Journal of Pharmaceutical and Biomedical Analysis 246 (2024) 116182

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Emerging technologies for quality control of cell-based, advanced therapy medicinal products

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ARTICLE INFO

Keywords:
Advanced therapy medicinal products (ATMPs)
Quality control (QC) of ATMPs
Microfluidic tools for QC of ATMPs
Quality-by-design (QBD)
Machine learning and AI

ABSTRACT

Advanced therapy medicinal products (ATMP) are complex medicines based on gene therapy, somatic cell therapy, and tissue engineering. These products are rapidly arising as novel and promising therapies for a wide range of different clinical applications. The process for the development of well-established ATMPs is challenging. Many issues must be considered from raw material, manufacturing, safety, and pricing to assure the quality of ATMPs and their implementation as innovative therapeutic tools. Among ATMPs, cell-based ATMPs are drugs altogether. As for standard drugs, technologies for quality control, and non-invasive isolation and production of cell-based ATMPs are then needed to ensure their rapidly expanding applications and ameliorate safety and standardization of cell production. In this review, emerging approaches and technologies for quality control of innovative cell-based ATMPs are described. Among new techniques, microfluid-based systems show advantages related to their miniaturization, easy implementation in analytical process and automation which allow for the standardization of the final product.

1. Introduction

1.1. ATMPs cell-based products

ATMPs offer personalized therapies to treat a wide range of different clinical applications like cancer, genetic disorders, and cardiovascular diseases [1] Cutting-edge technologies such as gene therapy, somatic cell therapy, and tissue engineering are used to generate different types of ATMPs. They include combined ATMPs, which are a combination of ATMPs and medical devices. ATMPs for gene therapy consist of a recombinant nucleic acid able to adjust, repair, replace, or delete a genetic sequence for genetic diseases. Somatic cell therapy is used to treat, prevent, or diagnose diseases. Somatic cell ATMPs involve manipulated cells or tissues with specific biological characteristics before being transferred to the patient. Unlike traditional transplant cells or tissues, which serve the same function in both the donor and the patient, cell-based ATMPs have a different biological function in the patient due to their manipulation. Tissue-engineered medicinal products consist of manipulated cells or tissues to repair, regenerate, or replace human tissues.

Cells or tissues for ATMPs can be classified based on their origin. They can be autologous, when derived from the patient, allogeneic, obtained from a donor, or xenogenic when derived from a donor of an animal species other than man. In cell-based ATMPs, blood-derived cells such as T-lymphocytes, B-lymphocytes, Dendritic cells (DC), and Natural Killer (NK) cells, are generally used in therapies, whereas non-blood cells, mainly stem cells, are used in tissue engineering products. Autologous or allogeneic adult cells, stem cells, and iPSC (Induced pluripotent stem cells) are the major sources employed in tissue engineering. Examples of established or highly developed products include Tumor Infiltrating-lymphocyte (TIL), Chimeric Antigen Receptor T-cell (CAR-T), and Engineered T-cell Receptor (TCR) therapy. Recently, several CAR T-cell therapies have been approved by the FDA and EMA (European Medicines Agency) for treating patients with lymphoma or leukaemia [2–4]. CAR-T technology consists of patient T lymphocytes modified by adding a gene encoding a specific receptor. This

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https://doi.org/10.1016/j.jpba.2024.116182
Received 30 November 2023; Received in revised form 22 April 2024; Accepted 25 April 2024
Available online 29 April 2024
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modification enables CAR-T to bind the specific antigens presented on the surface of the tumour cells and destroy malignant target cells.

In tissue engineering, ATMPs are created by combining cells with biological scaffolds and possibly adding biologically active molecules. Currently, there are two approved examples of these ATMPs: artificial cartilage and skin. Spherox® (CO.DON GmbH) is a medical product based on spheroids of chondrocytes recovered from the healthy cartilage of a patient's tissues. It is a tissue-engineered cartilage based on autologous chondrocytes combined with biomimetic cartilage scaffolds. It was developed to repair damaged cartilage in patients with symptoms (such as pain and problems moving the knee) and with affected areas smaller than 10 cm^2. The safety profile was considered acceptable by EMA and Spherox received a marketing authorization valid throughout the EU in 2017 [5]. The biotechnology company VERIGRAFT (Gothenburg, Sweden) developed the personalized tissue-engineered vein (P-TEV) for the treatment of patients with severe Chronic Venous Insufficiency (CVI) [6]. Currently, no synthetic products could replace the function of the bicuspid valves in the leg veins, and the risks associated with allotransplantation are too high. Through decellularization, donor cells and DNA are removed from a donated tissue resulting in a clean extracellular matrix scaffold. Subsequently, this scaffold is seeded with the patient́s cells derived from peripheral blood. The P-TEV protocols of decellularization and reconditioning have been used to produce porcine individualized tissue-engineered grafts now in phase I of clinical testing.

Among adult stem cells, mesenchymal stem cells (MSC) can be isolated from several adult tissues (primarily from bone marrow and adipose tissue) and one of the characteristics is their ability to differentiate mainly into osteogenic, chondrogenic, and adipogenic lineage, which makes them valuable in the healing process of damaged tissue [7]. Furthermore, the popularity of MSC-based therapy stems from their low immunogenicity and ability to regulate and suppress the immune system. Acceleration of MSC-based therapies and their standardization can be hastened by improving cultivation methods using xeno- and serum-free conditions, which results suitable for large-scale expansion, and evaluation of MSC from different sources. These actions can reduce variability among pre-clinical and clinical protocols [8] In 2018 the EU approved Alofisel® (Takeda Pharmaceutical Company Limited, Tokyo, Japan), an allogenic mesenchymal cell therapy for the treatment of anal fistula in Chron's patients. Stem cells are extracted from the adipose tissue of healthy donors, and they are used when fistulas have shown an inadequate response to at least one conventional therapy [9].

The first tissue engineering product approved by the EMA is Holoclar® (Holostem Terapie Avanzate S.r.l., Italy), a stem-cell treatment to replace damaged cells on the surface (epithelium) of the cornea. Autologous cells from the limbus are taken, and after a growing step on scaffolds to form corneal cells, they are implanted into the patient's eye [10].

1.2. *Development of ATMPs cell-based products: regulatory issues*

The development of ATMPs has rapidly grown since the 2000 s, and the number of treatments approved by the EMA has increased, mostly targeting orphan disease indications [11]. Based on orphan designation product reports until 2022, a total of fifteen ATMPs were approved in the EU for different clinical indications, whereas in the US, a total of nine therapies were approved (Fig. 1).

Although there is a high rate of innovation for ATMP products, only a small number have been approved and made it to the market. The scale-up of ATMPs presents various challenges, from manufacturing and patient safety to determining pricing. Additionally, the whole regulatory procedure have several obstacles [12]. The most important regulatory agencies responsible for the evaluation of medicines for human use are the EMA in the European Union, the Food and Drug Administration (FDA) in the USA, and Ministry of Health, Labor and Welfare (MHLW) in Japan. They cooperate through the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) to develop guidelines for the quality, efficacy, and safety of ATMPs. In the EU the procedure for ATMPs' market access requires the evaluation by the EMA Committee for Advanced Therapies (CAT). The EMA has proposed various strategies for faster access to innovative ATMPs. The EMA's Priority Medicines voluntary scheme (PRIME) offers support to medicines aimed at targeting unmet medical needs. Recently, authorized CAR T therapies, such as Yescarta® (GILEAD SCIENCES CANADA INC, Canada) and Kymriah® (Novartis Pharmaceuticals Corporation, New Jersey), have benefited from this accelerated evaluation procedure.

Typically, the Health Technology Assessment (HTA) body must wait

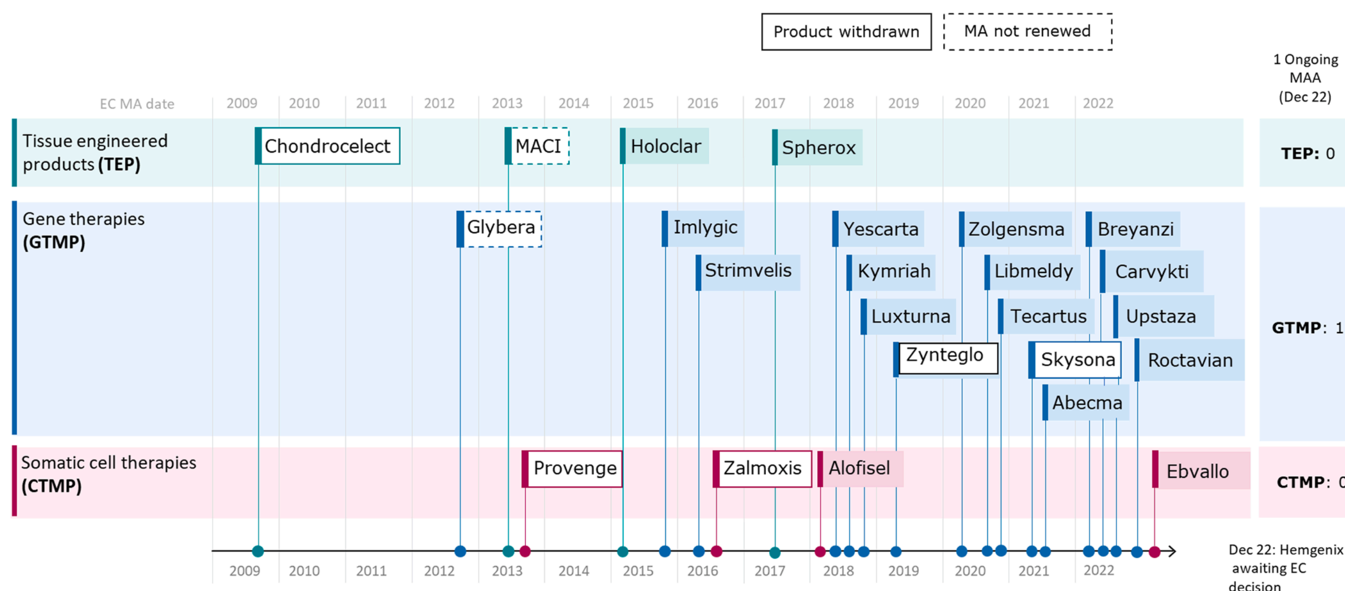

Fig. 1. Approved ATMPs from 2022. Abbreviations: ATMP: advanced therapy medicinal product; GTMP: gene therapy medicinal product; CTMP: cell therapy medicinal product; TEP: tissue engineered product; MA: Marketing authorisation. White box equal to MA withdrawn, dotted box equal to MA not renewed (From EMA/CAT/50775/2023. European Medicines Agency, January 2023, https://www.ema.europa.eu/en/documents/report/cat-quarterly-highlights-approved-atmps-january-2023_en.pdf, October 2023).

for the EMA opinion before assessing the risk/benefit of a therapy, which is the foundation for price negotiations. However, consultations between manufacturers, regulators, HTA bodies, and health insurers are already organized to define the therapy's development plan. In the development plan for ATMP products, the quality control (QC) system is essential to guarantee the quality and safety required of the finished product. QC includes strategies to assess the quality of raw and starting materials, intermediates, and finished products to comply with the requirements of good manufacturing practice (GMP) standards. It also must measure the stability and microbiology monitoring of production areas, instrumentation, and personnel [13–15].

Due to the great complexity and diversity of ATMPs, specific characterization tests are necessary for each individual product. Both European and American regulations require tests to confirm sterility, the identity of the cellular and non-cellular components, purity, viability, potency, and reproducibility. In addition, characterization, stability, and release testing must be conducted, requiring orthogonal analytical techniques able to deal with limited sample volumes in accordance with the best practices [14]. To ensure the safety and efficacy of the final product, tests are conducted to assess sterility, endotoxin levels, and mycoplasma contamination. Additionally, the identity and potency of the cells are evaluated through measures such as cell count, immunophenotype, and clonogenic assay. The presence of undesirable contaminants such as differentiated or senescent cells, non-cellular impurities, and cell debris should be minimized as they can negatively impact the function of the cell product. Cell viability, which is directly correlated to biological activity, is crucial for the efficacy and integrity of cell-based products. Live cells should constitute at least 70% of the products, and dead cells should be eliminated. Nowadays, the use of appropriate markers to identify membrane proteins is fundamental to standardize and validate cell isolation procedure and understand sample heterogeneity. Moreover, one of the requirements is the quantity of cell products suitable for cell therapy. Therefore, cells are expanded and successively cryopreserved until their use. The viability, purity, and homogeneity of cells must be validated also after thawing [16,17]. Automation based on standard operation procedure (SOP) is a strategy to maximize reproducibility, reduce costs, avoid labour-intensive procedures, and limit the generated errors. Automated cell culture systems are available and QC platforms, especially miniaturized QC, are used in cell factories [18]. These technologies show interesting advantages such as the reduction of manual steps and therefore costs generated using media and reagents and specific requirements.

Commercialization of ATMPs is a challenging task. The manufacturing process should be developed based on a comprehensive characterization of the ATMP at every stage of the development and production processes. Therefore, innovative manufacturing technologies and analytical tools are necessary for cell-based ATMPs to meet regulatory standards and ensure the required level of quality. Advancements in cell characterization technologies are happening quickly. This allows for rapid analysis, and it creates the opportunity to integrate lab-on-a-chip, sample miniaturization, multiparametric analysis, and high-content technologies (e.g. single-cell technologies) into the manufacturing process. New microfluidic tools and novel QC approaches could improve the safety and commercialization of ATMPs, and they will be discussed in this review.

2. Emerging microfluidic tools

The field of microfluidics is a relatively new area that combines technologies and principles from various domains, including chemistry, physics, biology, material science, and fluid dynamics [19]. Advanced microfluidic tools are increasingly being used to tackle current challenges in precision medicine and cancer research, as well as in the development of more predictive diagnostic assays [20,21]. According to George M. Whitesides, microfluidics is the science and technology of systems that process or manipulate small amounts of fluids (ranging from 10−9 to 10−18 litres) using channels with dimensions of tens to hundreds of micrometres [22]. Microfluidic systems operate with fluid samples and suspension of cells, and their high adaptability offers a useful tool for a vast range of applications.

Thanks to their versatility, microfluidic systems have emerged as a fundamental tool in GMP-compliant platforms, bioanalytical procedures, and the manufacturing of ATMPs. These systems offer numerous advantages, such as precise control over cell manipulation, scalable manufacturing, and reduced contamination risks. Microfluidic platforms enhance product quality and reproducibility, which are critical requirements of regulatory compliance, by supporting the generation of uniform cell populations. For instance, microfluidic devices are used for the isolation of white blood cells (WBCs) from blood [23] (Fig. 2A), T lymphocyte enrichment [24,25] (Figs. 2B, 2C), as well as for the delivery of the CAR gene and T cell activation [26] in CAR T cell-based therapies. Although these devices are miniaturized equipment and can be used for small quantities of cells, they are highly effective and efficient in their applications.

The unique architecture of these systems allows them to mimic the natural environment, which leads to improved cell viability and functionality. They are self-contained and offer precise control over the cellular microenvironment, making them ideal for uncovering how the environment affects cellular behaviour. Moreover, microfluidic systems can combine multiple processes, making production more efficient and reducing costs. Consequently, these advanced systems play a crucial role in the safe, compliant, and efficient manufacturing of cell therapy [27–29]. We will discuss the current understanding of microfluidic devices and their use in biomedical applications. Specifically, we will focus on microfluidic chips, devices designed for sorting cells, and tools for 3D culture and analysis of 3D structures.

2.1. Microfluidic chips

The use of microfluidic chips is revolutionizing medical research by allowing scientists to study individual cells in a precisely controlled environment. These compact devices offer fluid flows, precise separation, isolation, and in-depth cell analysis, making them an essential tool for medical advancements. One of the most revolutionary types of microfluidic chips is the Organs-on-chips (OoCs), which contain miniature tissues and a microfluidic network on a single chip. OoCs are in vitro microfluidic cell culture devices that recreate the miniaturized functional units of various organs, such as the lung, intestine, or neural networks. These highly advanced systems combine tissue engineering and microfabrication, providing a more reliable model for human pathophysiology and the development of new therapeutic approaches. [30]

Scientists have developed various microfluidic platforms for both 2D and 3D culture methods to meet the growing demands of biomedical research and cell therapy. Kim et al. successfully created a human Blood Brain Barrier (BBB)-like microvasculature using an angiogenesis microfluidic chip composed of human brain microvascular endothelial cells (Fig. 3 A). This in vitro model of stroke aimed to study the role of human pericytes and human astrocytes. Their research found that the infusion of human bone MSCs proved effective as perivascular pericytes in tight BBB reformation. These stem cells had a better vessel-constrictive capacity than that of pericytes, which provided evidence of reparative stem cells on BBB repair rather than a paracrine effect. [31]

As previously mentioned, one important aspect of evaluating the quality of stem cell therapy is ensuring that the cells retain their potency. However, traditional 2D culture systems have limitations when it comes to testing the potency of MSCs. A study by Schneider et al. [32] demonstrated the use of a low-cost microfluidic system that better mimics the physiological response of MSCs to IFN-γ, which produced more accurate results than the 2D culture method (Fig. 3C). Another study by Mykuliak et al. compared the vasculogenic potency of bone marrow MSCs to adipose derived MSCs using an OoC[33] (Fig. 3B). The

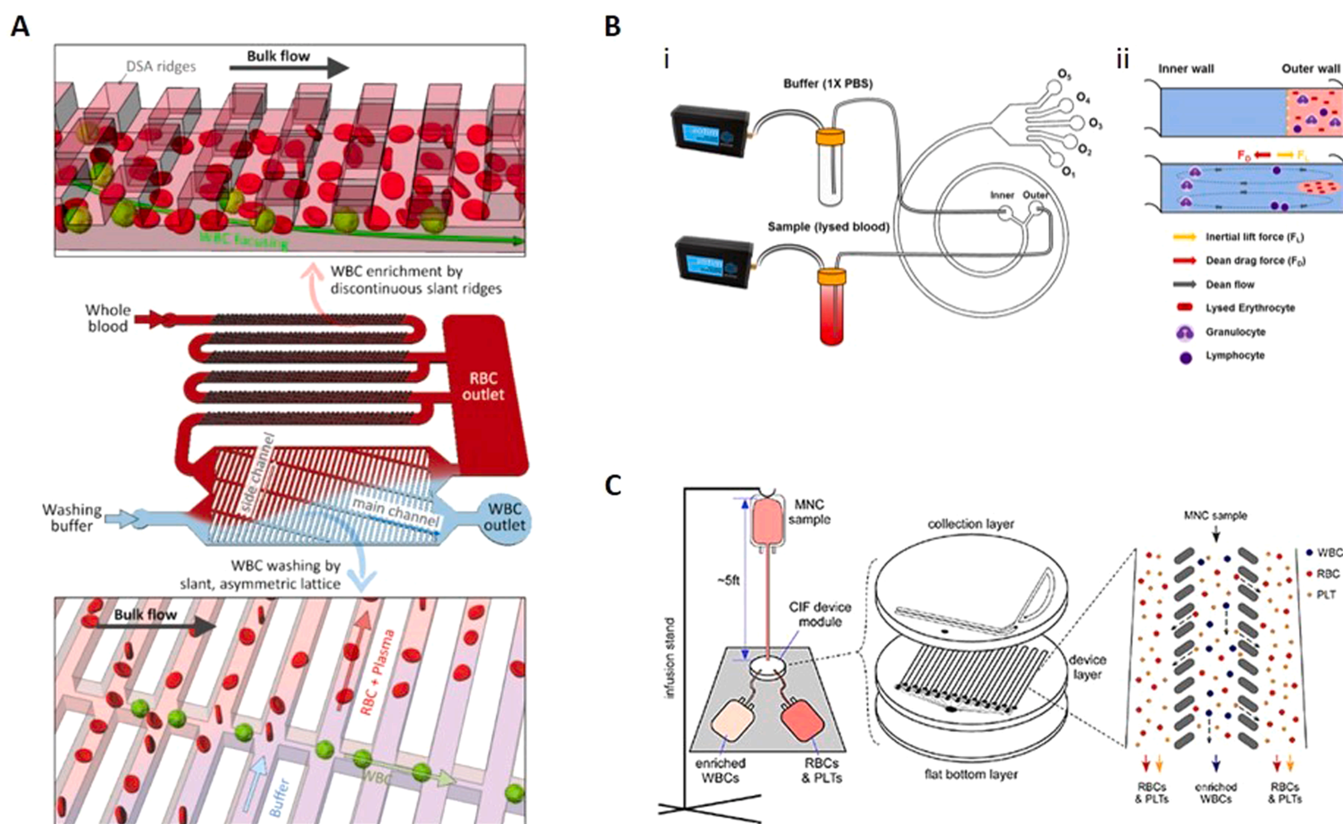

Fig. 2. Example of microfluidic tools. **A)** The microfluidic device efficiently purifies WBCs with high purity. DSA ridges enrich WBCs while slowing blood flow, producing a concentrated stream. SA lattice washes WBCs with a fresh buffer stream, removing residual RBCs and blood plasma while reducing the flow rate. *Reprinted with permission from [22]* ***B)*** *Design of CIF-based microfluidic devices enhances removal of RBCs. Schematic illustration of the revised CIF design, with posts slanted at 35. Reprinted with permission from [23]* ***C)*** *Illustration of the (a) setup and (b) concept of spiral microfluidic channels for isolating human PBLs rapidly and safely. Reprinted with permission from [24].*

bone marrow MSCs induced the formation of fully perfusable microvasculature with larger vessel area and length, while adipose cells resulted in partially perfusable microvascular networks. Furthermore, co-culturing with BMSCs led to significantly higher expression levels of genes specific to endothelial and pericyte cells, as well as genes involved in vasculature maturation.

2.2. Microfluidic tools for 3D cell culture

Moving from 2D to 3D cultures leads to significant changes in cellular growth behaviours, such as alterations in shape, architecture, and cell-cell adhesion organization due to variations in microenvironments. However, the introduction of microfluidic techniques in 3D cell culture greatly improves the co-culture of cells in a spatially controlled manner, more accurately representing tissue and organ organization, and enabling the generation and control of gradients and nutrient exchange [34]. Although 3D culture techniques are increasingly used in biomedical research, they are not yet able to fully capture the complexity of multicellular tissues, including vascularization, and lack proper control of gradients. Additionally, contrary to what happens in physiological conditions, traditional 3D cultures only undergo medium exchange at specific time points instead of continuously limiting the availability of nutrients and small molecules.

To effectively study drug metabolism and toxicity, as well as facilitate cancer research [35] and regenerative medicine, it is increasingly important to create stratified (co-)cultures with basal–apical access, gradient formation, and medium perfusion, utilizing cell patterning and extracellular microenvironment. There is therefore a high demand for microfluidic tools that can better simulate the in vivo environment. This opens opportunities for developing new devices that can analyze 3D structures over time with minimal manipulation. By directly measuring cell weight and mass density with microfluidic-based instruments, we can improve the monitoring of cell responses to external stimuli, which reduces the limitations of many imaging techniques. Changes in physical parameters during culture, such as the loss of density, could indicate cell damage and senescence. Therefore, we need increasingly accurate microfluidic devices that can monitor the physical characteristics of 3D models for better follow-up. Shin and Kim described a protocol for the culture of Caco-2 or intestinal organoid epithelial cells on microfluidic tools, for the establishment of functional intestinal microarchitecture. In particular, they developed a gut-on-a-chip device composed by two parallel microchannels and an elastic porous membrane in the middle, allowing to create a lumen–capillary interface. They also set-up the 3D culture using a hybrid chip, consisting of a single channel microfluidic device, that offers continuous basolateral flow below a polarized epithelial layer grown on a Transwell insert. Both these devices enables the regeneration of functional intestinal microarchitecture, with a control of the basolateral fluid flow, physiologically relevant shear stress and mechanical motions [36].

In the study conducted by Ahn and colleagues, a bone-mimetic microenvironment was developed using a microfluidic platform composed of hydroxyapatite and fibrin, in order to study the interaction between cells and tumour microenvironment in a 3D set up. Cancer cells, as well as fibroblast and endothelial cells were grown on the microfluidic device, to recreate an in vivo like tumour microenvironment. The result obtained indicated that the viability, proliferation and morphology of cancer cells was strongly influenced by the tumor microenvironment and by the hydroxyapatite concentration, and

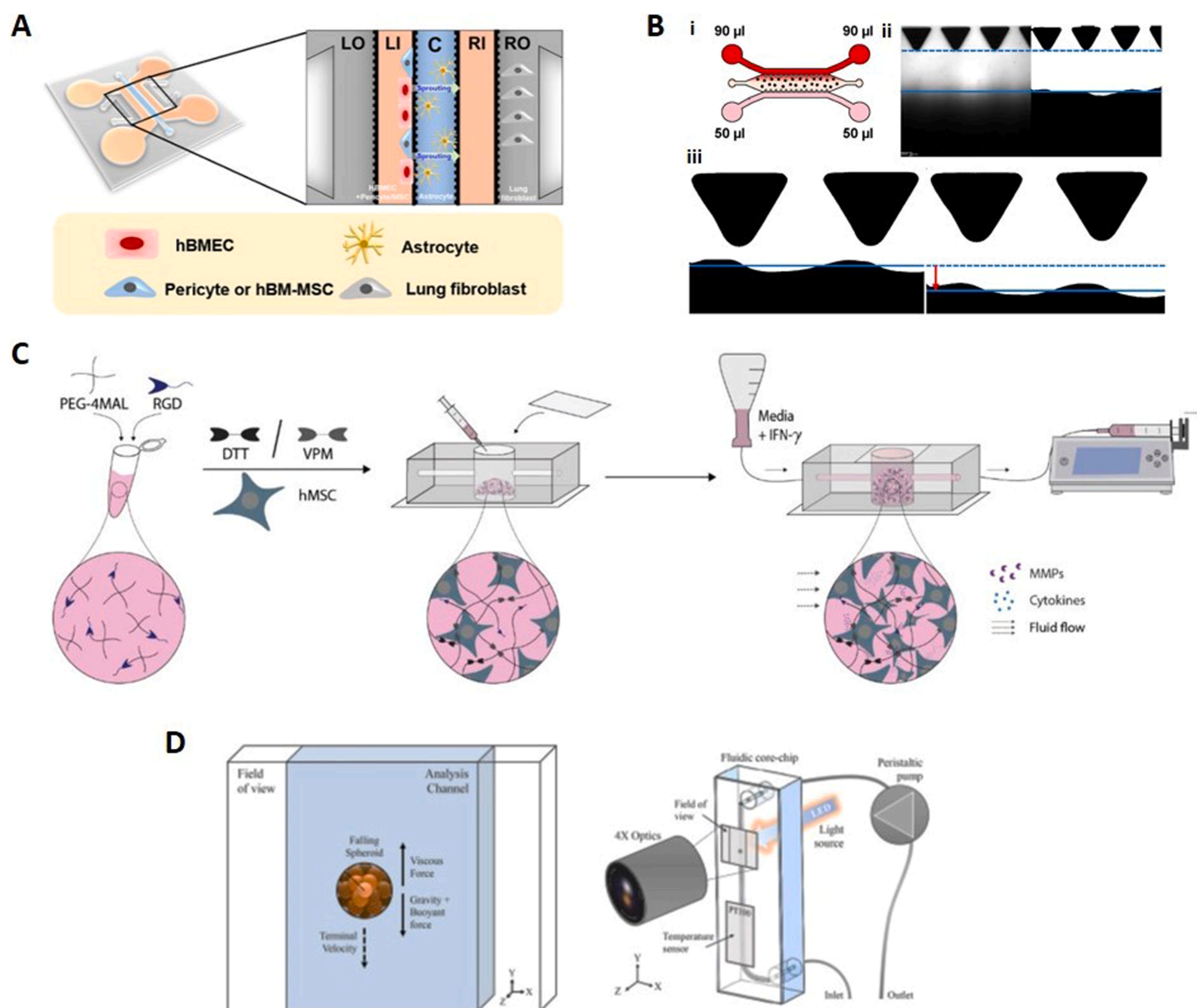

Fig. 3. Example of microfluidic chip and instrument for cell sorting. A) Reconstruction of human BBB-like microvasculature on an angiogenesis microfluidic chip and comparison of the role of each cell on the capillary network using two-colour live-cell imaging. Channel C is the main channel corresponding to the brain parenchyma, where human BBB equivalents are expected to form. To induce the directional angiogenesis from the left to the right direction, the angiogenic factor gradient was established from right to left across channel C by inoculating angiogenic factor-expressing human lung fibroblasts in channel RO, and angiogenic sprouting was initiated from the left side of channel C by inoculating hBMECs and hPCs or hBM-MSCs on the right lateral side of channel LI. *Reprinted with permission from* [30] **B)** Characterizing interstitial flow within the used microfluidic chip. (i) Gravity-driven flow across the hydrogel area was generated by applying DPBS to the medium reservoirs (pink) and Rhodamine B isothiocyanate–Dextran for the opposing medium reservoirs (red). The spatial change of the fluorescent wavefront was imaged sequentially. Cells are depicted as dots. (ii) Example of an original and binarized image used for estimating maximal flow rate. (iii) Example of the tracked waveforms of two consecutive images. Solid and dashed lines demonstrate the averaged waveform locations presenting the difference (red arrow) between averaged waveform locations between these two image indexes. The average change is then used to estimate the current flow rate. *Reprinted with permission from* [32] **C)** Microfluidic system informed from secretion of hMSCs delivered in vivo. Schematic of microfluidic synthesis including hydrogel crosslinking, cell encapsulation, and pressure-driven media perfusion. *Reprinted with permission from Schneider, Rebecca S et al. "High-Throughput On-Chip Human Mesenchymal Stromal Cell Potency Prediction." Advanced healthcare materials vol. 11,2 (2022)* **D)** Schematic representation of the technology system W8. Front view of the field of view within the analysis channel containing the analysis medium and the 3D spheroid. Representation of the forces involved and the terminal velocity. *Reprinted with permission from Sargenti, A et al. "Physical Characterization of Colorectal Cancer Spheroids and Evaluation of NK Cell Infiltration Through a Flow-Based Analysis." Frontiers in immunology vol. 11 564887. 23 Dec. 2020.*

affected the angiogenesis and vascularization. The use of this microfluidic tool ameliorates the monitoring of the 3D structure, and the interactions among cancer cells and the surrounding environment, thus suggesting the application of microfluidic devices in drug screening and studies regarding tumor growth and metastasis [37].

Boul and coworkers developed a device for the 3D culture of hepatocytes reproducing liver microarchitecture, adapting the device to the differentiation stage of HepG2 cells used for the development of 3D structures. The viability of cells cultured on the device was maintained for more than 14 days, moreover they succesfully aggregated in 3D structures and keep their differentiative ability during the culture. The 3D structure cultured on the chip was able to produce albumin at constant levels for up to 14 days [38].

Microfluidic techniques have already been developed for 3D culture, with one noteworthy example being acoustic levitation in an anti-gravity bioreactor. This innovative approach offers the benefits of

producing compact, uniform spheroids in a shorter timeframe. The MSC-derived spheroids created using this bioreactor have shown promising results, including upregulation of crucial growth factors, enhanced metabolic activity, and improved cell viability, all of which are essential parameters for cell therapy applications [39]. The W8 Physical Cytometer (CellDynamics Srl, Italy) is a flow device that accurately measures the mass density, size, and weight of 3D structures [40] (Fig. 3D). This innovative technique overcomes the limitations of traditional imaging methods for 3D spheroids, which typically require sectioning due to their thickness. The W8 instrument has been successfully used to analyze spheroids in mono- and co-cultures of perinatal cells, providing valuable information about their structure. Thanks to the analysis of the diameter in correlation with the mass density of the spheroid it was possible to confirm that monoculture spheroid of WJ-MSCs are stable during the time while co-culture spheroids composed of Wj-MSCs and AEC increase their compactness as the days pass by due to an increase of the mass density combined with a reduction in spheroid diameter. These considerations were then confirmed by histochemical analysis [41]. Researchers have also demonstrated the W8 ability to assess the effects of crizotinib (CZB) on LoVo cell lines spheroids, with CZB-treated spheroids exhibiting a significant increase in mass density and decrease in size and weight [42]. Overall, these microfluidic tools offer exciting new possibilities for studying 3D structures over time with minimal intervention, providing a more accurate representation of in vivo environments and improving our understanding of 3D models and cell responses to external stimuli.

3. Fluidic systems for cell sorting

The primary focus of biomedical research is the analysis and separation of various cell types found in complex biological samples that can represent the raw material for ATMPs. Cell sorting is an effective method to improve the efficiency of cell therapies by selecting more homogeneous cell subpopulations based on their phenotype and function. Modern techniques like FACS (fluorescence-activated cell sorting) and MACS (magnetic-activated cell sorting) involve the use of fluorescent or magnetic labelled antibodies to identify and sort specific cell types. While these methods are highly efficient in purifying known populations, they have certain limitations. Antibody binding can indeed activate a signalling cascade, altering the characteristics of the cell. Moreover, in some cases it is challenging to distinguish the subpopulation of interest or where multiple antibodies are required, such as in the case of MSCs.

Label-free techniques are characterized by several metrics, including throughput, purity or efficiency, yield of recovered cells, separation resolution, and enrichment. Separation criteria are fundamental and have a direct impact on the resolution. For instance, size is a commonly employed label-free separation criterion, and filtration is an intuitive approach. Some lab-on-a-chip platforms can separate cells without the use of antibodies and staining. These techniques are based on the unique characteristics of cells such as size, shape, electric polarizability, electric impedance, density, and hydrodynamic properties. To separate a specific type of cell from a population, these properties need to be linked to a separation force or method [43]. These methods are gentle and therefore encouraged to be studied further.

In a recent study, label-free cell sorting was applied for the detection of Circulating tumor cells (CTCs). To overcome the limitations and low specificity of conventional microcolumns, they first used a size-based two-array lateral displacement chip to first sort CTCs, followed by a stiffness-based cone channel chip to purify CTCs from dimensionally mixed leucocytes. Finally, cell types were identified using Raman approach. The use of the cone channel chip improved the purity of CTCs by 1.8-fold, suggesting an application of this multistage process for a highly pure, high-throughput and highly efficient cell sorting process [44].

Microfluidic channels were also tested as a possible time-saving and automated tools for the separation of peripheral blood mononuclear cells (PBMCs) from polymorphonuclear (PNM) cells and red blood cells (RBCs), which is commonly performed by density gradient centrifugation process. The establishment of density gradient in this device was based on the presence of a laminar flow within microfluidic channels which allows to layer of blood on a larger stream of Ficoll. The isolation yield of cell subsets using the automated device was similar to traditional approaches, with the advantage of a lower manipulation of blood samples [45].

Another important innovation in the medical field is the use of machine learning and artificial intelligence (AI) to support discovery and implement instrumentation, especially in recognising cell morphology from spectral signals like the one in flow cytometry analysis. Salek et al. recently presented COSMOS, Computational Sorting and Mapping of Single Cells, a platform based on AI and microfluidics to characterize and sort single cells based on real-time deep learning interpretation of high-resolution brightfield images [46]. The neural network developed was able to visualize deep morphological differences across biological samples and discriminate and enrich specific cells of interest with high efficiency in a label-free manner. Despite other image-based cell sorting methods, COSMOS can isolate viable and unaltered cells for downstream use and analysis and high-content pictures are captured and saved to generate an image database that can be successively used for reanalysis to detect additional phenotypes. A limitation of the platform is the lower throughput in comparison to conventional sorters.

The efficiency and use of microfluidic techniques can be however limited by the concentration of cells. On the macroscale, fibrous membrane filters contain a wide range of pore sizes, which can result in low separation efficiency for certain applications, such as fractionating blood into red blood cells, white blood cells, platelets, and plasma. One type of microfilter employs micro-posts spaced apart to create a critical size cutoff. Cross-flow filtration operates perpendicular to the micropost array or weir filter. To prevent clogging and filter saturation, these designs aim to separate rejected cells from selected ones by directing them towards different outlets. This technique has been used to separate plasma blood [47,48], WBCs and neonatal rat cardiac cell populations from whole blood. By adjusting the gap size, a critical size cutoff can be created [49,50].

In 1966, J. Calvin Giddings invented field-flow fractionation (FFF) [51]. This technology has proven to be capable of analysing, discriminating, and separating a wide range of biological samples based on their physical characteristics [52]. The sedimentation and the gravitational field-flow fractionation (SdFFF, GrFFF) variant was used for whole-cell analysis, and it separates samples based on the combined action of the mobile phase flow and a sedimentation field (either centrifugal or Earth's gravity field) in a capillary channel [53]. However, these systems showed limitations when it comes to analysing adherent cells, despite its simple instrumental set-up and low contamination risk.

The Non-Equilibrium Earth Gravity Assisted Dynamic Fractionation (NEEGA-DF) method (patent number: US8263359B2) has been invented to work with adherent cells without their attachment to capillary walls [54]. This method enables cells with different physical characteristics to acquire different velocities inside the capillary channel and elute at different times. Celector® (Stem Sel Srl, Italy) is a novel instrument based on a patented technology (patented technology (IT1371772, US8263359 and CA2649234) that implements the NEEGA-DF method. The instrumental setup consists of a fluidic system and a biocompatible capillary separation device. A camera with a microscopic objective is placed at the end of the separation channel and it monitors the elution process, generating a multiparametric fractogram that represents the number, size, and shape of the eluted cells as a function of fractionation time (Fig. 4). Celector® separates, characterizes, and sub-fractionates living cells from even complex samples, for further, downstream cell QC, characterization, and/or culture. In addition, it provides a complete biophysical characterization of the cell population. Celector® was shown able to select the most potent and homogeneous cell components

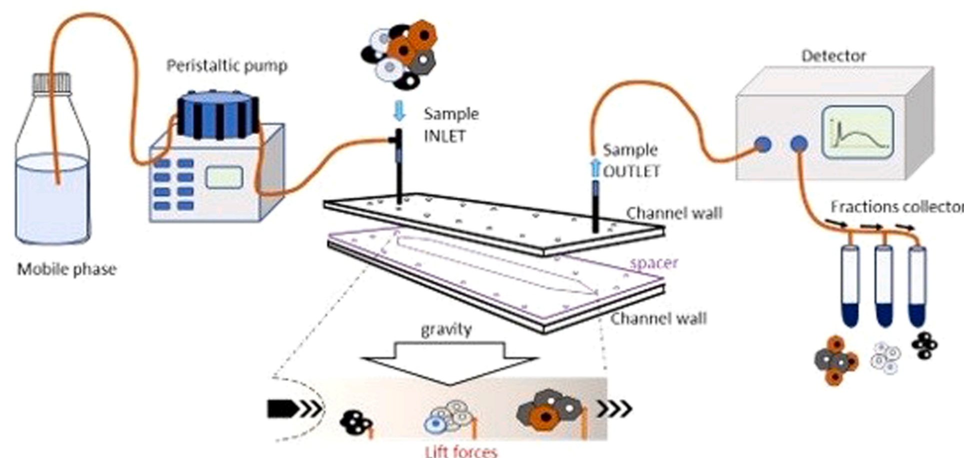

Fig. 4. Graphical representation of the separation device Celector®. The separation device is connected to a peristaltic pump at the inlet and a micro-camera detector next to the outlet. Separated cells were collected in different tubes. Cell samples were inserted and cells, based on their physical characteristics, reached a specific position across the channel. Bigger and denser cells were the first to exit, followed by the smaller ones. *Reprinted with permission from Zia, S et al. "Quality Control Platform for the Standardization of a Regenerative Medicine Product." Bioengineering vol. 9,4 142. 2022.*

from heterogeneous stem cell populations of different origins and to purify them from senescent and differentiated cells to improve the success rate of ATMP applications. It was exploited as an efficient tool for separating MSC from freshly aspirated bone marrow [55], and for quality control of different cell populations [56]. It also provides predictive data for defining successful isolation procedures of primary cells and optimization of cell culture procedures with interesting features for laboratories that isolate and cryopreserve stem cells for clinical applications. [57]

4. Quality by design for cell manufacture

The pharmaceutical industry has well-defined QC measures for drug development and manufacturing that have been in place for decades. There are strict regulations set forth by international scientific societies and companies to ensure the highest level of safety for medicines. The processes are complex and cover various aspects, from sterility to packaging. A new approach called Quality by Design (QbD) has been implemented to simplify this process. QbD uses statistical, analytical, and risk-management techniques to ensure the quality of medicines during their design, development, and manufacturing. The goal of QbD is to identify, explain, and manage all sources of variability that may affect a process through appropriate measures. This ensures that the finished medicine consistently meets its predefined characteristics (https://www.ema.europa.eu/en/human-regulatory/research-development/quality-design) (Fig. 4).

In recent years, the technique of using tumour cell lines to produce valuable proteins, DNA, and RNA has become increasingly popular. This method has been successfully applied in the creation of monoclonal antibodies and vaccines [58], including the COVID-19 vaccine. However, using living materials in these processes adds an additional layer of complexity. It is crucial to ensure that the cells are correctly identified and that their viability, proliferation, and sterility are accurately assessed. To address these challenges, QbD has played a critical role in the development of process monitoring and quality assurance approaches. This has helped to achieve high-quality products quickly and cost-effectively.

The QbD approach is a useful tool for addressing technical requirements and guidelines during the manufacturing process, particularly for accelerating ATMP development. Unfortunately, only 7% of ATMPs reach phase 3 of clinical trials [59], and a survey by Ten Ham et al.[60] found that 73% of companies producing ATMPs encounter manufacturing and quality assurance issues. These issues may be related to the scale-up phase, inconsistencies between the laboratory, or problems with quality standard definitions. The biological complexity of cells also slows down the translation from laboratory-scale experiments to industrial production of reliable cell-based therapies. For this reason, QbD focuses on quality control definition and verification from the beginning of process development, rather than just before product release.

The QBD method involves creating a Quality Target Product Profile (QTPP) to determine a product's characteristics based on patient and clinical considerations. Using the QTPP, critical quality attributes (CQAs) are chosen with both clinical and non-clinical data in mind, ensuring product safety and efficacy. To maintain consistency, each CQA is regularly tested using reliable and efficient procedures [61,62], with stability maintained within a specific range [62]. Critical Process Parameters (CPP) are used to monitor how the process affects the quality attributes, ensuring that the process remains within predetermined ranges for consistent product quality.

Biopharmaceutical companies have been encouraged by FDA for more than twenty years to adopt risk management techniques in their manufacturing process to increase safety and efficiency in quality control and regulation. In 2005, the International Conference of Harmonization published the ICH Q9 Guideline for pharmaceuticals and it was adopted by FDA, EMA and the Japanease Minister of Health, Labour and Welfare in 2006, to deliver methods for non-pharmaceutical companies to face risk and mitigate their effect. Risk analysis techniques mostly used in pharmaceutical companies are the following: failure mode and effect analysis/failure mode and critical effect analysis (FMEA/FMECA), fault tree analysis (FTA), hazard and operability analysis (HAZOP) and preliminary hazard analysis (PHA). The most suitable for cell manufacturing is the FMEA/FMECA because of its adaptability to processes that include extensive manual labour and context in which risk management is approached for the first time.

A method called Risk Priority Number (RPN) is often used for Failure Mode and Effect Analysis (FMEA) [63]. This approach prioritizes potential process parameter failures based on their severity, occurrence, and ease of detection. Every product and stage of the process is critically analysed to find the probability of failure mode and the severity of their consequences. RPN is obtained by multiplying the severity (S) x occurrence (O) x detection (D). Parameters with a high RPN value are considered critical and should be addressed first. C. Talarmin et al. showed its use in the risk assessment for the production of the chimeric antigen receptor T for the Car-T cell production and the criticality of each risk (minor, moderate, significant or major) was scored, and

corrective actions or preventive actions (CAPAs) for moderate, significant and major risks were proposed [64]. They identified five moderate, six severe and no major risks but thanks to the risk assessment were reduced to three minor and five moderate, showing the advantage of the method proposed. A detailed description of the use of FMEA and RPN analysis in the GMP production of embryonic pancreatic stem cells was published for the first time [65]. The authors compared a software-based (AHP) and direct estimation of the risk to identify the most efficient method for this innovative process. The authors concluded that the direct methodology was more efficient considering a reduction of around 80% in time of analysis with comparable results on RPN. FMEA in combination with RNP was also used to assess the collection of bone marrow which is a useful source for direct transplantation or to derive hematopoietic or mesenchymal stem cells for clinical application [66]. Parameters with a high RPN value are considered critical and should be addressed first. To ensure ongoing quality control, a defined control strategy for these critical process parameters (CPPs) should be implemented through Ongoing Process Verification (OPV). This will help detect any shifts or abnormalities that may affect the quality of the final drug product during routine manufacturing.

The quality of cell products is largely determined by two key processes: cell extraction and expansion. It is important to carefully select the appropriate tissue source, donor age, and extraction methods to ensure a homogeneous initial population. Despite efforts to optimize these processes, there may still be variations between donors and within the same population, which can be particularly challenging when working with allogenic ATMPs. Each donor has unique characteristics in terms of the number, type, and potency of their cells.

When producing cells for ATMPs, specifically MSC, certain key quality attributes (CQAs) are used to determine the purity, identity, genetic stability, cell quantity and viability, and potency of the cell product. Due to the typical therapeutic dosage is $1–2 \times 10^6$ cells per Kg of individual, cells must be amplified in a bioreactor. However, during this process, cells may change their original characteristics and experience senescence [67] just a few days after culture, differentiation, or even mutation due to errors in cell replication. Cell expansion using mono-layer flasks is a well-documented process, but it can be labour-intensive and presents a high risk of contamination. The use of a closed system, such as a bioreactor, is becoming more common as it offers a more standardized approach and reduces the risk of contamination. However, the high cost associated with these systems has led to limited usage in studies. It is important to consider critical parameters such as culture medium, supplements, oxygen levels, and pH when expanding cells to maintain their original characteristics.

Research has shown that the effectiveness of MSCs, which have powerful immunomodulatory capabilities [68,69] can be also influenced by the type and number of supplements used. MSCs are naturally found in specific areas of the body where the concentration of dO2 ranges from 1% to 7%. A study by Hung S.P. et al. found that BM-MSCs cultured at 1% O2 had increased gene expression related to growth and differentiation towards bone-forming cells [70]. When cells are cultured in a flask, the concentration of oxygen is not consistent due to various factors such as cell density, height of medium, oxygen consumption, and the type of incubator being used. These parameters, including oxygen levels, nutrients, donor age, and doubling time, all impact each other and are critical to the process [71]. Unfortunately, it is challenging to predict or analyze how they interact during MSC production.

Finally, ATMPs products are often produced in single-treatment batches for autologous or personalized therapies, and there may be limited or no material available for destructive release testing. Therefore, the analytical tools used must be able to measure complex biological attributes non-destructively and, ideally, non-invasively [72].

5. Process analytical technology (PAT) for cell manufacture

To ensure product's quality and prevent possible failures during manufacturing, the QbD approach must be validated through CQAs, which identify and control the impact of various factors. Quality risk management involves reviewing steps to systematically reduce the risk of failure and implementing an appropriate dynamic design space (DSp) [73] and Process analytical technology tool (PAT) to monitor the manufacturing process. However, ATMP validation is more complicated due to the lack of specific validation strategies [74–76]. The EMA divides validation into investigational ATMPs (early experimental phase) and authorized ATMPs (already on the market [77]). The former must demonstrate method suitability, while the latter requires complete validation for clinical use.

PAT is a valuable manufacturing tool that helps optimize production by preventing wasted time, materials, and excessive costs. This system allows for the design, analysis, and control of manufacturing processes by monitoring quality and attributes. The measurements can be taken online, in-line, at-line, or off-line. In-line and on-line approaches are preferred for cell monitoring as they provide real-time, automated measurements and avoid contamination. Cell viability and cell number (viable cell concentration, VCC) are key parameters in the cell manufacturing process because they inform the process progression and harvest readiness. Unfortunately, they are mostly measured off-line by an automatic cell counting system. In the market are present several tools for real-time measurement such as the Maestro Tray Z (Axion Biosystem), a live-cell impedance platform for real-time measurement of cell health and function. However, this tool is for small-scale cultures because can analyze only 96-well plates. Another interesting technology is the use of a holographic microscope connected to the cell culture that can provide cell counting in a non-invasiveness, dye-free way. The technology iLine F (Ovizio) provides continuous cell counting and monitoring without wasting material thanks to its closed-loop setup. Monitoring and controlling mammalian cell culture for protein production involves using standardized procedures, methods, and instruments. These procedures ensure that basic factors such as temperature, pH, dissolved oxygen, pressure, airflow, liquid flow, foam level, and stirrer rate are all maintained at appropriate levels. However, critical parameters such as cell counting and density, viability, proliferation, and potency are also important. To accurately count cells, the gold standard is an offline method that involves using an exclusion dye like trypan blue to differentiate between live and dead cells.

When it comes to manufacturing cells, it would be beneficial to have online monitoring in place to minimize measurement delays and keep a continuous flow of information regarding the cultivation system's state. There have been various methods used for this, but they primarily work with cells growing in suspension and not in an adherent mode like MSCs. Examples of these techniques include UV spectroscopy, near-infrared (NIR) and mid-infrared (MIR) spectroscopy, two-dimensional (2D) fluorescence, which monitor culture bulk composition [78–82]. However, these techniques don't focus on single components, instead, they provide large sets of correlated data from which meaningful information must be extracted. The NIR and MIR technologies can quickly provide a snapshot of a culture composition, including recombinant protein, glucagon, glutamine, lactate, and ammoniac. This information helps assess the growth status of cells. As an example, exploiting nuclear magnetic spectroscopy (NMR), now they are on commerce devices like the InsightCell (Bruker) which can monitor real-time cell activity of cell culture expanded in bioreactor and obtain the information about compounds consumed and produced by cells. In collaboration with the University of Bern, they showed a technical report in which they used this instrument to monitor cell health and metabolism by measuring lactate and glucose production in fibroblast culture after treatment with rotenone and 2-dehydroxy-D-glucose. These techniques are the most used PAT tool in the pharmaceutical industry and are integrated into their production line. Nonetheless, these techniques calculate cell concentration in suspension, including cell debris, and measurements can be interfered with by bubbles, stirring, high cell density, and viscosity of the medium. To solve this issue, an important in-line PAT tool is the

bio-capacitance probe that measures the electrical properties of cell membranes and can define cell viability without altering their properties [83]

Real-time measurement of specific parameters, such as cell characterization, can enhance cell culture control and minimize batch-to-batch inconsistencies. Optical and spectroscopic systems that employ automated methods have the potential to serve as monitoring tools in real-time for cell differentiation and viability [84]. Raman spectroscopy, with chemometric models and machine-learning algorithms, is an excellent non-destructive technique that is also available in miniaturized form [85]. In the context of ATMP, Raman spectroscopy has been utilized to evaluate phenotype properties of stem cells during in vitro differentiation and to track metabolites [86]

When it comes to expanding cells for allogenic therapies that use, for instance, MSCs the use of a bioreactor is essential. To increase production, 3D bioreactors are increasingly being used with fibrous scaffolds, microcarriers, and stacked plates to boost the available adherent surface area. However, counting the cells grown on microcarriers can be challenging due to the need to extract a sample from the culture and either detach the cells from the carrier or label them with fluorescent dye for counting. Odelaye et al. have developed an optical system that allows for the non-invasive monitoring of MSCs grown on microcarriers. The in-situ epi-illumination of MSCs provides quantitative measurements of confluence, aggregate recognition, and cell number without the need for cell manipulation.[87]

A microbead-based process control system is designed to monitor and control the concentration of TGF-β1, a signalling factor produced within the body. This system is integrated into a bioreactor to improve the ex vivo expansion of hematopoietic progenitor cells, even at higher input cell densities and over longer culture periods. Schwedhelm, I. et al. [88] created a suspension culture unit specifically for hiPSC that comprises a fully monitored continuously stirred tank reactors (CSTR) system, integrated into a custom-designed and fully automated incubator. It includes quality control measures such as in situ microscopic imaging that allows real-time visualization of hiPSC aggregation, without the need for time-consuming sampling.

The use of fluorescence spectroscopy is an effective and non-invasive method for monitoring biological processes and has been used for many years to measure biomass concentration. 2D fluorescence is particularly useful for in-line PAT applications, as it covers a wide range of excitation and emission wavelengths. Intrinsic fluorophores, such as aromatic amino acids, vitamins, and co-enzymes, are present in the bioprocess medium and their significant changes can be observed. A new LED-based 2D-fluorescence spectroscopy [89,90] sensor has been developed for bioreactors, which can track the cells' metabolic and growth state. This system has been validated through testing with cells cultured under oxygen limitation and has been shown to quickly detect deviations from a regular run. While certain technologies have proved successful in measuring the cell manufacturing process, certain parameters such as cell size, physical properties, and pluripotency still pose a challenge to define in dynamic cultures. To improve real-time monitoring with PATs, new non-invasive instrumentation must be developed to define such properties.

6. Concluding remarks

Microfluidics is a promising approach for the quality control of cell-based ATMPs products. This technology has demonstrated great potential to address some challenge in the manufacturing process. Tools integrating miniature tissues and microfluidic patterns were shown able to provide robust models for reliable cell culture in 2D and 3D formats for the development of new therapeutic approaches. Lab-on-a-chip platforms were created to sort and characterize raw materials. With advantages related to label-free approaches, fluidic systems guarantee the integrity of cellular materials. The fluidic tools can be integrated into the development process allowing for a standardization and automation. Although there have been many advancements in fluidic systems for cell analysis, efforts in systems fabrication and validation still need to achieve the complete integration of these systems in all phases of the process. The use of AI and machine learning (ML) presents promising opportunities for the optimization of fluidic structure and preparation. In addition, ML can manage data for monitoring and predicting the process state and could potentially aid in anticipating the therapeutic potential of cells in clinical applications. Due to the complexity of cell-based ATMP production process phases (cell isolation, cell expansion, cell analysis, cryopreservation and storing, administration and monitoring), which must consider several aspects (sterility, safety, pricing), the QBD approach is proving useful to simplify and accelerate the ATMP development process.

CRediT authorship contribution statement

Francesco Alviano: Writing – original draft, Data curation. **Francesca Paris:** Data curation, Writing – original draft. **Laura Bonsi:** Writing – review & editing, Conceptualization. **Pierluigi Reschiglian:** Writing – review & editing, Conceptualization. **Andrea Zattoni:** Writing – review & editing, Conceptualization. **Barbara Roda:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Valeria Pizzuti:** Writing – original draft, Data curation. **Silvia Zia:** Writing – original draft, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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