different sources, and even MSCs from the same source but different donors, have different surface-attachment requirements and properties [45]. This explains the broad range of microcarrier types, and the selection of appropriate carriers requires prior knowledge or attachment experiments.

Although porous microcarriers may imitate *in vivo* conditions more accurately, non-porous microcarriers allow more efficient cell harvesting. All microcarriers facilitate cell attachment and proliferation, but it remains challenging to harvest cells efficiently without damaging them [41]. The proteolytic enzymes used for cell passaging and tissue digestion may damage the ECM and thus affect the corresponding signaling pathways, ultimately affecting MSC behavior [84].

Microcarriers offer a simple and efficient way to expand MSCs and produce clinically relevant numbers of cells with the required characteristics [42]. Commercial microcarriers do not provide all the benefits of the natural MSC niche but can generate vigorous MSCs with potent therapeutic functionality. Several investigations have tailored microcarriers for MSC expansion, aiming to mimic the natural niche more precisely, for example by adjusting material stiffness, coating the surface with more natural ECM structures, and using dissolvable microcarriers to improve the efficiency of harvesting.

5.4 Equipment-Related Parameters for MSCs

Agitation in STRs is usually achieved by placing the impeller near the bottom of the vessel, generating the driving force for a convective flow regime that homogenizes the culture microenvironment, disperses gas and nutrients, ensures sufficient mixing, and reduces laminar boundary layers. These conditions are important for MSC bioprocessing, but agitation also generates shear forces and other forces that can have a profound effect on MSC growth or functionality. The influence of shear forces on cell proliferation and functionality has been described in 2D models (flow chambers), but with some contradictory results. Whereas some studies reported a positive effect on proliferation, others reported a decline [85]. This shows that every MSC product and manufacturing process must be characterized individually and no overall correlation between MSC products and the CPP "agitation" is valid. All STRs should limit the force experienced by cells to reduce the likelihood of cell damage and maintain CQAs such as functionality. The forces acting on cells growing on microcarriers are associated with hydrodynamic stress as well as cell–carrier and carrier–carrier collisions [86]. The resistance of MSCs to dynamic forces in a STR can be estimated using Kolmogorov's turbulence theory, which explains that stress acting on MSCs and microcarriers is caused by eddies similar in size to the cells/microcarriers and the distance between microcarriers [87]. These eddies do not cause cell damage if they exceed a critical size ($\geq 60\%$ of the cell or microcarrier diameter). Kolmogorov's theory is valid for a turbulent regime, but most MSC expansion processes in STRs are found within the transitional range, making this approximation inaccurate [46, 88].

Although cell stress must be limited, the power input must be sufficient to achieve a homogeneous microcarrier suspension. An agitation rate that is just sufficient to suspend the microcarriers means they do not remain on the bottom surface of the vessel for more than one second. A further increase can achieve the critical agitation rate (Nc) where microcarriers are homogeneously distributed. Nc can be approximated based on the microcarrier concentration, STR geometry and a stirrer-dependent constant [89]. The ability to achieve homogeneity while maintaining low cell stress is strongly influenced by the stirrer type.

Different stirrer types can be categorized by their fluid pumping characteristics. Radial impellers such as the Rushton turbine generally have high power inputs but low suspension capabilities. Axial pumping stirrers such as marine propellers or impellers are more suitable for MSCs grown on microcarriers. They facilitate bottom-to-top fluid movement and hence the Nc is low, which minimizes cell stress. However, many subtypes of stirrers have been developed by combining axial and radial fluid characteristics, such as the three-segmented pitched-blade stirrer. Fluid movement within the bioreactor is also affected by the interplay with the bioreactor setup. The stirrer diameter to tank diameter ratio (d_S/d_T) and the stirrer height to diameter ratio (h_S/d_S) are important parameters. The d_S/d_T ratio should be at least 0.4 to guarantee sufficient mixing characteristics, especially in large-scale processes, whereas h_S/d_S should be high to ensure homogeneous power dissipation in the bioreactor [90]. Nc can be reduced further using a fully baffled system [91] because baffles covert tangential flow to axial/radial flow, thus increasing suspension capabilities and homogeneity.

MSC biomanufacturing processes must ensure a sufficient oxygen and nutrient supply. Aeration systems can be divided broadly into headspace, membrane and bubble aeration setups. Headspace and membrane aeration systems are sufficient for small-scale experiments, but bubble aeration by means of a sparger is necessary at larger scales [92]. With the help of a sparger, a higher oxygen transfer rate (k_La) is achieved by increasing the interfacial area between the gas and liquid phases [93].

Bubble aeration can influence MSC growth because the high local velocities caused by rising and bursting bubbles generate shear stress and are responsible for foaming and cell entrapment. The sparger must therefore be chosen carefully. Macrospargers produce large bubbles with small interfacial areas and high local velocities, while microspargers produce smaller bubbles with a homogeneous size distribution and a large interfacial area, thus increasing the k_La [93]. However, excess oxygen induces oxidative stress by generating ROS, which disrupt biochemical processes [94]. The rational selection of aeration systems can be achieved by characterizing the oxygen demand of the cells. Primary MSCs consume oxygen at the rate of 90–100 fmol/(cell·h) whereas an immortalized cell line has a much higher demand of 300 fmol/(cell·h) [36, 95]. This highlights the importance of process design, in which STRs are customized and adjusted to specific MSC needs to ensure that CQAs are maintained.

6 Production of MSC-Derived EVs

The therapeutic effect of MSCs is mainly conferred by the secretome, particularly EVs, resulting in growing interest in the use of EVs as cell-free therapeutics. EVs were originally considered as waste products, but their therapeutic potential has been confirmed. The therapeutic application of EVs overcomes the drawbacks of manufacturing viable cells and the complexity of transfusion processes. EVs are more robust than cells, and more stable during storage and transport, thus maintaining their therapeutic efficacy [96, 97].

EVs are divided into three broad categories differing in size and therapeutic potential. Exosomes (30–100 nm) and microvesicles (50–1000 nm) are the most suitable as therapeutics, whereas apoptotic bodies have limited applicability [98]. Exosomes are derived from the budding endosomal membrane and are matured as intraluminal vesicles within the lumen of multivesicular endosomes (MVEs). The MVEs are transported within the endosomal system, and fuse with the cell surface for EVs release. Microvesicles are formed by the outward budding and fission of the plasma membrane and the release of EVs into the extracellular space. Both exosomes and microvesicles are positive for CD9 and CD81, whereas CD37, CD63, CD53 and CD151 are only found on exosomes [99]. The nomenclature and classification cannot be based on size and functionality alone because there are major differences in biogenesis, but the separation of EVs based on biogenesis is unrealistic. Therapeutically active vesicles in the size range 40–200 nm are therefore described as small EVs (MSC-sEVs), as recommended by MISEV2018 [100]. MSC-sEVs contain proteins, lipids, and various RNA molecules that can elicit responses from recipient cells. The positive effect of MSC-sEVs has clearly been shown over short and long distances. MSC-sEVs inhibit inflammation, apoptosis, and fibrosis, but enhance angiogenesis and tissue regeneration [98].

Like EVs in general, MSC-sEVs are communication vehicles that influence the state and functionality of neighboring recipient cells. The cargo of MSC-sEVs has been investigated to determine the bioactive molecules responsible for their therapeutic functionality, and has been classified based on molecular and cellular functions such as transcription factors, chemokines, cytokines, growth factors and miRNAs. However, the results of different genomic, proteomic, metabolomic and glycomic studies have differed considerably. This reflects the physiological diversity of MSCs, which respond to triggers in their environment (such as inflammation or hypoxia) by adjusting their metabolism and secreting MSC-sEVs representing the physiological state of the donor cell. The cargo is therefore highly sensitive to stimuli in the microenvironment and thus to *in vitro* process parameters. For the comparison of MSC-sEVs, it is therefore necessary to consider process parameters as well as the intrinsic nature of the donor cell [101]. The medium composition is also important, because the production of MSC-sEVs can be boosted by reducing the concentration of FBS and oxygen levels or increasing pro-inflammatory factors and shear rates [102].

The sensitivity of MSCs (and MSC-sEV composition) to the microenvironment is yet not fully understood. These first approaches to process design by triggering MSC-sEV production represent a milestone on the way to clinical applications. Furthermore, manufacturing processes could be specifically designed to develop individual treatments for each patient, bringing personalized medicine within reach [103].

7 Bioreactor Systems for MSC-sEV Production

As discussed above, bioreactors are required to control the microenvironment of MSCs *in vitro*, enabling the regulation of DO, pH, temperature, metabolite levels, and the concentration of viable MSCs. The production of MSC-sEVs is strongly dependent on the microenvironment, so the manufacture of MSC-sEVs for clinical applications requires robust and reproducible processes that comply with good manufacturing practice (GMP). The therapeutic potential of MSCs *in vivo* is determined by external triggers that arise following infection or injury. Similar triggers must be provided to produce MSC-sEVs *in vitro* by exploiting bioreactor design and equipment-related parameters. The STRs used to produce MSCs can also be used to manufacture MSC-sEVs, but hollow-fiber and fixed-bed systems are suitable too because there is no requirement for cell harvesting [6, 38]. The bioreactor types used for MSC-sEVs therefore include many commercially available disposable bioreactors (Table 1).

Hollow-fiber and fixed-bed bioreactors allow the continuous production and harvesting of EVs from the culture medium. MSCs grow densely on the fibers and macrocarriers because the 3D structure better represents the physiological cell niche. The benefits of 3D cultivation have been demonstrated by the aggregation of MSCs into spheroids, but the same advantages also allow the efficient production of MSC-sEVs and other EVs [104]. For example, HEK293 cells in a hollow-fiber bioreactor achieved a 40-fold increase in sEV production compared to static cultures [105], whereas ad-MSCs in a hollow-fiber bioreactor achieved a ten-fold increase in MSC-sEV production compared to static cultures [106]. The cultivation of bm-MSCs in a FiberCell Systems hollow-fiber bioreactor, a smaller version of the C2018 (Table 1) with a surface area of 0.4 m^2, led to a decrease in MSC numbers due to the use of a specific EV-collection medium, but continual EV production was confirmed by the detection of specific markers [107]. Fixed-bed bioreactors combine the advantages of hollow-fiber bioreactors (3D growth) with increased metabolite availability and exposure to moderate shear stress as a trigger for MSC-sEV production [36]. Large-scale disposable hollow-fiber and fixed-bed bioreactors are currently available with surface areas of up to 600 m^2 (Table 1). However, few studies have been published about the production of MSC-sEVs and further investigation is required.

Although hollow-fiber and fixed-bed bioreactors appear suitable for large-scale EV production, cell density cannot be controlled, leading to heterogeneous cell

distribution and zones with metabolite limitations and/or waste accumulation. The high cell densities in 3D-like structures combined with low diffusion rates can also lead to a general state of nutrient limitation. Although starvation can improve EV production and low metabolite concentrations/metabolite gradients are also found *in vivo*, the heterogeneous microenvironments in hollow-fiber and fixed-bed reactors hamper process standardization.

As stated above, suspension bioreactors such as STRs lack these disadvantages because they are homogenous systems that allow the online control of cell density, viability and size by dielectric spectroscopy [47]. STRs therefore provide an interesting alternative for the production of MSC-sEVs. Because the cells are not harvested, it is also possible to use porous microcarriers, which offer a larger growth surface for the cells and a 3D-like growth environment even in a STR. The cells on porous microcarriers are also protected from destructive shear effects. On the other hand, rationally designed shear forces can be used to trigger sEV production. The company EVerZom has developed a method that triggers massive EV release by applying turbulence/shear (www.everzom.com, data from 2021). The benefits of dynamic suspension cultivation have also been demonstrated by comparing uc-MSCs in static culture to those in spinner flasks on Star-Plus microcarriers, with the latter producing 20-fold more MSC-sEVs while maintaining the characteristic EV phenotype and size distribution [48]. Another dynamic culture system based on a vertical-wheel bioreactor was used to produce MSC-sEVs derived from three different MSC types. Compared to static cultures in T-flasks, dynamic cultivation resulted in a ~ three-fold increase of MSC-sEVs yields regardless of the cell type [108]. Although these small-scale processes using suspension bioreactors are promising, the hydrodynamic parameters affecting MSC-sEV production are unknown and detailed investigations are required. Once these aspects are understood, process development will be facilitated by the compatibility of suspension bioreactors with PAT and hence process standardization. Additionally, many single-use bioreactors are currently available for the analysis of process comparability. These bioreactors have a working volume of up to 2000 L providing 1080 m^2 of cultivation area with typical microcarriers.

8 CPPs Affecting the Production of MSC-sEVs

8.1 Cell-Related Parameters Influencing MSC-sEVs

Many process parameters that are critical for the production of MSCs are also critical for the production of MSC-sEVs. For the standardized and high-yield production of MSC-sEVs, an appropriate donor cell is required and the cell-related parameters must be characterized. For example, uc-MSCs not only proliferate faster than ad-MSCs as discussed above, but also produce four times as many MSC-sEVs per cell, and the EVs differ in size suggesting a difference in functionality

[48]. Although standardized MSC isolation methods are now available, this process is considered a bottleneck because the enzymatic treatment causes cell stress and affects the mechanotranscription profile [109]. Rather than processing their own MSCs, many groups working on EVs use commercial primary cells or develop immortalized MSC cell lines. However, as stated earlier, the properties of MSCs are highly dependent on age, and the functionality of MSC-sEVs is also age-dependent [110]. This was determined by comparing the gap closure ability of MSC-sEVs obtained from MSCs at various passage numbers (P2–P5), revealing that all MSC-sEVs promoted vascularization but the activity of the EVs from P5 was the weakest [110]. Cell passaging and the resulting increase in cell age is associated with the modulation of gene expression with effects on the cell cycle, protein ubiquitination, and senescence [111]. The comparison of MSC-sEVs secreted by primary bm-MSCs and the immortalized cell line hMSC-TERT (expressing the telomerase reverse transcriptase gene, and also originating from bone marrow) revealed that immortalization resulted in a slightly higher yield of CD63^{+} CD81^{+} sEVs [112]. All EVs were similar in morphology and size, as confirmed by phase-contrast transmission electron microscopy, but functionality was not evaluated. The effect of immortalization (transfection with lentivirus) has also been tested on human embryonic stem cell-derived MSCs (hESC-MSC) and cord-derived MSCs. The morphology of the hESC-MSCs changed and they were no longer MSCs according to the ISCT classification, but these cells produced a larger quantity of sEVs that significantly reduced the size of infarcts in mice. In contrast, cord-derived MSCs produced fewer sEVs post-immortalization but the cells retained their therapeutic efficacy [113]. These results clearly show that immortalization cannot serve as a universal strategy to enhance MSC-sEV production because of the diverse effects on different donor cells. Each cell and immortalization method must be evaluated to generate well-characterized cell lines that produce high yields of potent sEVs, representing an important step towards the standardized production of sEVs and more comparability in EV research.

8.2 Biochemical Parameters Influencing MSC-sEVs

Several triggers are already known that enhance sEV production *in vivo*, such as injury and infection, and the corresponding molecular signals must be provided *in vitro* to achieve the same therapeutic effect. The production of sEVs *in vitro* is often induced by adding cytokines such as IFN-γ and TNF-α, by starving the cells of serum, or depleting essential nutrients.

IFN-γ and TNF-α are pro-inflammatory mediators and thus mimic the behavior of damaged or infected tissues. MSCs and their sEVs therefore upregulate class I/II major histocompatibility complex (MHC) and stimulatory molecules to boost proliferation, enhance immunomodulatory and immunosuppressive functions, and increase the production of sEVs [114]. In the absence of IFN-γ, ad-MSCs released 281 sEVs per cell and hour, whereas those exposed to IFN-γ released 463 sEVs

per cell and hour, a 1.7-fold increase without changing the size distribution of expression of specific markers [115]. Additionally, a priming approach, using IFN-γ and TNF-α simultaneously, increased the production of ad-MSC-sEVs compared to the non-primed control group. These findings were confirmed by differences in protein expression, especially the upregulation of Rab27b, which represents a regulator for the release of exosomes [116]. However, the same combined treatment reduced the number of sEVs produced by bm-MSCs, indicating that cytokine treatment is not a universal solution for the production of MSC-sEVs [117]. Another challenge associated with the use of cytokines to stimulate MSC-sEV production is the impact on purification and the resulting safety concerns. GMP compliance requires that manufacturing processes must include steps to eliminate putative immunogenic and allergenic ingredients, which in this case would include steps to remove the cytokines that were deliberately introduced into the process, thus increasing process costs [118].

The production of EVs is also triggered by serum deprivation. FBS provides growth factors that support MSC proliferation, and these are often present in the form of FBS-EVs. Such EVs contribute to cell expansion and proliferation, but they are considered as impurities [119]. The starvation of MSCs by the depletion of FBS-EVs (or the complete removal of FBS) therefore prevents the isolation of FBS-EVs along with the target product. Serum depletion affects the three main MSC types in different ways, with limited impact on the abundance of uc-MSC-sEVs but a significant depletion of ad-MSC-sEVs and bm-MSC-sEVs [120]. The exosome fraction of the uc-MSC-sEVs also showed increased functionality (interacting with target neurons), whereas the functionality of the microvesicle fraction was reduced [120]. Nevertheless, starving cells is controversial. It is common practice to expand cells in serum-containing medium and transfer them to serum-free medium for EV production, but this approach may not be compatible with therapeutic applications. The transfer to serum-free medium triggers phenotypic changes in the donor cells, mirrored by changes in the protein and RNA content of the EVs [121], as well as growth inhibition and the induction of apoptosis [122]. Given that EVs represent their donor cell, it is important to keep these cells in an active and proliferative state so that the therapeutic potential is not affected. This does not mean that serum-free medium should be avoided. Indeed, serum-free or chemically defined media are recommended when cells do not change their characteristics in terms of proliferation and sEV production. It may be necessary to optimize the proliferation of MSCs by adding specific growth factors to the medium and selecting an appropriate growth surface in order to determine sEV characteristics under these culture conditions, thus taking a step toward standardized production [6].

Other biological triggers that enhance MSC-sEV production include hypoxia, which mimics the physiological microenvironment of MSCs (typically 2–7% pO_2) and provides appropriate conditions for the investigation of MSC proliferation, metabolism, and EV release. Hypoxic conditions of 1–10% pO_2 increase the proliferative capacity and survival of cells by limiting the generation of ROS [123]. Accordingly, the same approaches have been applied to MSC-sEVs. There was no difference in the production of ad-MSC-sEVs when switching from normoxic

(21% pO_2) to hypoxic (5% pO_2) conditions. However, the hypoxic sEVs showed significantly enhanced functionality in a tube formation assay [124]. Another study confirmed the enhanced functionality of bm-MSC-sEVs produced under hypoxic conditions in cell proliferation, cell migration and tube formation assays, and the simultaneous use of serum-free medium also significantly increased MSC-sEV yields [125–127]. Hypoxic conditions lead to the production of potent MSC-sEVs and no additional purification steps are required, but strict control of O_2 is necessary, which can only be achieved in bioreactors.

9 Conclusions

The development of MSCs and MSC-sEVs as novel APIs still involves many challenges. Both are complex products with unique manufacturing processes, in which the microenvironment needs to be strictly controlled because it has a huge influence on the final product quality. MSCs and MSC-sEVs are strongly dependent on cell culture parameters such as the origin and handling of the cells, the composition of the medium, the nature of the growth surface/matrix and the hydrodynamics in the bioreactor. A standardized environment is essential for the manufacture of clinical products. It is necessary to define this environment in order to determine the CPPs for individual MSC and sEV products. Large-scale biomanufacturing processes are needed and bioreactors facilitate MSC expansion *in vitro* (STRs) and the production of sEVs (STRs, hollow-fiber reactors and fixed-bed systems). We are only just beginning to understand the influence of the microenvironment on MSCs and MSC-sEVs, and further investigation is required to establish CPPs that will enable standardized GMP-compliant production.

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