STEM CELL BIOREACTORS

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Summary

Stem cells are very appealing for a wide range of potential applications in the fields of regenerative medicine, drug screening and toxicology. These unspecialized cells have extensive self-renewal capability and the remarkable capacity to produce mature cells with specialized functions, such as blood cells, nerve cells or cardiac muscle. In spite of generating great interest in the scientific and medical communities, cell-based therapies and other stem cell applications require the generation of high numbers of cells. However, the number of cells that can be obtained from available donors is very scarce. A possible solution for this question consists in the scale-up of stem cell culture *in vitro*. Herein critical culture parameters for stem cells are analyzed, possible bioreactor configurations are described, as well as recent developments in the cultivation of stem cells in bioreactors and integration of novel technologies in the bioprocess development stage. This chapter provides updated and comprehensive information focusing on the systematic production of stem cells in compliance with regulatory guidelines, using robust and cost-effective approaches.

1. Introduction

In the end of the 20th century, progress in biomedical sciences led to an increasing offer of biological origin products for the treatment of several diseases. A first generation of such therapeutics included recombinant proteins, antibodies, and molecular vaccines. The development of these products resulted in the accumulation of valuable expertise in the design of highly controlled and reproducible bioprocesses that would comply with the demands of regulatory agencies like the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA). Cell-based therapies are an emerging trend in this field, generating great interest in the scientific and medical communities. Although an increasing number of companies are developing novel cell-based therapies

for different diseases, the number of products that have already reached the market is still very small, being the majority still under preclinical development.

Stem cells, due to their unlimited self-renewal capacity, as well as their differentiated progeny, are most likely the starting materials of such manufacturing processes. Unfortunately, the typical number of cells needed to treat an average adult patient (~70 Kg) greatly surpasses the limited number of cells that can be obtained from the available donors. The success of cell-based products will thus require the development of novel technologies that allow the systematic production of clinically relevant numbers of high quality cells in a tightly controlled, standardized and cost-effective manner. This objective may be attained through the use of bioreactors, which are complex devices designed to sustain high cell density cultures, through the enhancement of mass transfer and control of culture conditions. The fact that the desired products are the cells themselves brings forward further challenges related to the required good manufacturing practices (GMP) and product safety. Donor-to-donor variability, microbiological contamination or potential tumorigenicity of the transplanted cells are examples of such issues. The *in vitro* propagation of undifferentiated stem cell populations thus remains largely undeveloped and is considered a major technical challenge because of, for instance, the heterogeneous nature of starting culture populations, the transient nature of the subpopulations of interest, the complex interactions between culture parameters as well as the complex biology involved in the stem cell state regulation, which is not yet fully understood.

Apart from cellular therapies, the development of stable *in vitro* systems for the expansion and differentiation of stem cells can also contribute for the study of the mechanisms controlling such events. For example, scaling out a given process by establishing high-throughput screening platforms may allow great improvements in the process itself as well as testing the effects of different molecules on cell behavior. Meaningful pharmacological studies can be performed using such systems. Furthermore, empirical and mechanistic modeling constitute additional means for achieving a clear understanding of the dynamics of cell differentiation and organ development, allowing the prediction and control of cell responses to changes in the cellular microenvironment. The culture of stem cells for research purposes is commonly performed, due to their simplicity, ease of handling and relatively low cost, on flat two-dimensional surfaces like tissue culture flasks (T-flasks), well plates or gas-permeable blood bags consisting of a single unstirred compartment where nutrients diffuse to cells. Gas exchange (*e.g.* oxygen and carbon dioxide) occurs at the medium/gas interface.

Nevertheless, these systems where culture is performed under static conditions have serious limitations. First, they lead to concentration gradients (pH, dissolved oxygen, nutrients, metabolites, etc.) in the culture medium. The on-line monitoring of culture parameters such as pH or pO_2 is today possible in these culture systems, but the tight control of these variables is much more difficult. Scale-up is also difficult since these culture platforms present reduced surface area/volume ratios, limiting the number of cells supported per surface area. Multiple plates or flasks (or flasks with multiple trays) are required to obtain high numbers of cells, but require repeated handling in order to feed cultures or obtain data on culture performance, making this solution laborious and prone to contamination. Automation and robotics could minimize the impact of the last

issue, but the culture still would not be under dynamic conditions. The use of three-dimensional culture systems that more closely resemble the *in vivo* environment provides increased surface area for cell adhesion and growth thus leading to higher cellular concentrations but the mass transfer limitations also increase.

Mass transfer limitations and other problems can be minimized with the use of bioreactors that can accommodate dynamic culture conditions. This text will describe the most common bioreactor systems that are used (or can be used) for large scale stem cell culture as well as some advances already achieved with different kinds of stem cells in this field.

2. From Bioprocess Selection to Bioreactor Design and Operation

In order to select an adequate bioprocess for clinical or pharmacological grade production of cellular products, a few important considerations should be taken into account. In principle, when compared to molecular therapeutics, cell-based therapies have the potential to provide superior clinical outcomes because of the broad biological activity of cells (growth factor release, contribution to tissue regeneration, release of morphogens, etc). However, this will only be true if cells are produced in a reproducible way, a requirement for consistent clinical outcomes. Therefore, it is possible to foresee some major hurdles related with scientific, technical, regulatory, and commercial aspects. In fact, the development of bioreactors for stem cell expansion and differentiation is clearly dependent on the ability to mimic the physiological, biochemical and mechanical cues of the *in vivo* microenvironment - a scientific and technical challenge - in addition to the need for complying with strict regulatory guidelines, while keeping a robust, competitive and cost-efficient process. These objectives are mainly challenged by the intrinsic variability of cells obtained from different batches or donors, as well as by the response of cells to variations in the culture environment.

2.1. Bioprocess Development and Optimization based on Microscale High-Throughput Profiling

The ability to explore various conditions in a rapid and parallel mode can also help enhancing our understanding of a given system, and potentially contribute to accelerate bioprocess development. Microscale high-throughput profiling approaches, such as spatial patterning of cells or microfluidics, can contribute to speed up the transition from biological observation to optimized, clinical-scale bioreactor systems. Thus, the development of *in vitro* high-throughput screening methods for evaluating the effects of new growth factors and cytokines, as well as other culture conditions in cell models might assist in the rapid and cost-effective development of novel bioprocesses, and also increase the knowledge on conditions that selectively control cell fate. Consequently, the use of these technologies will ultimately support the production of cellular products in a more reproducible and cost-effective manner. Initial efforts regarding the application of micro-engineered systems in stem cell research focused mainly on the discovery of combinations of signaling environments that direct stem cell fate. In fact, signals emanating from the stem cell microenvironment, or niche, are crucial in regulating stem cell functions. In this context, advances in microfabrication and

microfluidics have also driven the generation of multiple platforms that allow bioprocess optimization. For example, micro-bioreactor arrays, containing independent micro-bioreactors perfused with culture medium, have been fabricated using soft lithography. These systems supported the cultivation of cells, either attached to substrates or encapsulated in hydrogels, at variable levels of hydrodynamic shear, and automated image analysis detected the expression of different cell differentiation markers. Various conditions and configurations have been validated for different cell types, including mouse myoblasts, primary rat cardiac myocytes and human pluripotent cells. Cell growth monitoring, design parameters, mass transport phenomena and shear stress issues are examples of parameters that can be examined in such devices.

These high-throughput cell culture platforms are thus efficient in the analysis of multiple parameters and parameter interactions that might prove important for bioprocess optimization. However, one major limitation of such systems is the ability to quantify specific cellular responses in an accurate and straightforward manner. Methods to alleviate such limitations have been developed and include, for example, immunofluorescence-based assays for high-throughput analysis of target proteins *in situ*, or microscale platforms with integrated multifunctional sensing elements (*i.e.* sensors) that allow immunodetection of secreted proteins.

The development of consistent, high-throughput, high-content screening approaches for depicting stem cell fate can thus contribute with valuable information regarding mechanisms controlling cell proliferation, differentiation or death. This can greatly benefit process development, as the underlying aspects of the stem cell biology are becoming further understood. Additionally, microscale strategies can also be directly employed for parameter measurement and optimization, and ultimately lead to the development of an integrated process for clinical-scale production of stem cells.

2.2. Critical Parameters for Bioreactor Design

For the successful implementation of stem cell culture in bioreactor systems several critical parameters need to be addressed. These include physicochemical variables, such as pH, dissolved oxygen, or temperature, and biochemical input, including the levels of key nutrients and metabolites, or growth factors.

2.2.1. Aeration and Oxygen Tension

In early stem cell culture efforts, attention was given to aspects such as culture medium composition, in terms of nutrients, growth factors or pH buffers. However, not many studies focused on the effect of oxygen tension as it was assumed that the oxygen tension in atmospheric air was appropriate for cell growth. However, direct measurements of oxygen tensions in embryos or tissues known to contain stem cells revealed values much lower than expected. Oxygen tension is thus an important component of the stem cell microenvironment and appears to influence stem cell self-renewal/maintenance and differentiation. Mesenchymal stem cells (MSCs), neural stem cells (NSCs), hematopoietic stem and progenitor cells (HSPCs) and pluripotent stem cells are known to be influenced by the oxygen tension to which they are exposed. Indeed, the manipulation of oxygen tensions, for instance towards more physiologic

oxygen tensions (2-9%), in culture leads to different outcomes in terms of cellular proliferation and cell-fate commitment. In terms of bioreactor design, oxygen mass transfer is thus a critical parameter. In small vessels, aeration obtained through surface aeration is often sufficient. However, when scaling-up to larger volumes, or to higher cell densities, surface aeration may not be enough to provide oxygen to all the cells in the vessel (for instance, those closer to the vessel bottom). This problem may be minimized with well known strategies from traditional biochemical engineering like increasing the agitation rate of the impeller, enriching the headspace gas for oxygen (oxygen tensions above 21%), sparging the medium with sterile air or addition of compounds to increase oxygen solubility in the culture medium, providing that they have no effect on the cells.

Determining the oxygen requirements of the system is thus an important step for bioreactor design. To avoid oxygen exhaustion in the culture medium, the oxygen transfer rate across the medium should be enough to meet the oxygen uptake of the cells. In general, the mass conservation equation, which can be applied for oxygen within the culture medium, is

$$Input + Generation = Output + Consumption + Accumulation \quad (1)$$

Without sparging, oxygen input in the culture medium, or oxygen transfer rate (*OTR*), is provided by surface aeration. In this case, the *OTR* is proportional to the oxygen concentration gradient, being the volumetric mass transfer coefficient ($k_{\mathrm{L}}a$) the proportionality constant. The OTR can therefore be estimated by

$$OTR = k_{\mathrm{L}}a\left(C^{*}_{\mathrm{O}_2} - C_{\mathrm{O}_2}\right) \quad (2)$$

where $C^{*}_{\mathrm{O}_2}$ corresponds the saturation oxygen concentration in the culture medium in equilibrium with the headspace oxygen (may be determined with Henry's law) and C_{O_2} is the measured oxygen concentration in the bulk culture medium. The oxygen consumption for a particular cell line, or oxygen uptake rate (*OUR*), is given by the specific oxygen uptake rate (q_{O_2}) and the cell density in the bioreactor (X):

$$OUR = q_{\mathrm{O}_2} X \quad (3)$$

Considering that no oxygen is released from the system (output = 0) or generated (generation = 0), Eq. (1) can be written

$$k_{\mathrm{La}}\left(C^{*}_{\mathrm{O}_2} - C_{\mathrm{O}_2}\right) = q_{\mathrm{O}_2} X + \frac{dC_{\mathrm{O}_2}}{dt} \quad (4)$$

The specific oxygen uptake rate can be determined, for instance, using a culture vessel with the top portion of the flask truncated and sealed, so that no oxygen is transferred from the headspace. Thus, since the dissolved oxygen (*DO*) in the medium is the only source of oxygen for the cells, it may be assumed that the oxygen uptake by the cells equals the rate of oxygen depletion in the medium, which can be measured with a probe:

$$\frac{dC_{O_2}}{dt} = -q_{O_2} X \tag{5}$$

From the slope of the "*DO* versus time" plot obtained the specific oxygen uptake rate can be calculated. Furthermore, Eq. (4) can be simplified with the following assumptions:

- at maximum cell density, in steady-state operation (accumulation = 0), cells consume oxygen instantly upon contact
- the oxygen concentration in the bulk culture medium is zero in this moment

$$k_L a \cdot C^*_{O_2} = q_{O_2} X \tag{6}$$

Thus, once the q_{O_2} for a cell type is known the limiting $k_L a$ value for a desired cell concentration can be determined. The oxygen volumetric mass transfer coefficient is influenced by aspects such as aeration type, vessel type, design and dimensions or culture medium properties. The presence of cells is also assumed to have impact on mass transfer. Different methods exist for the experimental determination of $k_L a$ as well as several empirical relationships that have also been developed for the determination of this parameter. Such correlations take into account different variables, for instance, the power input per unit volume (P/V), impeller geometry and position inside the bioreactor or other properties of the system, but considerable variation exists between them and thus the choice of the best correlation for a give system must be done carefully. In order to avoid oxygen limitations in a bioreactor, the "real" $k_L a$ in the system must thus be above the limiting value, calculated with the method described above.

Oxygen mass transfer has also a very important role when scaling-up the system. In fact, one of the methods often used for bioreactor scale-up consists in keeping the volumetric mass transfer coefficient constant when moving to a higher volume vessel.

2.2.2. pH

pH has an important role over the metabolic activity of cells, and is carefully regulated *in vivo* by complex mechanisms, which can be influenced by carbon dioxide and production of lactate or other substances. Suboptimal pH conditions will lead to growth inhibition and may influence cell differentiation.

Most culture media has been formulated with components designed to work with a specified CO_2 concentration, usually ranging from 0 to 10% CO_2/air mixtures, to give a pH of 7.0-7.4. In computer-controlled bioreactors, pH can be measured with a probe and controlled by adjusting CO_2 gassing. Although this strategy may be efficient at the initial stages of cultivation, when cell densities increase, pH control may become more complicated and addition of acid or base solutions may be necessary.

2.2.3. Agitation and Hydrodynamic Stress

A crucial factor that has to be taken into account in bioreactor culture of stem cells is the hydrodynamic shear stress. Stem cells, being animal cells, lack a cell wall and thus these cells are more sensitive to hydrodynamic forces than fungi or bacteria. In stirred bioreactors, an impeller provides agitation of the culture medium, increasing mass transfer of oxygen and nutrients, minimizing concentration gradients and allowing the creation of a homogeneous environment for the cells. In addition, mixing is necessary for suspending cell aggregates or microcarriers, when anchorage dependent cells are cultured on these surfaces. However, agitation results also in transfer of energy from the impeller to the culture medium, causing the formation of areas of intense turbulence, termed eddies, which dissipate the energy. The largest eddies are generated near the impeller region and start a cascade of eddies decreasing in size. The smallest of the eddies are called Kolmogoroff eddies and can be characterized by their size (η) and velocity (u), which can be estimated, according to Kolmogoroff's theory, with the following equations:

$$\eta = \left(\frac{\nu^3}{\varepsilon} \right)^{\frac{1}{4}} \tag{7}$$

$$u = (\varepsilon \nu)^{\frac{1}{4}} \tag{8}$$

where ν is the kinematic viscosity and ε is the rate of dissipation of energy per mass unit, given by.

$$\varepsilon = \left(\frac{P}{V_{\mathrm{L}} \rho} \right) \tag{9}$$

$$P = N_{\mathrm{P}} \rho n^3 d_{\mathrm{i}}^5 \tag{10}$$

where P is the power input, N_{P} is the dimensionless power number (which can be obtained with appropriate empirical correlations), ρ is the fluid density, n is the impeller rotational rate, d_{i} is the impeller diameter, and V_{L} volume where energy dissipation takes place.

These eddies generate localized shear on particles in the bioreactor, including the surface of cell aggregates, cells attached to microcarriers or single cells in suspension. When a large eddy forms in a region occupied by a particle, the particle may be completely surrounded by the eddy and could rotate and translate along with it, without creating excessive stress on the surface, either the surface of a microcarrier or of a cell aggregate. If the eddies are smaller, the particle motion will be restricted and the cells experience damage created by the full force of the eddy. Thus, cell aggregates and cells on microcarriers are affected at lower agitation rates than single cells, as the Kolmogoroff eddy size decreases with increasing agitation.

In a fluid system shear stress is caused by a velocity gradient in a direction perpendicular to the direction of flow. This gradient is called the shear rate and has units of velocity/length. Assuming that the maximum shear stress is the result of flow through Kolmogoroff eddies, the maximum theoretical values can be estimated (in dyn/cm^2) with Eq. (11).

$$\tau_{\max} = 5.3\rho(\varepsilon v)^{\frac{1}{2}} \quad (11)$$

Beyond agitation rate, other aspects have implications in the bioreactor shear stress, like impeller diameter, geometry and location or the presence of probes or other vessel internals. Impeller geometry may also be critical for avoiding stagnation regions and to minimize cell damage near the tips. It is thus possible to conclude that an equilibrium must be found, when designing bioreactor protocols, between the high levels of mass transfer provided by higher agitation rates and the low levels of shear forces on the cells observed with lower agitation rates.

Cell damage may also occur due to sparging with gas bubbles. As already referred, in some cases the bioreactors used for stem cell culture can fulfill the oxygen requirements of the cells with aeration from the headspace only. However, larger volume reactors, which are likely to maintain higher cell densities, may require additional aeration to supply appropriate concentrations of oxygen. Cell damage may occur if cells attach to gas bubbles and are exposed to high shear stress when the bubbles collapse. Sparging of protein containing media may also lead to the problem of excessive foam production, a problem that may be minimized with the addition of low concentrations of anti-foam products providing that they have no cytotoxic effect.

Although shear stress may have a detrimental effect on cell culture, it can also be a stimulus for cell differentiation. In fact, fluid shear stress may have a role directing stem cell differentiation pathways and evidence exists, for instance, that mesenchymal stem cells may have enhanced differentiation along the endothelial lineage in response to fluid flow *in vitro*.

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Biographical Sketches

Carlos A.V. Rodrigues is currently a Post-doctoral research fellow at the Institute for Bioengineering and Biosciences, Lisbon, Portugal. Carlos graduated in Biological Engineering (2007) at Instituto Superior Técnico (IST), Universidade de Lisboa and received his PhD degree also from IST in 2011. His research interests are focused on the development of bioreactor systems for the large-scale production of pluripotent stem cells and neural stem cells, using tailored microcarriers and biomaterials to support cell adhesion, proliferation and/or differentiation, which will enable the application of these cells in novel cell-based therapies as well as for drug discovery.

Dr. Rodrigues has been involved in several research projects financed by the Portuguese Foundation for Science and Technology (FCT), as well as by the European Community, in the field of stem cell bioprocessing.

Tiago G. Fernandes is currently a Post-doctoral research fellow at the Institute for Bioengineering and Biosciences, Lisbon, Portugal. He received his PhD degree from Instituto Superior Técnico, Universidade de Lisboa, in 2009. His research interests are focused on the development of artificial cellular niches for studying the mechanisms that affect human stem cell pluripotency, and aim at contributing to a better understanding of the cellular and molecular events that regulate stem cell functions. During his doctoral studies he also worked at the Rensselaer Polytechnic Institute, NY, USA, in the development of microscale platforms for high-throughput studies of stem cell fate. His work has earned him the prestigious Malcolm Lilly award, presented to a promising young scientist or engineer working in the biochemical engineering field by the European Symposium on Biochemical Engineering Science (2008). Dr. Fernandes is currently involved in several research projects financed by the Portuguese Foundation for Science and Technology (FCT), as well as by the European Community, related to the development of different systems for pluripotent stem cell culture envisaging further applications in regenerative medicine and drug screening.

Maria Margarida Diogo received her PhD degree in Biotechnology from Instituto Superior Técnico in April 2004. Her thesis was dedicated to the development of Bioprocesses for Production and Purification of Plasmid DNA for Gene Therapy and DNA vaccines. As a result of this work, a United States Patent was published in 2007. From April 2004 to March 2008 she received a post-doctoral fellowship to work in the field of Stem Cell Bioengineering and she is presently a Research Scientist at the Stem Cell Bioengineering Laboratory at Instituto Superior Técnico. She is involved in several research projects financed by the Portuguese Foundation for Science and Technology (FCT) as well as by the European Community related to development of large-scale bioprocessing strategies for human Induced Pluripotent and Neural Stem Cell culture and purification envisaging further applications in regenerative medicine and drug screening. As a complementary approach, she is the Principal Investigator of a project for development of microscale technologies for stem cell culture.

Cláudia Lobato da Silva (1978) graduated in Chemical Engineering (Biotechnology), at Instituto Superior Técnico (IST), Universidade Técnica de Lisboa (UTL) (2001). Cláudia got her PhD in Biotechnology (2006) at IST/UTL in collaboration with the University of Reno, Nevada, USA. Presently, Cláudia is an Assistant Professor at Department of Bioengineering, IST, Universidade de Lisboa. She has published 50 peer-reviewed papers, 11 book chapters and has 1 licensed patent. The objective of her current research in the Stem Cell Bioengineering and Regenerative Medicine Laboratory, Institute for Bioengineering and Biosciences (IBB) at IST, is to contribute for a better knowledge of the ex-vivo expansion of human stem cells, namely hematopoietic stem/progenitor cells and mesenchymal stem/stromal cells, in controlled bioreactor systems for Cellular Therapies.

Joaquim M.S. Cabral is Professor of Biological Engineering, in the Department of Bioengineering at Instituto Superior Técnico (IST), Universidade de Lisboa, Portugal. He was the founder and current Head of Department of Bioengineering, and the Director of the Institute for Bioengineering and Biosciences. He obtained his Diploma in Chemical Engineering from IST in 1976. His Ph.D. and "Habilitation" degrees in Biochemical Engineering were obtained from IST in 1982 and 1988, respectively. After a post-doctoral study at Massachusetts Institute of Technology in 1983 - 1984, he joined IST Faculty as Assistant Professor and became a Full Professor in 1992. Joaquim Cabral's research aims at contributing for biochemical engineering science through novel developments in Bioprocess Engineering and Stem Cell Bioengineering. Stem Cell Bioengineering interests address the ex-vivo expansion and controlled differentiation of stem cells in bioreactor systems and downstream processing, using hematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and pluripotent stem cells (PSC), as model systems. Dr. Joaquim Cabral has been establishing an international research track record across the Stem Cell Engineering field, greatly expanding the understanding of stem cell expansion in bioreactors, and contributing to the national leadership role of IST-IBB in the field. This leadership has been recognized over the last decade also at the international level with many publications in international peer review journals and several invitations to Dr. Joaquim Cabral to Chair scientific sessions in international conferences focusing on stem cell processing. Very recently, IST-IBB got the global recognition has one of the main players in the field of Stem Cell Engineering, being part of the final report of the International

Assessment of R&D in Stem Cell Engineering (http://www.wtec.org/SCE/). Dr. Joaquim Cabral is member of several Editorial Boards of scientific journals and of Organizing and Scientific Committees of conferences in the areas of Biotechnology and Stem Cells, Tissue Engineering and Regenerative Medicine. He has published over 400 research papers and co-authored or edited 6 books.