Human and animal cells as factories for therapeutic molecules

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Human and animal cells as factories for therapeutic molecules production: applications and culture processes

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1. Introduction

Animal and human cells have progressively become a versatile platform to produce various therapeutic biomolecules. This category of expression systems mainly includes mammalian (essentially hamster, mouse and human cells) and insect cells. From a general point of view, non-human mammalian cells are still the most used at the industrial scale (production of glycosylated recombinant proteins and of adenoviral vectors among others) but some continuous human cell lines can be used *in vitro* in a bioreactor to obtain a supernatant enriched in a specific protein or in extracellular vesicles after selective isolation. Moreover, human stem cells can be used *in vitro* as a source of differentiated cells, as with induced pluripotent stem cells (iPS).
Considering that stem cells can be the source of more differentiated cells, their use *in vivo* make them cell factories. The use of human cells as an *in vivo* factory has been performed for decades with hematopoietic stem cells (HSC) for the reconstitution of hematopoietic blood cells and immune cells. On the same model, specific T cells like Viral specific T cells (VST) can proliferate *in vivo* and differentiate into cytotoxic anti-viral T cells. Mesenchymal stromal cells (MSC) when used in regenerative medicine, can also be considered as cell factory able to produce differentiated cells *in vivo*.
From a process engineering point of view, the success of the development and strengthening of biotherapies using animal and human cells should be based on an advanced knowledge of the cell behavior in their culture system, namely the deciphering of the couplings between cells and their culture environment.
In view of the above, we will first present hereafter the operative and expected applications of animal and human cells as *in vitro* factories but also as *in vivo* factories, focusing on mesenchymal stromal cells. Then, the current and future strategies of optimization of the interactions between the cells and their biochemical and hydromechanical environments will be discussed.

2. Human and animal cell culture for the production of therapeutic biomolecules: main applications

2.1. Industrial applications of continuous cell lines

Compared to primary cells, which are derived from extraction and/or digestion processes of biological tissues, continuous cell lines theoretically have an unlimited capacity to divide, thus allowing for a major expansion of the use of animal cells in industrial biotechnology processes. These continuous lines are either cells that have undergone a mutation of genes involved in the cell cycle,

or cells transformed by an oncogene, or cancer cells taken from an organism. Thus, as early as 1975, the fusion of rodent lymphocytes and myeloma cells allowed the creation of hybridomas and the production of monoclonal antibodies, which are mainly used today in diagnosis.

Gradually, in the 1980s, the use of these cells to produce therapeutic biomolecules was accepted by the regulatory authorities; the scaling up of culture processes to ensure a significant reduction in production costs was facilitated by obtaining (i) cell lines that were robust to agitated conditions and (ii) optimized culture media. The technology of cloning a gene coding for a protein of interest into an expression vector introduced into the cell has opened broad horizons for the production of customized recombinant proteins, making mammalian cells sophisticated platforms for the industrial production of these biomolecules. For example, in 1987, tissue plasminogen activator (tPA) was the first therapeutic molecule produced by Chinese Hamster Ovary (CHO) cells to be approved for marketing. Since these breakthrough innovations, the development and marketing authorizations of molecules produced by animal cells and in particular by mammalian cells have continued to rapidly increase (Figure 1). The market for recombinant proteins dedicated to therapeutic use, and especially that of monoclonal antibodies, is the major application sector driving the industrial use of animal cells. Monoclonal antibodies are used in the treatment of cancer, cardiovascular diseases, respiratory diseases, infections, ophthalmic or inflammatory diseases. They are currently finding new uses in the treatment of severe infections such as SARS-CoV2 (antibodies bamlanivimab and etesevimab).

The most common cell line is the CHO cell, which alone seems to account for approximately 75 % of recombinant protein production. In addition to mammalian cells, insect cells, infected with a baculovirus carrying the gene coding for the protein of interest, are also attractive tools for the transient production of proteins on a smaller scale. A list of the main mammalian cell lines and their recognized applications is given in Table 1.

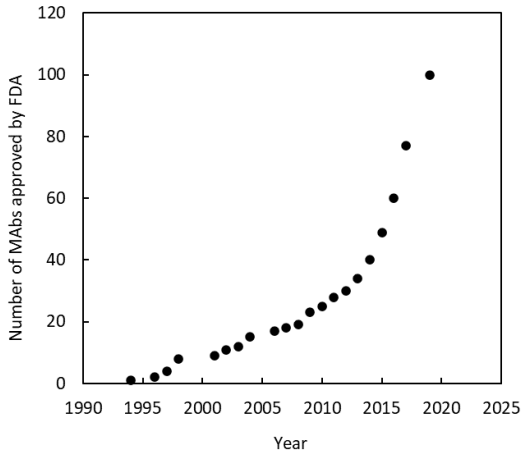

Figure 1. Number of monoclonal antibodies approved by FDA.

More recently, new lines of human origin are emerging for the production of adenoviral vectors or recombinant proteins (HEK293, PER.C6)[1]. They present a higher advantage as they allow the production of a non-immunogenic glycan. The main drawback is that they are susceptible to human virus contaminations requiring viral inactivations. HEK293 (Human Embryo Kidney 293) and its different variants, transfected with viral DNA, is the most prominent human cell line used for protein expression. Other cell-lines, including HT-1080 (human fribrosarcoma), CAP (from human amniocytes), PER.C6 (from human retinoblast), and HuH-7 (from human hepatoma) are used for the generation of factor VII, adenoviral vectors, immunoglobulins, or factor IX, respectively[2].

[1] O'Flaherty et al (2020). Biotech Adv, 107552.
[2] Dumont et al. (2015). Crit. Rev. Biotechnol. 18:1.

Table 1. Most common mammalian cell lines and related applications.

Cell	Biological source	Some common uses
CHO	Chinese Hamster Ovary cells	Recombinant proteins
VERO	Kidney epithelial cells extracted from an African green monkey	Human and veterinary viral vaccines (rotavirus, rabies, poliovirus)
BHK	Baby Hamster Kidney fibroblasts	Factor VIII, veterinary viral vaccines
HEK 293	Human embryonic kidney	Adenoviral vectors (SARS-CoV2)
Hybridomas	Murine hybrid cell line	Monoclonal antibodies
PER.C6	Derived from human embryonic retinal cells	Recombinant proteins, adenoviral vectors (SARS-CoV2)
NS0	Derived from the non-secreting murine myeloma	Recombinant proteins
MDCK	Madin-Darby canine kidney cells	Viral vaccines (flu)
MRC5	Human fetal lung fibroblast cells	Human viral vaccines (flu)

2.2. Human cells for the production of extracellular vesicles

Extracellular vesicles (EV), including exosomes, micro-vesicles, and apoptotic bodies display different sizes (from 30 nm for exosomes to 5 µm for apoptotic bodies), derived from most of cell types, and implicated in intercellular communication participating in physiological but also pathological processes like initiation and progression of tumor[3]. Among EV, exosomes are generated secondary to the invagination of the endosomal membrane, harboring a unique composition, and are further released from the cell by membrane fusion[4]. Exosomes have been described as micro-carriers releasing proteins and lipids, non-coding and coding RNAs, micro-RNAs (miRNA) for the regulation of biological processes, genomic DNA and transference of antigens or immunogenic molecules for the activation of T-cells during immune responses[5]. They exhibit many characteristics from the originated cells with which they share properties, making them relevant (i) as biomarkers for the diagnosis of a wide variety of health disorders and (ii) for cell-free therapeutics as they could present a better safety profile than the original cell therapy[6]. For example, they can be generated from cells interfering with immune response like dendritic cells, macrophages and MSC inducing either immunostimulating or immunomodulatory effects. Monocyte-derived-exosomes appear to be able to escape immune phagocytosis, allowing their increased circulation and efficacy compared to other exosome types[7]. Interestingly, they can also be derived from tumor cells, and they can elicit an anti-tumor cellular immune response and easily target the tumor due to their membrane homology with tumor cells. However, there are controversial reports showing that tumor cell-EV promote cancer progression through different mechanisms like induction of angiogenesis, escape to apoptosis, and intensification of tumor cell proliferation[3]….

Thereby, due to their high biocompatibility and ability to penetrate through biological barriers, their enhanced stability and limited immunogenicity, exosomes are very promising tools for targeted drug-delivery going beyond the limits of liposomes[3]. Many kinds of chemotherapeutics drugs have been

[3] Meng et al. (2020), Drug Delivery, 27 :585.
[4] Bunggulawa et al. (2018) J Nanobiotechnology 16:81
[5] Gurunathan et al. (2019) Cells 8 :307.
[6] Donoso-Quezada et al. (2020) Crit Rev Biotechnol 40:804.
[7] Luan et al. (2017). Acta Pharmacol Sin 38 :754.

loaded into exosomes like paclitaxel or doxorubicin, allowing an increased uptake by tumor cells compared to the liposomal form of the drug and even overcoming drug resistance mechanisms. The cargo can also be therapeutic RNA or proteins.

Despite the advantages and promising interests of EV, many challenges remain for bringing exosomes into the clinic[8]. First, cell source selection is a critical step depending on the targeted tissue and on the elucidation of potential deleterious effects, as mentioned previously for tumor cell-EV. Exosome generation from standardized cell lines would be of great interest. The scale-up of the culture systems of cells must take into account serum-free medium, stirred tank bioreactors. Second, the definition of a purification process for achieving high yield and purity is a limiting milestone. Ultracentrifugation is the well-known method for exosome isolation. Improvements are needed to reach a large-scale manufacturing of exosomes and new methods are currently emerging like size-exclusion chromatography, ciliated micropillars nano-traps, acoustic wave separation and flow field-flow fraction[9]. Third, specific quality controls (QC), including potency assays, have to be sought and developed. New technologies are necessary to allow an on-line fast QC. Finally, while overcoming those production concerns, a key issue will be to determine the optimal dose, the maximum tolerated dose, the minimum efficacy dose and the route of administration.

Different early phase clinical trials have been initiated and, for some of them, completed in different indications like tissue repair, cancer, Graft versus Host Disease (GVHD) or chemotherapy delivery, confirming the great interest for this promising therapy and that there is still a long way to move on and bring them to the bed side[9].

2.3. Induced Pluripotent stem cells: a cell factory for the generation of more mature derived cells

Induced pluripotent stem cells (iPSCs) were first obtained by Yamanaka and colleagues while reprogramming adult cells, transferring 4 limiting transcription factors (Oct3/4, Sox2, c-Myc, and Klf4)[10]. Compared to embryonic stem cells (ESC), iPSC could present molecular anomalies (epigenic traces of their somatic origin, genomic instability possibly due to the reprogramming process, or alteration of mitochondrial DNA) but also different advantages, mainly the capacity to be generated in an autologous setting avoiding immunological problem. Those pluripotent stem cells can then be differentiated in many types of cells (neurons, MSC, …). However, although the autologous setting is attractive to overcome immune conflicts, a large-scale production of stable and controlled iPSC lines is only possible in an allogeneic setting. Some iPSC banks for research or clinical trials are currently developed in different countries but many challenges remain to meet the medical needs[11]: (i) overcoming immune rejection is an issue by developing large HLA typed banks to allow HLA matching with the recipient or by gene editing, (ii), implementing QC to ensure safety and efficiency of cells (especially genomic sequencing). Finally, although iPSCs are considered devoid of ethical problems compared to embryonic stem cells, there are currently raising ethics proceedings in France considering iPSC could be potentially differentiated into gametes (Ethic law in project).

[8] Song et al. (2020) Am J Reprod Immunol 85:e13329.
[9] Willis et al. (2017) Front Cardiovasc *Med* 4 :63.
[10] Takahashi and Yamanaka et al. (2006). Cell 126 :663.
[11] Umekage et al. (2019). Inflamm Regen 39:17.

2.4. MSC: a cell factory producing soluble factors depending to the inflammatory environment

Discovered in 1970s in bone marrow (BM), Mesenchymal Stromal/Stem Cells (MSC) have since been found in multiple tissues like adipose tissue, dental pulp, menstrual blood or extra-embryonic source such as Wharton's Jelly (WJ) or placenta[12]. In 2006, the International Society for Cellular Therapy (ISCT) defined Mesenchymal stem/stromal cells as (1) adherent to the cell culture plastic; (2) able to differentiate into osteocytes, chondrocytes, and adipocytes; (3) with a phenotype that is positive for the CD73 CD90 CD105 mesenchymal markers and negative for the CD34 CD45 HLA-DR hematopoietic markers (MSC can modulate both innate and adaptive immunity by cell contacts, secretion of cytokines, chemokines, growth factors, and release of extracellular vesicles[13]. Briefly, immune properties of MSC rely on two main mechanisms: a direct interaction of MSC with immune cells and a paracrine effect of MSC. The main factors expressed by MSC in response to immune cell interactions are: microRNAs, PD-L1, HLA-G, Prostaglandin E2 (PGE2), cytokines (transforming growth factor-β (TGFβ), Interleukin (IL)6, IL10, HGF, VEGF…), and one enzyme (Indoleamine 2,3–dioxygenase: IDO). Immune properties of adult MSC have been widely reported[14]. Two MSC phenotypes - immunomodulatory or immunostimulatory- depending on the inflammatory context and the stimulation of their TLR were described[15]. In a cell anergy context, they could exhibit a pro-inflammatory phenotype, MSC1, making it possible to reduce apoptosis and promote T-cell survival. On the other hand, in case of inflammation, they could adopt an immunosuppressive and anti-inflammatory phenotype, called MSC2. This dual phenotype according to environment brings new opportunities for therapeutic uses. However, these immunomodulatory cells are slightly impacted by the host immune system (lack of expression of MHC class II antigens and CD80/CD86 costimulatory molecules, weak expression of MHC class I antigens) allowing there use in an allogeneic setting without considering HLA compatibility. Current data suggest also that MSC exert strong antimicrobial effects both indirectly, across their role in the host immune response against pathogens and directly, by the secretion of antimicrobial peptides and proteins (AMPs), but also by the expression of molecules such as IDO and IL17[16].

Up to now, more than 800 clinical trials have been performed with MSC from different sources in various indications, highlighting their safety and, in some of high phase clinical trials, their efficacy. Market authorization has been obtained in USA and Europe in GVHD treatment or in Crohn's disease fistulas.

In an allogeneic setting, high numbers of MSC have to be generated. In academic laboratories, MSC production is mainly performed in 2-dimension (2D) culture flasks which is time consuming and yields not compatible with the medical need. Currently, industrialization of 2D models begins with the marketing of bioreactors. Bioreactor increases cell culture surface and limits steric hindrance. A more versatile and scalable technology of culture has also arisen last recent years: the 3-dimensional model, consisting in using microcarriers as adherence support, in stirred tank bioreactors, while reducing the operating cost of the process. Regardless of differences due to MSC source, different challenges remain: (i) the recovery of MSC from microcarriers to achieve a good yield, (ii) the maximum volume of culture while keeping stable MSC characteristics (most of the cultures in stirred

[12] Friedenstein et al. (1970). J Embryology Exp Morphology 16:381.
[13] Dominici et al. (2016). Cytotherapy 8 :315.
[14] Laroye et al. (2017). Stem Cells 12 :2331.
[15] Waterman et al. (2010). PLoS One 5 :10088.
[16] Laroye et al. (2019). Stem Cell Res Ther 10:192.

bioreactors operate today at a scale of few liters [17,18] (iii) and on-line monitoring of culture parameters.

As previously mentioned, exosomes are generated from MSC as they exhibit anti-inflammatory and immunomodulatory capacities and would allow a cell-free therapy. However, although the production of standardized homogeneous batches would be an improvement compared to whole MSC, the main advantage of the whole cells would be lost. The strength of MSC relies on their main drawback: to be a "living drug". The use of EV instead of cells induces the loss of adaptability capacities (dual phenotype MSC1 and MSC2). In multifactorial immune pathologies with concomitant inflammation and lymphopenia as described in sepsis, could it be possible to do without this main benefit?

2.5. Adoptive T cell immunotherapy: T cell factories allowing in vivo cell expansion and differentiation into cytotoxic T cells

The recovery of virus-specific T-cell immunity is crucial for patients after hematopoietic stem cell transplantation (HSCT) to avoid viral infections or reactivations. Based on donor lymphocyte infusions experiments, *ex vivo* virus-specific T cells (VST) were generated for patients with viral infection, refractory to anti-viral drugs[19]. VST can be expanded *ex-vivo* but also isolated *in vitro* from a donor leukapheresis using an immunomagnetic separation system (Miltenyi Biotec). In this last situation, a very small number of isolated specific T cell is obtained and infused into the patient, requiring an *in vivo* expansion when T cells interact with antigen-presenting-cell presenting viral peptides. As mentioned before, such VST are generated for a dedicated patient. Thus, large scale manufacturing is not an issue. The Prodigy platform, a closed, automated system, compliant with GMP guidelines developed by Miltenyi Biotec, allows bringing this medicine to the bed side. On a clinical point of view, randomized controlled studies are still missing to conclude on efficacy and safety of these allogeneic VST. Such a phase III clinical trial is currently including patients on a European scale (sponsor: Munich university).

2.6. Comparison with other expression systems

Today, most of recombinant glycoproteins produced by biotechnology are produced in mammalian cells. Other expression systems can be used, such as bacteria (Escherichia coli), yeast (Pichia pastoris, Saccharomyces cerevisiae), insect cells (Sf9, Sf21, BTI 5B1-4) or plants. These systems have clear advantages as they allow higher product concentrations; their production processes are considered as more easily scalable with reduced production costs (particularly in terms of culture media costs) compared to mammalian cell production processes. However, they lack the ability to implement complete post-translational modifications of the protein, notably glycosylation. Thus, their use is generally restricted to the production of small proteins or to the vaccine industry. Notable examples are the production of human insulin by the yeast *Pichia Pastoris*, the production of pseudo-viral particles for the design of the vaccine against the Papilloma virus (GARDASIL9) by *Saccharomyces cerevisiae* or viral vectors by Sf9 insect cells. The main advantages and disadvantages of mammalian and non-mammalian cells as expression systems are reported in Table 2.

[17] Ferrari et al. (2014). Appl. Biochem. Biotechnol 172:1004.
[18] Sion, C. et al. (2020). Biochem Eng J, 157, 107521.
[19] Qian et al. (2018). Bone Marrow Transplant 53:114.

Table 2. Advantages and drawbacks of mammalian and non-mammalian expression systems adapted from O'Flaherty et al. (2020)[20].

Cell source	Advantages	Drawbacks
Mammalian (human or animal)	Excretion of proteins; growth in suspension or adhered on microcarriers in mixed bioreactors; scale-up robustness	Risk of viral contaminations; high culture costs; moderate yields and cell concentrations (except in perfused or optimized fed-batch modes of culture)
Non-mammalian (yeasts, bacteria, plant, insect)	Higher yields; fast growth; less risk of viral contaminations; lower production costs; scale-up robustness	Partial post-translational modifications of therapeutic proteins; risk of protein aggregation and inclusion body formation

3. Industrial processes at the service of cell factories

A production facility needs to interact with its socio-economic environment, with infrastructures allowing the routing of raw materials and people, and the management of flows leaving the factory (waste, products). The optimization of internal flows (communication, goods, etc.) is also one of the keys to the efficient running of the production means. Like an industrial plant, the animal cell can be described as a "factory" not so much because of the production it generates but essentially because of the complexity of the extra- and intracellular exchanges and interactions observed. To describe this complexity of flows, modelling tools are used, such as metabolic modelling, which represents the cell factory in the form of a "road map" and quantifies the distribution of flows within this mesh. For instance, a recent study modelled a CHO cell with 2101 genes and 4527 metabolites interconnected by 7436 reactions[21] ! Even if, in the near future, we can hope that this type of model will be used to control the production process, the approach favored in recent years by the engineering sciences to size and optimize culture processes is a systemic approach, which incorporates the intracellular machinery without precisely describing it. Thus, the determination of optimal culture conditions is based on the empirical or semi-empirical control of extracellular environments (that we will call the inlets of the cell factory) promoting the targeted performances of the cell (that we will call the outflows). By extension, the successful scale-up of animal cell culture processes to an industrial scale implies tacitly, but resolutely, that the maintenance of bioproduction performance from one scale to another can be guaranteed by the conservation of the input flows into the cell factory (the same causes promoting the same effects), provided that the intrinsic biological characteristics of the cell are also maintained (Figure 2). With the progress of knowledge and modelling tools, this systemic approach is progressively moving towards a multi-scale modelling that integrates all the length and time scales of the production process: metabolites/reactions, cell/physiological state, cell populations, bioreactor/hydrodynamics, process/pilot (see paragraph 4).

[20] O'Flaherty et al. (2020). Biotech Adv, 107552.

[21] Fouladiha et al. (2021). Biotech Letters, 43(1), 73-87.

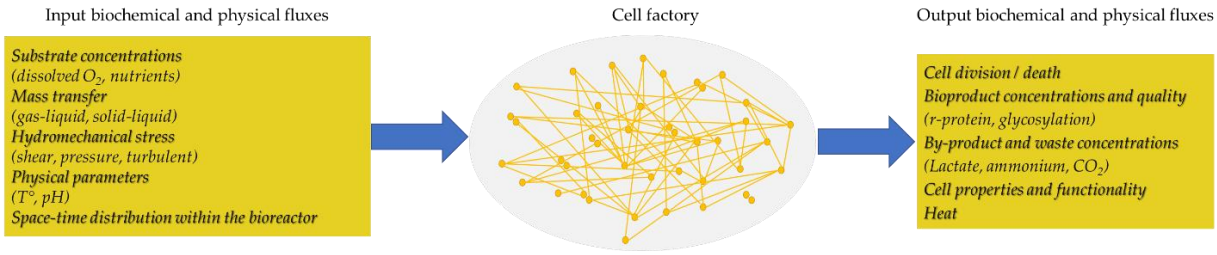

Figure 2. The concept of cell factory: the key integrated scale for process optimization.

Controlling the microenvironments of the cell factory relies on the deployment of experimental and numerical methodologies that will aim to (i) strengthen the understanding of cell/environment couplings and (ii) predict, in a sufficiently anticipated manner, the temporal evolutions of cellular responses, knowing the dynamics of these at a given time. Among the challenges taken up or to be taken up, let us mention in particular:

Control and optimization of substrate and nutrient concentrations. To achieve this goal, the culture processes are or should be based on the use of culture media, if possible, chemically defined, the composition of which will have been optimized during preliminary screening phases; this strategy makes it possible, in particular, to eliminate possible batch-to-batch variability in the culture medium. While the use of such media is gradually standardized for the industrial production of recombinant proteins (for these applications the use of compounds of plant origin or recombinant compounds in place of animal or human sources is also very frequent), the addition of compounds of animal or human origin (fetal calf serum, human platelet lysate, growth factors) remains classic for the culture of primary human cells (MSC). However, as the kinetics of nutrient consumption or generation of metabolic products modifies this initial biochemical environment, it is also necessary to have methods for measuring, as far as possible "on-line", the concentrations of substrates and metabolites. Thus, the 2010s have seen the emergence and concretization of spectroscopic sensor technologies implemented in bioreactors (dielectric, RAMAN, near infrared, etc.) including on industrial production lines. Once combined, these sensors can not only measure in real time the concentrations of glucose, lactate, and glutamine but also the concentration and quality of the monoclonal antibody produced[18]. They are also required to improve the performance of fed-batch or continuous-perfused modes of culture.

Knowledge of hydromechanical environments. Most of the time, the use of agitated bioreactors imposes a turbulent, multiphase (gas-liquid, liquid-solid or gas-liquid-solid) hydrodynamics, notably characterized by its temporal variability and spatial heterogeneity. The description of the local hydrodynamics imposed on the cells during their culture makes it possible to anticipate the appearance of cellular damage in case of too strong agitation, to predict modifications in the size of cellular aggregates, or to generate mechanical environments suitable for the differentiation of stem cells, for example[18]. It can also contribute to a better understanding of the phenomena of mixing and mass transfer (oxygenation, desorption of CO_2) or even to optimize bioreactor designs for a targeted application[22]. However, it requires the deployment of sophisticated experimental and numerical methodologies (numerical fluid mechanics, particle image velocimetry) which still have limitations in the case of complex multiphase flows. To overcome these limitations, the use of macroscopic scale-up criteria (mechanical power, mean Kolmogorov scale, volumetric oxygen transfer coefficient, etc.)

[22] Loubière, C. et al. (2019). Chem Eng and Technol, 42(8), 1702-1708.

makes it possible to maintain the hydromechanical stresses within acceptable limits or to dissolve the oxygen at a controlled rate.

4. Strengthen (further!) the interactions between the cell factory and the culture processes

The versatility and industrial operability of continuous cell lines or primary cells will be all the more strengthened that the constraints of cell factory operation are integrated early into the culture process optimization strategies and/or by using advanced modelling approaches. Thus, the prediction of the evolution of the physical cell environments during the transition to an industrial production scale (mechanical constraints, concentration heterogeneity) must be carried out during the process development phases, using mechanistic simulation approaches (CFD, compartments) and, if necessary, dedicated scale-down methodologies (two-stage systems, bioreactor screening).

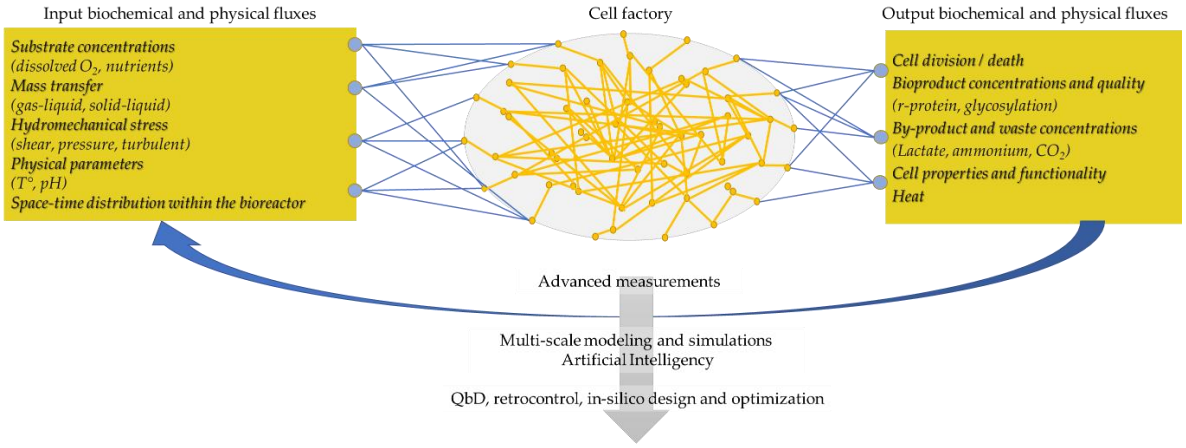

Figure 3. Towards in-silico design and optimization of the cell factory microenvironments.

The construction of predictive multiscale models, helping not only to predict cell performance under given culture conditions but also to optimize them, is currently underway and should be strengthened in the coming years (Figure 3). It requires the use of online measurement technologies to characterize cell dynamics and predict the actions needed to maintain cell productivity through implementation of optimal control strategies. On-line monitoring of cell functionality is also an important challenge to be met in the coming years to allow the application of these advanced understanding approaches. In this general framework of Quality By Design applied to animal and human cell cultures[23], the contribution of Artificial Intelligence and in particular Deep Learning by Neural Networks, numerical simulation of flows coupled to cell kinetics and new generations of on-line sensors will be essential to "close the loop".

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[23] Maillot et al. (2021). Biotechnol Adv, doi.org/10.1016/j.biotechadv.2021.107765.