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Technological advances towards extracellular vesicles mass production

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Abstract:

Extracellular vesicles (EVs) are becoming essential actors in bio-therapeutics, as much for their regenerative or immunomodulatory properties as for their potential as cargo delivery vehicles. To enable the democratization of these EV-based therapies, many challenges remain such as large-scale production which is necessary to reduce costs of treatment. Herein, we review some advanced works on high-yield EV manufacturing. One approach consists in developing large-scale cell culture platforms, while others focus on cell stimulation to increase particle yield per cell. This can be done by moderate physico-chemical stresses or by disrupting cell membrane towards autoassembled vesicle-like particles. We critically compare these different techniques, keeping in mind that the field still lacks shared characterization standards, underline the importance of therapeutic potency assessment and discuss mass production strategies that have been identified in current clinical trials.

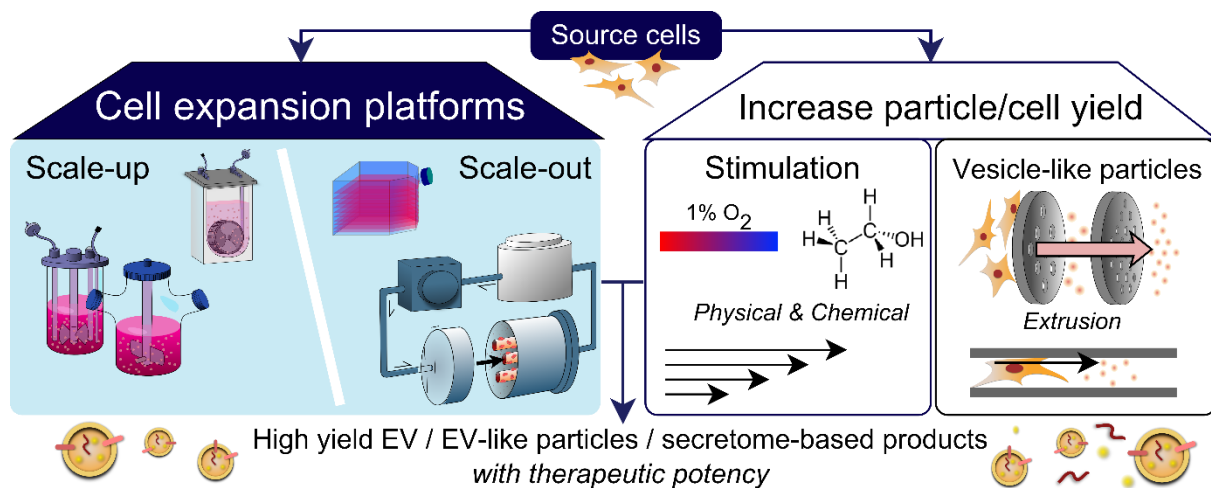


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1. Introduction

Recently, extracellular vesicles (EVs) have emerged as promising therapeutics, offering a potential cost-effective cell-free and *off-the-shelf* alternative to cell therapy. EVs, especially from mesenchymal stromal cells (MSCs), hold many promises as they have been regarded as direct mediators of the paracrine effect inducing MSC regenerative properties^{1,2}. MSC-derived EVs (MSC EVs) are thus considered as intrinsically bioactive vehicles, which are safely taken up by the organism, mitigating the risks associated with cell injection such as unwanted replication, differentiation and vascular occlusion³. Their therapeutic potency in animal studies has been reported in multiple models for the treatment of skin⁴, bone⁵, cartilage⁶, heart⁷, liver⁸, kidney⁹, lungs¹⁰ and brain^{11,12} among others. Furthermore, EVs have been able to regulate the immune system, enhancing or inhibiting the immune response depending on their parental cell source and of the immune context of the application site¹³, demonstrating a potential use in immunotherapy. In addition to EVs' intrinsic bioactivity, they can also be engineered with specific loading or targeting abilities, adding applications in drug delivery to the list of therapeutic possibilities¹⁴. For instance, genetic materials^{15,16} or chemotherapeutic drugs¹⁷ have successfully been loaded within EVs and, as a result, were efficiently delivered to tumors leading to cancer growth inhibition *in vivo*. EVs from other cell sources than MSCs have also been considered for their clinical applications¹⁸, both harnessing specific properties of EVs, and the ease of culture or harvest compared to stem cells (e.g. suspension cell lines or plant cells).

In the wake of all these findings, research on EVs has increased almost exponentially these last few years. Since the 1st of January 2020, over 20 % of all EV related articles

have been published¹⁹, more than 3,000 patents in English containing “extracellular vesicles” or “exosomes” in their claims have been published²⁰, and 23 new clinical trials with EV-products as treatments have been posted²¹. Notably, the active search for COVID 19 treatments also involved EVs since they have been reported as potential regenerative cell-free medicine for acute respiratory syndrome^{22,23}.

As a relatively new and expanding field, new scientific guidelines have recently appeared to standardize the report of EV research and advances²⁴. However, hurdles have concurrently emerged when beginning the translation of EV-therapeutics to clinical practices²⁵. Indeed, high-yield and scalable production or isolation methods still remain rare. Different techniques and processes have recently been proposed to scale up cellular culture^{26,27}, such as 3D culture on microcarriers²⁸ and the use of hollow fiber bioreactors²⁹, or to increase EV yield per cell, including stimulation with chemical agents³⁰ or mechanical stress³¹. Another approach is to totally disrupt cell membrane to form biogenic vesicles, which partially mimic EVs regarding membrane composition and intracellular content, and are easily manufactured in much larger quantities^{32,33}. In this paper, we will use the term EV-like particles for such vesicles. Tangential flow filtration (TFF) has also been proposed as a promising enrichment technique for large volumes of conditioned media³⁴ and size exclusion chromatography (SEC) as a straightforward technique to purify EVs and EV-like particles from soluble factors³⁵. Yet, difficulties arise when comparing these techniques because of a lack of standardization and reproducible characterization methods. The need for robust assessment of purity, stability and potency standards is growing rapidly, aiming at developing quality controls for future clinical trials under Good Manufacturing Practices (GMP) requirements.

In this review, we provide an overview of the recent advances that have been proposed to scale up EV production to manufacture safe and cost-effective EVs or EV-like particles. Cell culture platforms allowing massive expansion of source cells are critically discussed and compared, as well as innovative processes to boost the production yield of EVs and EV-like particles. The scale-up strategies in ongoing clinical trials are then presented.

2. Challenges of EV large-scale production

For EV-products to be able to replace cell therapy as a safer and more affordable biological medicine, enabling large-scale production of EVs is a major challenge to reduce the costs of EV manufacturing. As an example in cell therapy, dosages between 3 million and 240 million MSCs were reported to have a beneficial effect in preclinical models on big animals (swine and sheep) and clinical models of heart disease depending on the route of administration³⁶. One could estimate that an EV dose would be the amount of EVs naturally secreted by the same number of cells which would require the culture of between one and one hundred 150 cm² flasks. In the optics of treating large cohorts of patients, different strategies can be adopted and combined to obtain the required quantity of EVs.

The most straightforward way is to directly scale up or scale out cell culture platforms, which has already been done in other fields such as monoclonal antibody manufacturing³⁷. This includes other 2D supports such as hyperflasks or roller bottles,

and 3D culture methods generally referred to as bioreactors (e.g. perfusion, fixed bed or spinner flasks), represented in **Figure 1**. In these approaches, the stakes are not only to obtain the maximal number of cells, but also to reduce general costs by minimizing manipulation time, culture time and consumables spent. This can be achieved by increasing surface area available, with hollow fibers or microcarriers, and may have as a consequence to decrease the culture medium used per cell.

Another strategy is to increase the number of EVs secreted per cell. Indeed, if most (if not all) cells constitutively release EVs, this process can be enhanced by stimulating them through serum starvation, hypoxia, or physical and chemical stresses³⁸⁻⁴¹. On the other hand, large amounts of EV-like particles per cell can also be obtained by disruption of the cellular membrane. These approaches may produce vesicles, or vesicle-like particles, with other phenotypes than the ones of spontaneously produced EVs^{42,43}.

Ultimately, EV final yield, nature and purity also results from the purification and enrichment strategies employed. To date, ultracentrifugation remains the gold standard to concentrate EVs⁴⁴ for research purposes, despite a poor scalability and low recovery rate. Size-based isolation and enrichment techniques are thus rising, including size exclusion chromatography (SEC) or flow field fractionation (FFF). While density gradient ultracentrifugation is still the method of choice to purify EVs from soluble components, some researchers also developed affinity-based purification techniques, yet introducing issues regarding specificity and ubiquity of EV markers⁴⁵. Other methods have been studied for the isolation of EVs, among which polymer precipitation or ion exchange chromatography, as reviewed elsewhere⁴⁶⁻⁴⁸. Finally, to translate to large-scale EV manufacturing, chromatography and ultrafiltration have been used for some time in order to concentrate and purify bio particles²⁶ (e.g. viral particles, small proteins and antibodies), and are beginning to be implemented for EVs^{49,50}. Considering the difficulty of isolating and characterizing EVs, another strategy would be to prioritize therapeutic efficacy over purity⁵¹, manufacturing a secretome-based product or an EV-enriched secretome product, also acknowledging that bioactivity may also be due to soluble factors co-isolated with EVs⁵².

Overall, EV manufacturing is a process which encompasses a considerable number of input parameters, each of them possibly generating an impact on the final product, either on its physical and chemical properties (size, number, zeta potential...), its biological content (proteins, RNAs, lipids, metabolites...), purity, stability or on its therapeutic potency. Indeed, what is often noted for any change in the upstream process of EV manufacturing is the largely unknown potential effects on the nature of the EVs⁵³. Thus, a major challenge is to characterize the effect of the variation of these parameters on the final product identity and therapeutic activity. Yet, the lack of standardization of characterization and isolation methods impedes the comparison between studies, making it difficult to assess if differences are due to manufacturing strategies or to characterization techniques.

Additionally, regulations require the choice of GMP-compliant raw materials (xeno-free culture media, cells...), storage and administration strategies. GMP EV manufacturing processes should follow guidelines from the International Council for Harmonization of

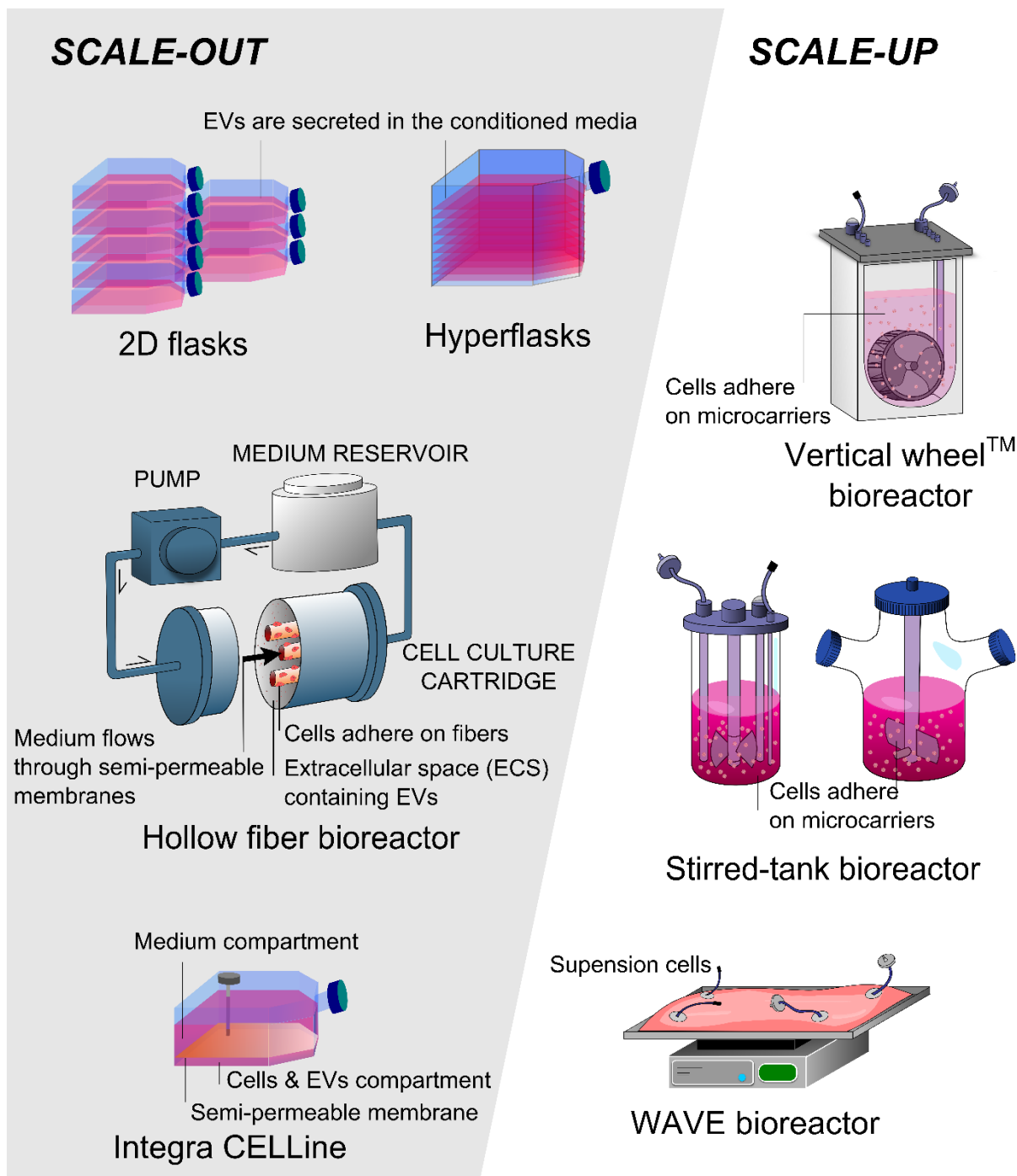


Figure 1 : Cellular platforms : principles of the different methods of cell expansion

Technical Requirements for Pharmaceuticals for Human Use (ICH), more specifically the Common Technical Document (CTD) for the registration of pharmaceuticals (available online : <http://www.ich.org/products/guidelines>). This document covers the quality, safety, and efficacy of pharmaceuticals manufacturing, addressing the topics of producer cells, raw materials, manufacturing and quality control. These recommendations are discussed more in depth in other papers, among which in a position paper on regulatory concerns published in this issue.

Finally, many companies and laboratories develop EV engineering strategies, optimizing loading and targeted delivery of EVs. While this is also a major stake in this

field, it will not be detailed in the present paper which focuses on comparison between recent technologies for EV large-scale production, more specifically the scale-up of cell culture and the improvement of EV yield per cell.

3. Optimized cellular culture: scaling out *versus* scaling up

Most strategies to expand cells *in vitro* with minimal costs rely on the increase of surface area while minimizing the volume of culture media per cell. This induces the concentration of EVs in conditioned media and thus shortens the isolation process. Different platforms used for cell expansion and EV production are summarized in **Table 1**. In the cases where cell number was not reported at the end of the expansion phase, an estimated maximal cell number was indicated according to the literature^{54–56} or technical sheets obtained from the manufacturer's website.

3.1 Scale-out

The scale-out of the culture system consists in the optimization of the cell culture on a platform of limited size or scalability, but which can be parallelized to further expand the process. For instance, the use of the “flask” bioreactor Integra CELLine was proposed in 2008⁵⁷. In this set-up, the cells are separated from the culture media by a 10kDa molecular weight cut off (MWCO) membrane, allowing the nutrients to be exchanged but not the larger molecules. EVs, which are produced in the cell compartment, are thus concentrated in a very small volume. In average, 180 mL of conditioned media, pooled from several harvests, yielded 1.5 mg of proteins quantified by bicinchoninic assay (BCA) after sucrose gradient ultracentrifugation. The most common CELLine bioreactor can hold 1 L of media and 15 mL for the cell compartment, allowing a concentration of cells up to $1E7 - 1E8$ cells/mL. This technology has been used by Jeppesen *et al.* to produce large quantities of EVs from cancerous bladder cells for proteomics analysis⁵⁸. Although this platform has been adapted to adherent cell lines, the maintenance of cells over extended period of times at high densities makes it not appropriate for stromal cells and this may explain why its use with MSC is not recommended by the supplier.

EV production in hyperflasks has also been recently reported by Madel *et al.*⁵⁹, in which between $1.2E11$ and $3.8E11$ particles (measured by Nanoparticle Tracking Analysis, “NTA”) were obtained by precipitation and ultracentrifugation (UC). These EVs were produced from $4E7$ bone marrow derived MSC (BM-MSC) cultivated in 4-layer flask Cell Factory™ (Nunc™ Thermofisher™) and in media supplemented with human platelet lysate not depleted of EVs. According to the manufacturer, the volume per hyperflask would be between 600 mL and 800 mL, for a surface area of 2,528 cm², which means there is no real economy in media required compared to 2D flasks, just a reduction of manual operations during cell culture and media harvest. Andriolo *et al.*⁶⁰ obtained in average $3E13$ particles from cardiac progenitor cells (CPC) cultivated for 14 days without serum in 16 hyperFLASKS® (Corning®) each containing 500 mL media and 1,720 cm² surface area. This work also evidences the feasibility of isolating EVs from a large volume of conditioned media (8 L) by filtration and tangential filtration.

One of the method of choice for scalable EV production is spontaneous release of EVs in hollow fiber bioreactor. Developed by FiberCell Systems and by Terumo (Quantum bioreactor), these bioreactors are perfusion bioreactors in which cells are grown on the

surface of semi-permeable fibers through which the supplemented media flows. EVs released by cells accumulate in the media external to the fibers (termed extracellular space “ECS”), usually serum-free, which can easily be harvested every 4 to 14 days. Five hundred million adipose derived stromal cells (ASC) seeded in a C2011 (4,000 cm² surface area, 20kDa MWCO) yielded between 1E12 and 3E12 particles in 4 to 7 weeks, corresponding to 12-14 mg of proteins. One harvest of conditioned ECS consists in 40 mL with about 50 µg/mL of proteins⁶¹. However, it was observed that after 6 weeks of culture, ASC formed embryoids in the bioreactor, raising the question of the differentiation state of cells after several months of culture. In 2016, Watson *et al.*⁶² reported the utilization of the same cartridge to produce EVs from human embryonic kidney 293 cells (HEK293) expressing high levels of interleukin 15 in heterodimeric form (hetIL-15), obtaining similar yields. Interestingly, when using the particle over protein ratio and the protein concentration measured in the supernatant, and according to the estimated number of cells in the cartridge in order to calculate the total number of particles produced from one harvest (20 mL), one finds a 1,200 particle/cell yield in 48 to 72 h, while it was in the 7,500 particle/cell range for T-flask culture in 48 h. Our hypothesis would be that, as Patel *et al.*⁶³ described, the higher density of cells on the fibers leads to lower EV yield per cell. Even then, the specific set-up allowing cells to access nutrients through the semi-permeable fibers permits to have continuous culture and production of EVs over several weeks, which adds up to a large amount of EVs. To boost even more production output, collection frequency could also be optimized, as it has an effect on final EV yield⁶³. Importantly, ratios of particles over proteins obtained from hollow-fiber EV production are repeatedly higher than for classic 2D culture. It is often regarded as a purity assessment, however it may also be linked to the overall protein content of EVs, which seems here to be reduced.

Nonetheless, Yan *et al.*⁶⁴ showed that EVs from umbilical cord derived MSC (UC-MSC) produced in hollow fiber bioreactor had an improved osteochondral regeneration activity than 2D-EVs. Indeed, it increased proliferation of chondrocytes and promoted more effectively their migration than 2D-EVs *in vitro*, and had a superior repair activity on cartilage defects *in vivo*. While this may not be true for all therapeutic applications, it indicates that hollow fiber EVs can be equally, if not more, potent than 2D-EVs. Moreover, the hollow fiber platform offers the possibility to engineer EVs, as was demonstrated by the large-scale production of EVs enriched with mir202 and mir-133a by overexpressing these miRNA in mother cells⁶⁵. Finally, combining the hollow fiber technology and TFF or SEC enables the production and enrichment or purification of clinical grade EVs at moderately large scales³⁵. Currently, Fibercell cartridges can contain up to 1.2 m² of surface area available for cell culture⁶⁶ [fibercellsystems.com], corresponding up to 5E10 cells per cartridge, depending on the cells. Mendt *et al.*¹⁹ reported the use of the Quantum Bioreactor (Terumo) containing 11,500 hollow fibers with a surface area of 2.1 m² for BM-MSC, seeded with 20 million cells, and it yielded about 1E13 vesicles in 12 days after 9 days of expansion. These quantities are already considerable and among the highest reported to this day, yet this technology would still need to be parallelized in order to deliver treatment to hundreds/thousands of patients, in Phase III /Phase IV trials, which will require, as was already mentioned, rigorous quality control checks to ensure batch to batch reproducibility.

3.2 Scale-up in stirred tank bioreactors and other up-scalable bioreactors

Inspired from the development of monoclonal antibody production³⁷ or viral vectors²⁶, the culture of cells in stirred tank bioreactors, with the eventual use of microcarriers for adherent cells, has been suggested as a scale-up strategy for EV production²⁷. Recently, first results of yields obtained in these conditions have been published^{28,34,67}. Human UC-MSC were cultured on Star-Plus Solohill® beads in a 250 mL spinner flask, and EV production was initiated after 48 h of culture in xeno-free StemPro™ medium from 4.6E7 cells³⁴. EVs were isolated by UC or TFF and it was calculated that with TFF 1E5 particle/cell were obtained and 1.4E4 particle/cell with UC in 3D cultures, whereas it was 2E4 particle/cell and 7E2 particle/cell for TFF and UC respectively for 2D flasks. While it appears that 3D culture alone increases the number of particle/cell, the final yield is mostly increased by the use of TFF over UC. Furthermore, the authors evidenced that EVs produced by all four processes contained similar proteins, even though the particles over proteins ratio was decreased 2 to 4 fold with 3D culture compared to 2D. 3D EVs were also more efficient than 2D EVs at delivering silencing siRNA that had been loaded onto the EVs post production.

In an application note from Eppendorf, Escobar Irvico and Sha⁶⁷ described the process by which they produced EVs from induced pluripotent stem cell derived MSC cultured on collagen coated microcarriers in a single use stirred tank bioreactor vessel, controlled in pH, dissolved oxygen and temperature, seeded with 9E6 cells. They quantified EVs with the ExoELISA-ULTRA CD63 Kit (System Biosciences), reporting between 2.6E10 EVs at day 5 and 8.6E10 EVs at day 16.

On the other hand, de Almeida Fuzeta *et al.*²⁸ proposed the Vertical-Wheel™ bioreactor technology as an alternative to the conventional spinner flask in order to mix more gently and homogeneously the cellular suspension. Their approach also consisted in demonstrating the feasibility of this technique as a scalable xeno-free microcarriers based system. They compared the EV yield measured by NTA between 2D and 3D conditions for three cell lines: BM-MSC, ASC and UC-MSC. Overall, it was observed that MSC produced around three times more particles per cell upon 3D condition, and as the ratio of media over surface area was decreased, the final concentration of EVs in conditioned media was multiplied by almost 6. They obtained 2.8E11, 3.1E11 and 4.1E11 particles from approximately 25 million BM-MSC, ASC and UC-MSC respectively. No significant difference was found in the particle/protein ratio between 2D and 3D. As a proof of concept, this study was performed in a 100 mL bioreactor and isolation of EVs was performed with Total Isolation Kit (Invitrogen), but the isolation strategy will need to be revised for scale-up to a volume of 3 to 500 L.

3.3 Adaptation to suspension cells

Diversifying the source of cells may be a promising approach to optimize EV yield with any technology. Notably, for drug delivery purposes, cells easier to cultivate have been investigated, such as THP-1⁶⁸ or U937⁶⁹ suspension cell lines, for which the issue of scaling up cell culture becomes minimal. Indeed, similarly to bacteria or yeasts, suspension cell lines are easily cultured in stirred tank bioreactors that are the most straightforward for large-scale culture. Culturing 1000 L volume and over were already implemented in the early 2000s for the production of monoclonal antibodies with the

Table 1 : Medium to large scale cellular platforms

Type of culture	Cells	Surface area (cm ²)	Seeding cell density	Max cells growth	Culture time	Working volume	Production media	Harvests	EV supernatant in	Isolation method	EV isolated	
CELLine bioreactor	mesothelioma cells NKL cells	~300 cm ²	30E6/15mL	<i>Estim.</i> 400E6	>6 months	15 mL cell CM / 500 mL outer volume	RPMI + 10 % FBS	15 mL every 4-7 days	10.06 µg/mL of CM 6.76 µg/mL of CM (BCA)	UC + sucrose gradient	1,5 mg for 180 mL of CM	⁵⁷
Hyper-flasks	CPC	16x1,720cm ² = 2.75 m ²	3E4/cm ²	<i>Estim.</i> 2.7E9	-	500x16	Serum free	After 2 weeks	-	TFF	3E13 part (NTA)	⁶⁰
	BM-MSC	2,528 cm ² per flask	1000 cells/cm ²	<i>Estim.</i> 1E8 per flask	>48 h	600-800 mL per flask	DMEM + 10% hPL	Every 48 h from 50% to 80% confluence	-	PEG precipitation	2.4E11 part / 40 million cells (NTA)	⁵⁹
Hollow fiber C2011 20kDa MWCO Quantum bioreactor	ASC	4,000 cm ²	1.25E5 cells/cm ²	<i>Estim.</i> 1-2E9	4-8 weeks	20-40 mL	Serum free	6-7 harvests (every 4 to 7 days)	x	UC	2.1E12 part (NTA)	⁶¹
	HEK293		7,5E4 cells/cm ²		16 weeks	20 mL	Serum free	3 harvests / week	x	Filtration & UC	1.2E12 part or 1.1mg per harvest (NTA)	⁶²
	BM-MSC	2.1 m ² (11,500 HF)	~1000 cells/cm ²	<i>Estim.</i> 7E8 cells	21 days	250 mL	Platelet free	6 harvests (every 48 h)	9.8 – 15.6E12 part (NTA)	UC	x	²⁹
Spinner flasks	UC-MSC	1,150 cm ² (3.2 g of beads)	3,000 cells/cm ²	4.6E7 cells	48 h + 4x48 h	250 mL	Serum free	4 harvests (every 48h)	-	TFF	4.6E12 part (NTA)	³⁴
Vertical wheel	BM-MSC	720 cm ² (2g)	7,000 cells/cm ²	12-53E6 cells	7-11 days	60 mL	Serum free	1 after 48h	2.8E11 part	Total Isolation kit	-	²⁸
	ASC			29E6 cells	7 days				3.1E11 part			
	UC-MSC			20E6 cells	10 days				4.1E11 part (NTA)			

Chinese Hamster Ovarian (CHO) cell line which has been engineered for suspension culture³⁷. This strategy to modify the cell line to make the suspension culture possible can also be implemented for EV production⁷⁰. However, as culture in spinner flasks has been shown to inhibit the expansion of primary T lymphocyte⁷¹, it may also affect the nature of EV produced. On the contrary, regulatory T cells yield was increased almost 20-fold in a hollow fiber Quantum reactor in the presence of a soluble activator complex compared to 2D flasks. Ultimately, it goes back to the lack of standardized characterization methods for potency, because despite the fact that EV complex nature was shown to be affected by changes in culture conditions⁷², the current challenge is to demonstrate the potency of such EVs.

Finally, blood-derived cells such as red blood cells (RBC) or peripheral blood mononuclear cells (PBMC) have also been regarded as an interesting source of EVs⁷³⁻⁷⁵ for their relative ease of manufacture in very large quantities. Specifically, RBC do not need to be cultured and can be stored at 4°C up to 120 days, which means that cell expansion platforms are not needed.

Considering that even among MSCs, some cells produce more than others³⁴, systematic preliminary studies to select parental cells based on their intrinsic EV secretion rate and the therapeutic goals expected would maximize EV final amount.

4. Stimulating cells to increase EV yield

4.1 Physical & chemical stimulation

Another strategy to scale up EV production is to increase the yield of EV produced per each single cell. The final EV yield is not only the result of release but it also involves the recapture by cells. It is then difficult to assess if one process affects the former or the later action. Anyway, several treatments have been shown to increase the number of EVs compared to spontaneous release from equivalent number of cells⁴⁰. These are summarized in **Table 2**, in which yield is reported as an enhancement factor compared to control, consisting in treating the same number of cells for the same duration in the indicated production media.

The most widely used stimulation is certainly serum deprivation⁷⁶, which not only was reported to boost EV yield⁴² but also brings the advantage of avoiding EV contaminants brought by the serum even when depleted of particles. As the most common starting material, EV produced in serum-free media are often used as a reference to compare yields obtained after other types of stimulation (see **Table 2**). Other physico-chemical stresses improve the EV production, such as hypoxia^{39,77,78}, low pH^{79,80}, heat shock⁸¹ or ultrasounds⁸². In the later, Zhao *et al.*⁸² have evidenced that exposition of ovarian cancer cells to ultrasound for 60 to 90 minutes multiplied the protein yield of vesicles preparation by two. Increased vesiculation from endothelial cells was also observed with transmission electronic microscopy (TEM) after low level irradiation (80 J/cm²) using diode laser at a wavelength of 675 nm while investigating the mechanisms behind angiogenesis modulation after photodynamic therapy⁸³. Particle concentration and protein amount were not indicated but the level of CD63 in supernatant was measured at 0.9 ng/mL compared to 0.5 ng/mL in the control.

Additionally, EV yield can be enhanced by the use of chemical agents. For example, 100 mM ethanol conditioning for 48 h multiplied particle concentration measured by NTA by a factor 10 for primary hepatocytes⁴¹ and a factor 4 for primary monocytes⁶⁸. Other chemical stimulations include the use of anti-inflammatory drugs such as acetylsalicylic acid, celecoxib and chloroquine⁸⁴. Similarly, activated neutrophils, incubated 20 min with TNF- α , IL8 or LBT4, produced between two and three times more EVs than control cells³⁰, characterized by imaging flow cytometry. In the same article³⁰, Headland *et al.* also reported that cytochalasin D, an actin polymerization inhibitor, increased twice the number of EVs measured.

Finally, exposition of cells to several types of exogenous particles have also been reported to increase EV secretion. Internalization of positively charged iron oxide nanoparticles encapsulated in PLGA-PEI polymer by MSCs increased secretion of extracellular vesicles, which appeared to be linked to increased autophagosome formation⁸⁵. In Emam *et al.*⁸⁶, four cancerous cell lines were incubated for 48 h with different concentration of neutral, cationic-bare or PEGylated liposomes solutions. Neutral (NL) and cationic bare liposomes (CL) enhanced protein concentration in isolated EVs for all four cell lines, by a factor of about 2 for NL and ≥ 3 for CL at 2 mM. This approach may however require more purification steps, and the possible underlying mechanisms remain at the moment unknown.

Another physically-derived strategy to trigger EV release is to rely on mechanical stress for the stimulation. In 2017, our team demonstrated the feasibility of EV release from endothelial cells induced by a laminar flow in a microfluidic chip³¹. As this fluidics-based work is not easily scalable, we next developed shear stress induced vesiculation in a turbulent flow in spinner flasks by increasing the stirring speed, combining a large-scale culture platform and a 10-fold increase of particle/cell yield compared to classic starvation^{89,90} (WO2019002608). Typically, about 1E13 particles were obtained from 3.9E8 murine MSCs cultured on Cytodex 1 beads in a 1 L spinner flask after 4 h of turbulence stimulation^{91,92}. Administered in a thermoresponsive gel, these EVs were shown to decrease fistula output and external orifice diameter in a rat model of colcutaneous post-surgical fistula⁹¹. These EVs were also loaded with meta(tetrahydroxyphenyl)chlorin (mTHPC), a photosensitizer used for photodynamic therapy and injected intraperitoneally in mice with colorectal and ovarian carcinomatosis, showing enhanced tumor specificity⁹². After laser irradiation, survival was improved compared to free drug but also to the liposomal formulation of mTHPC. Patel *et al.*⁸⁸ also used shear stress to stimulate EV production in a 3D printed perfusion bioreactor, which they infused with a 4 mL/min EV-depleted media flow (corresponding to shear stresses between 1.5E-2 dyn/cm² and 1.3 dyn/cm²), and obtained a 20-fold increase in particles after isolation from human dermal microvascular endothelial cells (HDMEC) compared to the classic flask culture, going from 4E9 particles to 8.7E10 particles in 48 h from 1 million cells.⁸⁸ Interestingly, they also stimulated the endothelial cells with 100 mM ethanol and compared the results to the non-stimulated samples. The number of particles produced in the presence of ethanol was similar, but the pro-vascularization activity of EVs produced with ethanol conditioning was superior. Although this study was not yet adapted to large scales, it evidenced the feasibility and potential of perfusion bioreactor for production of therapeutic EVs.

Table 2: Recapitulative table of stimulation methods to increase EV produced per cell

Stimulation		Cells	Treatment duration	Production media	Isolation method	Measurement method	Enhancement factor (stim/control)	Ref
Hypoxia	1% O ₂	MCF7	48h	EV-depleted	UC	Particles in isolated EVs (NTA)	1.44	39
	0.1% O ₂		24h				1.77	
	0.5% O ₂	mMSC	24h	Serum-free	x	Particles in supernatant (NTA)	1.2	
Low pH	pH 4	HEK293	72h	EV-depleted	Exoquick	Extracted proteins in supernatant (BCA)	5	80
	pH 6	Melanoma cells	48h/72h/96h	EV-depleted	UC + sucrose gradient	Proteins in isolated EVs (BCA)	2 / 3 /7.5	79
Glucose deprivation		H9C2	48h	Serum free	UC	CD9/ CD81/ CD63 band intensity in WB	~3	87
Heat shock	42°C 10min-1h	3LL	4h	Serum-free	UC	Proteins in isolated EVs	2.3	81
Shear stress	Medium flowing in 3D printed bioreactor	HDMECs	72h	EV-depleted	UC + spin column	Particles in isolated EVs (NTA)	22	88
Ethanol	100 mM	Hepatocytes	48h,72h	EV-depleted	Exoquick	Particles in supernatant (NTA)	7 / 5	41
	50mM, 100 mM	monocytes	48h	EV-depleted	Exoquick	Particles in supernatant (NTA)	4	68
Cytokines	TNF- α (50ng/mL)	Neutrophils	20min	PBS	x	Particles in supernatant (Imagestream)	3.5	30
	IL8 (50ng/mL)						3.25	
	LTB4 (10nM)						3	
Cytoskeleton disruption	Cytochalasin D						3.4	
	ML7						2.8	
Anti-inflammatory drug	Aspirin 2.5mM	MDA-MB-231	24h	Serum free	x	Particles in supernatant (NTA)	5.6	84
	Celecoxib 20 μ M						23.7	
	Chloroquine 20 μ M						10.2	
Low intensity ultrasound	0.5W/cm ² 1h	A2780	6h	Serum-free	Exoquick	Proteins in isolated EVs	1.75	82
Laser irradiation	80J/cm ²	HUVEC	48h	EV-depleted	X	CD63 in supernatant	1.8	83
Incubation with SPIOs encapsulated in PLGA-PEI polymer		hMSC	24h	Complete	Exoquick	CD9/ CD81/ CD63 band intensity in WB	~2.5	85
Incubation with liposomes	Neutral	C26 / B13BL6 /MKL45/DLD1	48h	EV-depleted	Exoquick	Proteins in isolated EVs	~2.5	86
	Cationic						~3.5	

It is to be noted that different stresses may have different effects depending on the type of cells. For example, ethanol conditioning induced increased EV release in monocytes and hepatocytes whereas it did not change EV yield for endothelial cells, although the set-ups were very different. The rationale for the stimulation of the cells usually comes from physiological or pathological conditions, such as the use of shear stress on endothelial cells⁹³, or the treatment of hepatocytes with ethanol to mimic binge drinking⁴¹. Notably, stress-mediated EV production have often been reported on cancerous cells as a proof-of-concept, because they are often easier to cultivate than primary cells or stem cells. Besides, it was also shown that EV release from cancer cells can be triggered *in vitro* and *in vivo* by the cancer therapy itself, such as chemotherapy⁹⁴ or photodynamic therapy⁹⁵. Interestingly, increased vesiculation in response to stress was also demonstrated in Gram negative bacteria, as a defensive mechanism to improve survival in a hostile environment⁹⁶. For example, *Staphylococcus aureus* EV production, quantified by protein yield, was increased approximately three times by low temperature (30°C), oxidative stress, in iron-depleted media, or at sub-inhibitory concentration of antibiotic, which are stresses that may occur as the pathogen is infecting a new host⁹⁷.

Although EVs produced by stimulation are often reported to have similar physical characteristics (size, zeta potential, topology observed by TEM) compared to spontaneously secreted EVs, their protein and RNA contents may differ^{42,43}. Nevertheless, the main stake of manufacturing EVs is to produce therapeutically potent vesicles, which is why most works report the efficacy of their stress-mediated produced EVs in potency assays, that we will describe later.

In the optics to potentiate the EVs produced, some strategies have been adopted which may be contrary to the dogma that cells must remain alive during EV production. Indeed, while some authors warned that apoptotic bodies contamination may hinder therapeutic effect of EV preparations⁵³, HJ Ankersmit's team has shown that the secretome of apoptotic peripheral blood mononuclear cells (PBMC) are efficient for skin regeneration and angiogenesis⁹⁸, and also has immunomodulatory capabilities⁹⁹, which makes this EV-containing product a very promising treatment, with possibility of scale-up as the source cells are readily available in large quantities. Although not fully elucidated, inducing apoptosis in blood cells has already been used in clinical settings for decades to treat inflammatory diseases when using the extracorporeal photopheresis method¹⁰⁰.

4.2 EV-like particles

To address the issue of production and reproducibility, some works reported other strategies to create EV-like particles by completely disrupting the cell membrane integrity, releasing self-assembled particles with a yield up to 100 times superior to EV, which was recently reviewed in Lu et Huang¹⁰¹. Yet, direct comparison between yields is not as straightforward as for chemical and physical stimulation because EV are secreted in culture media and often purified in a different way than EV-like particles, that are produced directly in PBS. Protein and particle amounts measured for production techniques of EV-like particles, all using PBS as production media, are reported in **Table 3**.

Table 3: High-yield production of EV-like particles

Treatment	Cells	Nb of cells	Volume	Isolation method	Total protein	Total particle	Ref
Extrusion through 10-5-1µm filters	Murine ESC	1E8	1mL	Density gradient	952µg	X	32
	U937	1E7	1mL	Density gradient	203µg	2.1E11	69
	MCF-10A	1E7	1mL	Density gradient	379µg	3E11	105
	BM-MSC	4E6/mL	X	Density gradient	161µg	5.18E10	102
	mouse hepatocytes	5E6	1mL	Density gradient	110µg	x	106
Spin cups	U937	2E7	1mL	SEC	540µg	x	104
Microfluidic channels	Murine ESC	1.5E6	1mL	Density gradient	115µg	x	103
Slicing blades	Murine ESC	1E7	1mL	SEC + Amicon	3.5µg	1E11	33
Hypotonic shock + homogeneization + sonication (Nanoghosts)	hMSC, rat MSC and hSMC	x	x	UC	x	x	107
2mM PFA + 2mM DTT 2h (extracellular blebs)	Mouse lymphoma cells	1E7cells/mL	x	30Kda centrifugal tubes	100µg/mL	x	108

The most common way to disrupt cells is by extrusion, which consists in sequential filtration through smaller and smaller pore size (from 10 µm to 1 µm), and this has been done with suspension⁶⁹ or adherent cells^{32,69,102} to form particles termed nanovesicles (NVs). Other studies have reported the use of microfluidic chips, in which cells are forced through channels smaller than their diameter¹⁰³, or containing micro fabricated silicon nitride blades slicing the cell membrane³³. All these processes can be seen as a trade-off between high yield, purity, production time and therapeutic potency. Indeed, cells can be highly concentrated within a small volume of particle-free PBS and thus will yield a considerable amount of self-assembled particles in a very short time as they are extruded, also releasing their intracellular content. Purification has then to be more thorough to separate contaminants from NVs, such as density gradient or SEC, but is usually easier to perform because of the smaller volumes. Goh et al¹⁰⁴ advocated that the production time reduced from 48 h to 1 h combined to the use of affordable spin cups and a simple step of size exclusion chromatography (compared to differential ultracentrifugation to isolate the particles) render their technique a cost-effective method to produce EV-like particles at large scale.

NVs can be used as drug delivery vehicles which can encapsulate siRNA¹⁰⁵ or chemotherapeutics, such as doxorubicin or paclitaxel^{69,102} and have also shown therapeutic potential *in vivo* for immunotherapy^{109,110} and liver regeneration¹⁰⁶.

Alternatively, EV-like particles can be manufactured from the cellular membrane of cells deprived of cytoplasm and nuclei. These empty nanovesicles, named nanoghosts, were historically produced from MSCs¹⁰⁷. They have recently been also produced from other cell types such as macrophages¹¹¹ or monocytes¹¹². Oieni *et al.*¹¹³

evaluated the scalability and robustness of the nanoghost technology. The first step consists in the manufacture of ghost cells, by hypotonic treatment for 5-20 min followed by an important homogenization step to remove cytosolic content. Then, the membranes are downsized by extrusion or sonication to release the nanoghosts. Coefficients of variance of the main parameters were indicated for over 40 productions: 57 % and 49 % for the lipids and proteins yield respectively, 59 % for the purity and 8% for the size, which were considered correct if we consider the sources of variability introduced during the process such as the origin the cells or the operators. According to the authors, this robust production platform could be scalable, by using high pressure homogenization systems and microfluidics as well as continuous flow ultracentrifuge for isolation.

Finally, extracellular blebs (EBs) were also described as a promising alternative to EVs. Cellular blebbing has been studied since the 1970's, but it has only been recently proposed as a way to produce EV-like particles by irreversible blebbing¹¹⁴. EBs represent an interesting option because they are produced in the presence of a chemical agent such as paraformaldehyde (PFA) or N-Ethylmaleimide (NEM), as quickly as EV-like particles, but without release of intracellular contents. On the other hand, their isolation will require careful removal of these products. In 2018, Ingato *et al.*¹⁰⁸ reported a nearly ten-fold increase in protein yield between EB and EV, as well as an enhanced stability.

5. Comparability between technologies

After the description of the numerous strategies to address the issue of scaling up EV production, the remaining task is to find a way to compare these technologies. Indeed, the lack of standardization in the processes of isolation and characterization generates a significant variability in the reporting of EV yield in terms of protein content or particle numbers at different stages of EV isolation. This amounts to poor comparability between technologies as differences in EV yield and purity may highly depend on those aspects. Nonetheless, after a more detailed discussion on EV quantification methods, we will introduce the calculation of a yield factor from only a selected number of publications which quantified EVs in a similar way, and comment on its relevance. We also provide a flowchart of possible production methods in **Figure 2**.

5.1 Quantification methods

Among a variety of techniques that have been developed to detect and quantify EVs¹¹⁵, Nanoparticle Tracking Analysis (NTA) and total protein dosage, using colorimetric techniques such as Bradford reagent or bicinchoninic acid (BCA) are the most commonly used to report EV yield. However, these techniques are not specific to EVs. For instance, NTA measures any particle in suspension, including agglomerate of proteins possibly originating from soluble components. Although it is possible to perform labeling with specific fluorescent markers in some set ups of NTA, the lack of report of such data in EV production papers shows this is not a widespread method, contrary to non-specific light scattering of all particles in suspension. Moreover, results obtained from two NTA instruments by the same operator were found different unless settings were optimized¹¹⁶, underlying the variability of particle concentration

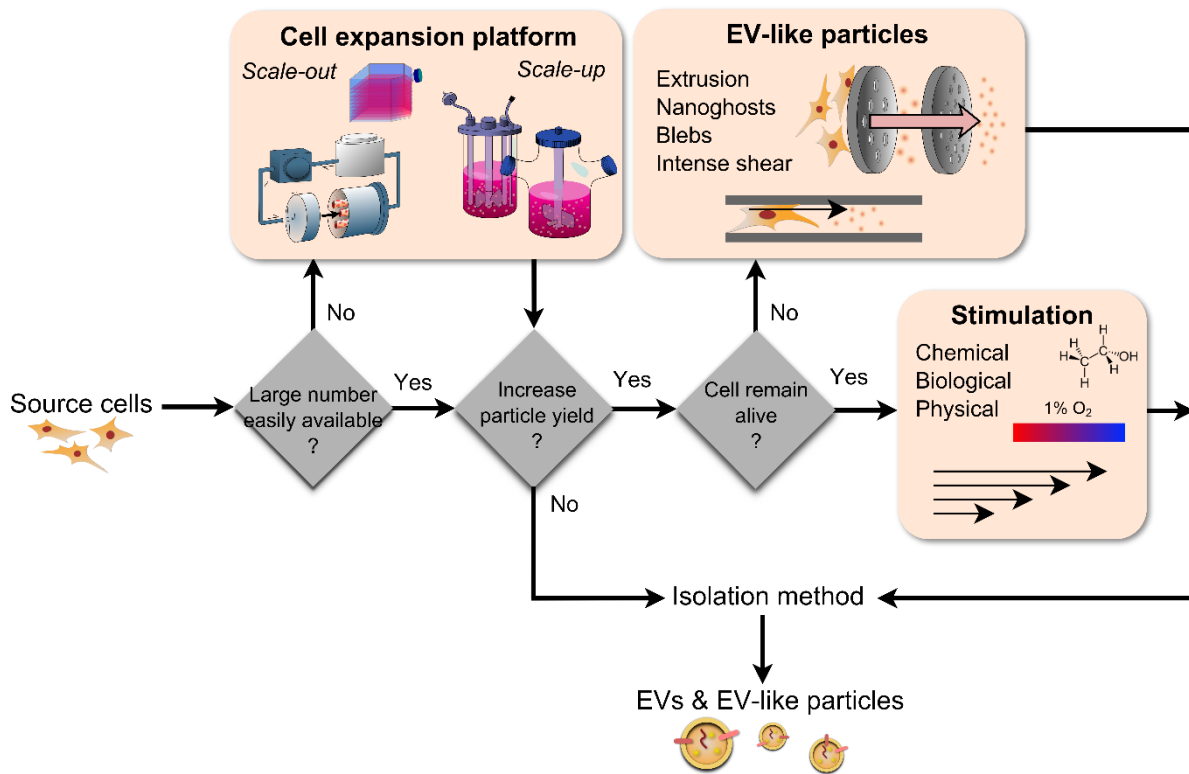


Figure 2 : Flowchart of high-yield EVs and EV-like particles production strategies

measurements between laboratories. Calibration according to commercially available bead standards may improve reproducibility but accurate EV concentration standards would be ideal. By contrast, protein dosage in reference to a calibration curve may be more reproducible from one laboratory to another, but contaminating proteins and interfering agents, such as phenol red or glucose for BCA and detergents for Bradford reagent, may lead to an over-estimation of protein concentration. Absolute values measured for particle or protein concentration thus need to be critically evaluated and compared to control condition. Even then, as these control conditions may have been performed using different media (EV-depleted or serum-free), cell density, etc. such strategy is still limited for inter-studies comparisons.

Another interesting parameter to systematically report is the ratio of particles measured over proteins. However, this parameter is not easily interpreted, as was previously observed. Indeed, considered as a purity parameter for some time for EVs isolated from biological fluids¹¹⁷, it is not so relevant as a purity estimator in the case of starvation EVs because the media in itself does not contain many proteins. In this case, increase of particle over protein ratio would rather indicate a loss of protein content from EVs. As this ratio can depend on the production method⁸⁸, it also means that fold change in protein content or in particle concentration compared to control may not be the same, and should probably be both reported separately.

Additionally, quantification of markers enriched in EVs such as CD63, CD9 or CD81 with Western Blot or ELISA tests have been reported. As these techniques are less common, it is difficult to compare between studies, but these results can mitigate and complete a yield obtained in particles or proteins⁸⁸. Similarly, even though many new

technologies have emerged to quantify EVs, including flow nanocytometry or tunable Resistive Pulse Sensing, reviewed elsewhere¹¹⁸, these tools are not yet widely used and hindsight is lacking regarding their reproducibility across laboratories. Moreover, considering affordability, NTA and protein assays might remain the gold standard for quantification of EVs for some time, hence the importance of establishing more standardized procedures with controls and calibration solutions. Combination of different methods to characterize EVs might also continue to be necessary to analyze with better accuracy the identity and properties of EVs.

Taking all this into account, it appears that a yield parameter integrating the many variables at play could be a useful tool to compare large-scale production techniques. The estimation next provided is a tentative approach that may provide valuable insights for comparison.

5.2 Definition of a yield parameter and application to large-scale approaches

From a strictly quantitative point of view, EV production is a process which takes as an input a certain amount of cells and yields a number of EVs in a given time, as represented in **Figure 3**. Thus, one can calculate EV yield as

$$(1) \quad r = \frac{\text{Total isolated EV}}{\text{Initial cell number} \times \text{Time}}$$

Here, a particle yield factor has been calculated in amounts of particles per cell per day and a protein yield factor in micrograms of proteins per cell per day.

Depending on the strategy used, cells can be expanded on a culture platform or directly used as raw materials, for instance for extrusion, but in our definition of yield this will only impact the total duration of the process. For example, cell expansion time was calculated for hyperflasks according to seeded cell number (1E8 cells), final cell number measured (700E8 cells) and doubling time (52 h) of CPC.

Table 4 first contains representative yield factors calculated for studies that disclosed sufficient data, with time expressed in days. Importantly, yield factors do not only depend on the production method but is also impacted by the downstream process. This underlines the importance of reporting EV number before isolation in order to better compare different approaches. According to the literature, we can estimate that UC and Density gradient are low yield isolation methods¹¹⁹ while SEC is usually reported to have better yield than density gradient while obtaining similar purity¹²⁰ and retains a degree of flexibility with a possible trade-off between higher recovery and more efficient isolation from soluble factors¹²¹. On the other hand, TFF enrichment process can recover between 7-fold and 20-fold more particles than UC³⁴. Considering this, the 3D printed perfusion bioreactor, turbulence in spinner flask as well as most extrusion techniques seem to be promising in terms of quantity of particles obtained at the end of the process, and the 3D printed bioreactor specifically for protein yield. Interestingly, Quantum Bioreactor and Fibercell hollow fiber bioreactors, despite being based on the same technology, have very different yields. This is due to the high number of cells seeded in Fibercell systems compared to Quantum bioreactor, which is seeded with only 20 million cells. Moreover, harvests are performed every 48 h for Quantum bioreactor, which may lead to highest yields.

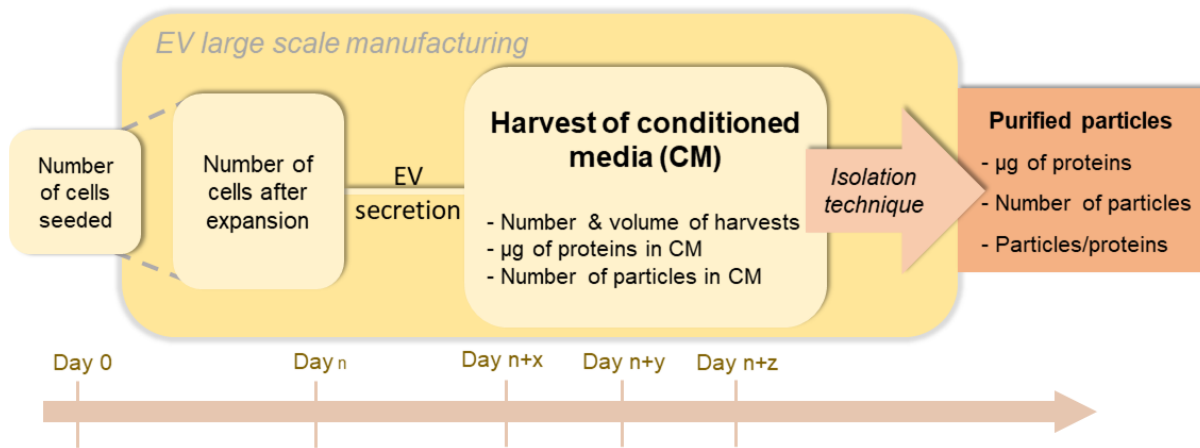


Figure 3 : EV large-scale manufacturing process from a quantitative point of view and suggested information to be reported

Despite the fact that measurements are very difficult to compare from one study to another, because of variations in instruments, settings and operators, as well as different isolation processes, we proposed here the calculation of a factor that would enable us to compare different scale-up strategies solely on particle and protein yield. To implement this tool to future studies, the careful report of parameters listed in **Figure 3** would be required. Moreover, as basal protein concentrations in EV-depleted and serum free production media may add a bias in the results, reporting the initial number of particles in the medium at the beginning of EV production would be critical.

However, particle and protein yields are not sufficient to assess the best process for clinical translation. Indeed, adaptation from 2D to 3D culture and physico-chemical stimulations, which impact EV release, also affect EV identity and function probably by activating specific pathways within the mother cells before EV secretion. Besides size, concentration and in a way purity, these changes can only be assessed with additional tools than NTA and protein assays, including flow cytometry, ELISA, omics technologies and potency assays. Further reports of such results would allow meta-analysis of data, giving an insight into the link between production process, EV identity and functionality.

5.3 Evaluation of functional activity of EVs

In the end, the foremost concern when evaluating production strategies from a translational point of view is probably EV therapeutic potency. Therefore, **Table 5** introduces potency assays performed for scale-up approaches, with the aim of providing a more complete description of the prospects of the different methods.

Recognized as biological vectors and actors of paracrine effects, EVs may have several types of bioactivity, mainly their potency as delivery vehicles, their regenerative activity or their immunomodulatory capability. While the internalization of EVs or EV-like particles inside recipient cells is reported at minima in most studies^{62,79,82,86}, Jo *et al.* also demonstrated the transfer of endogenous RNA content from EVs to cells to propose their NVs as EV-mimetic alternatives for drug delivery^{32,103}. Another approach is to load EVs with exogenous content in order to study the delivery of this specific cargo, as opposed to the use of naïve EVs. For instance, siRNA have been

Table 4 : Comparison of yield factors for large-scale production strategies

Technology	EV number	Protein mass (µg)	Cells seeded	Time	Particle yield factor (part/cell/day)	Protein yield factor (µg/cell)	Isolation method	Volume processed	Ref
3D printed perfusion bioreactor	8.7E10	1E3	1.13E6	3 days	26,000	3E-4	UC	50 mL	88
Extrusion	2.1E11 – 3E11	2.1E2 – 3.8E2	1E7	<1 day	21,000-30,000	2-4E-5	Density gradient	1 mL	69,105
Quantum bioreactor	9.8E12 – 1.56E13	2E4 – 3E4	2E7	9 + 12 days	23,000 – 37,000	5-7E-5	No isolation	1.5 L	29
Hyperflasks	3E13	2.8E5	1E8	20 + 14 days	9,000	8E-5	TFF	8 L	60
Vertical Wheel	2.8E11 – 3.1E11 – 4.1E11	1.4E3 – 1E3 – 1.3E3	5E6	11 – 9 – 12 days	4,000 - 7,000	2E-5	No isolation	60 mL	28
Fibercell	1.2E13	8E3	2E8	30 days	2,000	1E-5	TFF	0.5 – 1 L	65
Turbulence in spinner flask	1,4E13	Not disclosed	3.9E8	<1 day	36,000	Not disclosed	UC	< 1 L	92

electroporated into EVs produced from Quantum Bioreactor²⁹, or into extrusion NVs¹⁰⁵, and successful transfer can be easily detected through the silencing of the target gene.

Overall, EV-like particles appear to be a very attractive option for drug delivery applications, such as targeted cancer therapy. In addition to a simpler and higher yield manufacturing process, they seem to deliver cargo as efficiently as 2D EVs, at least *in vitro*^{32,69,105}. *In vivo*, chemotherapeutics such as doxorubicin⁶⁹, paclitaxel¹⁰² and sTRAIL¹⁰⁷ as well as siRNA¹⁰⁵ loaded into NVs, nanoghosts or EBs¹⁰⁸ were conveyed more selectively to tumor sites than free materials and inhibition of tumor growth and prolonged survival was thus observed in several mice cancer models. Moreover, plasmid DNA loaded in nanoghosts was transferred to recipient cells *in vitro* and *in vivo*¹²³, evidencing the potential of EV-like particles in gene therapy. On the other hand, despite the common conception that EV-like particles are not as intrinsically bioactive as EVs, Wu *et al.* also demonstrated that primary hepatocyte derived NVs produced by extrusion possessed intrinsic liver regeneration properties after partial hepatectomy on mice¹⁰⁶, which only adds further potential to this approach.

Furthermore, 3D culture platforms or chemical and physical stimulation of mother cells have notably been reported to enhance EV potency compared to control 2D EVs in some studies, notably for regeneration of cartilage⁶⁴, kidney¹²², skin⁸⁸ and heart⁷⁷, and for immunostimulation^{68,81}. This is why despite the fact that EV production in Fibercell bioreactor or induced by chemical and physical stresses may not yield as many EVs as other strategies, they remain full of potential to obtain specific phenotypes in EVs. In this perspective, Patel *et al.* combined the two approaches, manufacturing EVs in a scalable platform which have enhanced therapeutic efficacy due to chemical stimulation by ethanol⁸⁸. Similarly, our team used stirred tank bioreactors to stimulate cells *via* shear stress and produce MSC EVs with regenerative properties for fistula therapy⁹¹, and potential as drug delivery vehicles with enhanced tumor specificity for peritoneal carcinomatosis⁹². Ultimately, the overview of the functional activity of large-scale produced EVs confirmed the interest towards these approaches. Among them, EV-like particles strategies and 3D culture potentially combined with priming of mother cells emerge as really promising. Having considered this, we will next present large-scale production platforms which are being implemented in current clinical studies.

6. Production platforms for clinical studies

As of now, 23 Phase I / II clinical trials are registered on clinicaltrials.gov for testing the treatment of a pathology using human cells derived EVs. The university of Kentucky also has 3 ongoing trials testing plant EVs, and furthermore, there are 9 trials registered with EV-containing products (conditioned media for instance). For most of them, only dozens of patients were treated, with reported doses of EVs in the range of 1E8 – 1E10 EVs per injection per patient, which requires medium-scale manufacturing and no specific scale-up strategy was used. However, while this may be feasible for up to about a hundred participants, depending on the dose of EVs, for later stages of trials, large-scale manufacturing processes need to be developed. Ongoing clinical trials for EV-based treatments with more than 90 patients or clear scale-up strategies are indicated in **Table 6**, EV number being reported without disclosure of measurement method.

Table 5: Functional activity of EVs or EV-like particles produced by large-scale approaches. Blue: Delivery potency; Pink: regenerative properties; Orange: immunomodulatory effect.

Approach	Naïve or engineered EVs	Potency in vitro	Potency in vivo	Comparison 2D EVs	Ref
Cell culture platforms					
Hollow fiber	hetIL15 on HEK293-EV surface	Uptake and Natural killer cell activation	Uptake in tumors in mice with breast cancer	-	62
	Naïve UC-MSC EVs	Stimulation of chondrocyte proliferation, migration, matrix synthesis	Regenerative effect in a cartilage defect model in rabbit	Enhanced	64
	Naïve UC-MSC EVs	Uptake and increase of viability in tubular epithelial cells treated with cisplatin	Renoprotective and anti-inflammatory effect in a cisplatin induced model of acute kidney injury in mice	Enhanced	122
Hyperflasks	Naïve BM-MSC	Decrease in T lymphocyte activation (dependent of donor)	Improvement of graft-versus-host disease symptoms in mice (dependent on donor)	-	59
	Naïve CPC EVs	Anti-apoptotic effect on staurosporine treated CPC and pro-angiogenic activity	Regenerative efficacy in rat model of permanent coronary artery occlusion	-	60
Spinner flasks	UC-MSC EVs loaded with siRNA targeting <i>Huntingtin</i> gene	Efficient <i>Huntingtin</i> silencing by siRNA transfer in neurons	-	Enhanced	34
Quantum bioreactor	MSC EV electroporated with siRNA targeting oncogenic <i>Kras</i> gene	Induction of apoptosis in pancreatic cancer cells with <i>Kras</i> mutation and downregulation of <i>Kras</i> expression	Improve survival and decrease metastatic burden in pancreatic ductal adenocarcinoma model on mice	Similar	29
CELLine	Naïve mesothelial cell derived EVs	Anti-proliferative effect on lymphocytes	-	Similar	57
Chemical & Physical stimulation of cells					
Shear stress in perfusion bioreactor	Naïve and ethanol treated HDMEC EVs	Increased gap closure for dermal microvascular endothelial cells	-	Enhanced	88
Shear stress in spinner flasks	Naïve murine MSC EVs administered in thermoresponsive gel	-	Decreased fistula output and orifice diameter in rat model of colo-cutaneous post-surgical fistula	-	91
	mTHPC loaded murine MSC EVs	-	Increased tumoral selectivity and prolonged survival in mice colorectal carcinomatosis model	-	92
Hypoxia	Naïve murine MSC EVs	Uptake and decreased apoptosis in cardiomyoblasts, pro-angiogenic activity	Cardiac protective activity and prolonged survival in a mice infarct model	Enhanced	77
Low pH	Naïve melanoma cells EVs	Uptake by tumor cells	-	Enhanced	79
Glucose deprivation	Naïve H9C2 EVs	Pro-angiogenic activity	-	Enhanced	87
Heat shock	Naïve 3LL EVs	Chemoattraction and activation of dendritic cells and T cells	Increased infiltration of T cells and dendritic cells into tumor, inhibition of tumor growth an prolonged survival in mice lung cancer model	Enhanced	81

Ethanol	Naïve hepatocyte EVs	Uptake in monocytes and transfer of miRNA-122, increasing pro-inflammatory effect in the presence of LPS	-	Enhanced	41
	Naïve monocyte EVs	Stimulation of naïve monocytes towards M2 polarization	-	Enhanced	68
Low intensity ultrasound	Naïve ovarian cancer cell EVs	-	Biodistribution of labeled EVs in mice, no immunogenicity	Similar	82
Incubation with liposomes	Naïve EVs secreted in the presence of cationic bare liposomes	Uptake in cancer cells	-	Enhanced or reduced (dependent on liposome)	86
<i>EV-like particles</i>					
Extrusion	Naïve mES EVs	Uptake in epithelial cells and transfer of endogenous RNA	-	Similar	32
	MCF-10A EVs electroporated with CDK4 targeting siRNA	Uptake, downregulation of CDK4 and arrest of cell cycle in MCF-7 cells	Increased tumoral selectivity and tumor growth inhibition in subcutaneous MCF-7 tumor xenograft mice	Similar (in vitro)	105
	U937 and Raw264.7 NVs loaded with doxorubicin	Delivery of doxorubicin to TNF- α endothelial cells inducing cytotoxicity	Increased tumoral selectivity and anti-tumoral effect on mice colorectal cancer model	Similar (in vitro)	69
	BM-MSC NVs loaded with paclitaxel	Delivery of paclitaxel to breast cancer and normal breast cells inducing cytotoxicity	Reduced tumor growth mice breast cancer model	-	102
	Naïve murine primary hepatocytes NVs	Increase proliferation of primary hepatocytes	Liver regenerative effect after partial hepatectomy in mice	-	106
Microfluidic channels	mESC NVs	Uptake in fibroblast and transfer of endogenous RNA	-	Similar	103
Slicing blades	mESCs NVs loaded with polystyrene fluorescent beads	Delivery of encapsulated beads to fibroblasts	-	-	33
Nanoghosts	sTRAIL loaded MSC-NGs	Uptake and cytotoxicity in prostate cancer cells	Accumulation of NGs in tumor and in liver, inhibition of tumor growth in mice prostate cancer model	-	107
	cancer-toxic gene encoding pDNA loaded MSC-NGs	Gene delivery of pDNA inducing cytotoxicity in prostate cancer cells	Inhibition of tumor growth and metastasis, prolonged survival in mice prostate and lung cancer	-	123
PFA + DTT (extracellular blebs)	Doxorubicin loaded EL4 EBs	Release of doxorubicin from EBs inducing cytotoxicity on EL-4 cells	Reduced tumor growth and prolonged survival in mice lymphoma cancer model	-	108

Table 6: Scale-up strategies adopted in clinics

Company / Institute	Phase /III clinical study	Nb of patients	Number of EVs (total)	Treatment per patient	Source of EVs	Scale-up strategies reported in patent or publication by main investigator
Codiak Biosciences	A First-in-Human Study of CDK-002 (exoSTING) in Subjects With Advanced/Metastatic, Recurrent, Injectable Solid Tumors (NCT04592484)	180	Not disclosed	Not disclosed	hMSC	Quantum bioreactor, Terumo WAVE bioreactor 50L fed batch bioreactor
Ruijin Hospital	A Clinical Study of Mesenchymal Stem Cell Exosomes Nebulizer for the Treatment of ARDS (NCT04602104)	169	2E12	2E8 / 8E8 / 16E8 EVs inhaled every day for 7 days	hMSC	Not disclosed
Samara Regional Medical Center Dinasty	Safety and Efficiency of Method of Exosome Inhalation in COVID-19 Associated Pneumonia (NCT04602442)	90	2.5E13	0.5 – 2E10 EVs inhaled twice a day for 10 days	hMSC	Hollow fiber or microcarrier based approach
Exopharm	PLEXOVAL II : safety and benefits in wound healing of platelet derived EVs	15	Not disclosed	Not disclosed	Platelets	EVs isolated directly from donors platelets with patented isolation technology LEAP
James Graham Brown Cancer Center	Plant Exosomes and Patients Diagnosed With Polycystic Ovary Syndrome (PCOS) 17 (NCT03493984)	176	Not disclosed	Not disclosed	Ginger EV-like particles, <i>Aloe vera</i> EV-like particles	Raw materials available in large quantities
Aposcience	A Study to Evaluate Safety and Efficacy of APO-2 at Three Different Doses in Patients With Diabetic Foot Ulcer (NCT04277598)	132	EV-like particles produced by 50E10 PBMC	0.5 mL per cm ² of wound of secretome from 12.5 / 25 / 50 million PBMC 3 times a week for 4 weeks	PBMC	Raw materials available in large quantities

The production platforms which have been identified are reviewed in the following, acknowledging however that many companies prefer not to disclose details about their production methods and their yield. Moreover, advancement of different cell expansion platforms towards clinical application is represented in **Figure 4**.

After a successful evaluation of safety in 25 healthy volunteers of exoIL12 (EVs exposing an active form of IL12 on the membrane)¹²⁴, Codiak BioSciences has started to recruit 180 participants to test their second engineered EV product for the treatment of solid metastatic tumors (NCT 04592484). Codiak Biosciences is one of the major pharmaceutical companies leading research on EVs and has a large portfolio of patents on EV technology. They have identified two EV-associated membrane proteins, PTGFRN (facing the exterior of the membrane) and BASP-1 (facing the lumen), which they use as scaffolds to link molecules of interest and engineer EVs to promote a specific therapeutic application. It is from this engineering platform, termed engEx™, that they created two of their more advanced products, exoSTING (EVs enriched in stimulator of interferon genes in the lumen), and exoIL12. Recently, the use of a Quantum bioreactor was reported to produce 1E13 particles in Pr. Kalluri's team²⁹, who is a scientific advisor of Codiak Biosciences, suggesting the use of perfusion bioreactor from the company to scale up MSC-EV production. Moreover, Codiak patented the production of EVs in single-cell suspension within chemically defined culture media (US20190085284A1) in which they showed that they obtained 3E15 particles from a 2.4 L perfusion bioreactor containing 40E6 HEK cells/mL in 12 days, preferentially than from 50 L fed batch culture. On the other hand, the company also reported the use of Wave bioreactor for the large-scale culture of HEK293 and production of EVs, with 10 or 25 L working volumes¹²⁵.

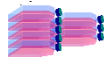
In the wake of the COVID19 sanitary crisis, the Ruijin Hospital, attached to the University of Medicine of Shanghai, has initiated several trials to study the safety, tolerability and efficacy of aerosol inhalation of allogenic MSC EVs for the treatment of pulmonary infection and Acute Respiratory Distress Syndrome in collaboration with Cellular Biomedicine Group (NCT04544215, NCT04276987, NCT04313647). Their largest trial so far (NCT04602104) will involve 169 participants divided in 6 groups. The manufacturing conditions of EVs are not detailed, however, as the inhalation dose proposed does not exceed 1.6E9 EVs, even with 7 inhalations planned per patient, this would at most require 2E12 EVs which is still manageable to produce with 2D flask culture. The Health Facility "Samara Regional Medical Center Dinasty" in Russia also completed a first trial evaluating safety and efficacy of inhaled MSC-EVs in Sars-CoV2 associated pneumonia. Their next trial, enrolling participants by invitation, may require up to 2.5E13 EVs which is more challenging to produce by conventional methods. Although no manufacturing method is described either, according to the patent filed by O. Tyumina concerning the process of obtaining and concentrating MSC-EVs, they could consider hollow fiber bioreactor or microcarriers approaches to scale up their culture¹²⁶ (RU2710368C2).

EV-like particles are also investigated in clinical trials, proposing an alternative to scale up with the use of more available sources of cells. For instance, three clinical studies from the James Graham Brown Cancer Center in Louisville, USA, are testing the efficacy of plant EV-like particles for the treatment of pathologies associated with

Strategies

SCALE-OUT

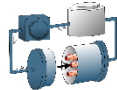
2D flasks



Hyperflasks



Hollow fiber bioreactor

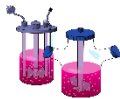


Integra CELLline

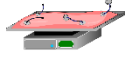


SCALE-UP

Stirred-tank bioreactor



WAVE bioreactor



Vertical wheel™ bioreactor

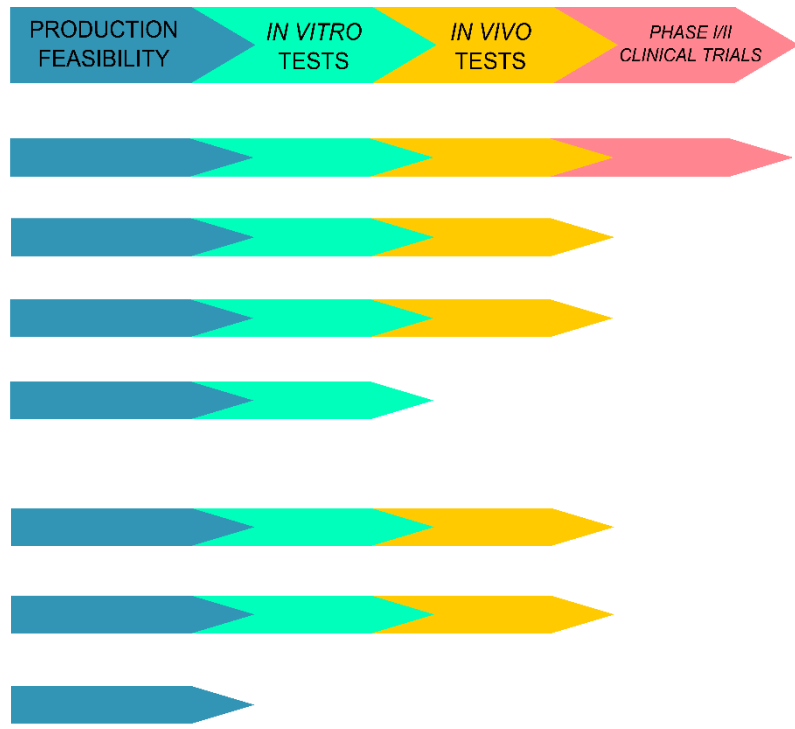


Figure 4: Pipelines of cell expansion platforms

cancer (NCT01294072, NCT03493984, NCT01668849), more specifically particles isolated from *aloe vera*, ginger or grape. While plant EVs may not have similar bioactivity than human cell derived EVs, they still can be used as drug delivery vehicles¹²⁷. On the other hand, the company Exopharm has initiated its second clinical trial, PLEXOVAL II, a Phase I study evaluating safety of platelet derived EVs for wound healing treatment with 20 participants¹²⁸. The company has patented their Ligand-based Exosomes Affinity Purification (LEAP) technology, which allows them to isolate EVs directly from large volumes of pooled platelet packs at a low cost and in less than one day. They manufactured 40 doses of EVs from 2 L of platelets in 8 h from purification to final product, and propose a scale-up strategy from 40 to 4,000 doses by upscaling the amount of raw materials, the equipment size and automated vial filling. The company also plans to develop their MSC EV product, isolated from stromal cell conditioned media by LEAP, and initiate Phase I clinical study. In a similar approach, Aposciences is developing a secretome product obtained from the irradiation of peripheral blood mononuclear cells, which has been shown to promote wound healing¹²⁹. The results of a Phase I study, MARYSAS I, showed the safety of administrating the secretome produced by 25E6 PBMC. MARYSAS II, now recruiting 132 participants with diabetic foot ulcer, will evaluate the safety and efficacy of APOSEC. The treatment will consist in topical administration of APOSEC at different dosages three times per week for 4 weeks. Because the raw material can be easily procured, the scalability of this approach is promising.

Finally, one can note that, except for Codiak BioSciences, the most advanced scale-up strategies which have been disclosed preferentially use large quantities of available raw materials and produce EV-like particles or secretome based products. This could be explained, as was pointed out in Beer et al.⁷⁵, by the fact that access to blood products entails a collaboration with blood banks, which already possess expertise in

GMP processes and certifications, facilitating and accelerating the clinical translation of EV-based products. Overcoming challenges of EV production and increasing robustness of characterization methods will hopefully pave the way towards a multiplication of large-scale EV clinical trials.

7. Conclusion

Several strategies have been tested to develop the large-scale manufacturing of EVs these last few years. Depending on the availability of cell source, expansion may be required on cellular platforms such as hollow fiber or stirred tank bioreactors. Chemical or physical stimulation can also be considered, as a way to increase EV yield. To drastically increase particle yield, another approach is to disrupt cell membrane and create auto-assembled EV-mimetic particles which retain EV drug delivery potency. Due to the lack of characterization standards, comparison between studies is complex and better comparison tools must be developed, which is why we proposed here a tentative approach to estimate yield factors that could be used to evaluate production methods. To this day, most large-scale clinical studies employ plant vesicles or EV-based products obtained from blood-derived cells, as they are more easily available source of vesicles. Most recent technological advances in production methods which report similar or enhanced EV therapeutic potency compared to EVs produced in flasks should facilitate the implementation of phase III clinical studies and ultimately the transition from cells to EV therapy.

Abbreviations

EV: extracellular vesicles
NV: nanovesicles
EB: extracellular blebs
MSC: mesenchymal stromal cells
MSC EV: mesenchymal stromal cell derived extracellular vesicles
UC MSC: umbilical cord derived mesenchymal stromal cells
BM MSC: bone marrow derived mesenchymal stromal cells
ASC: adipose tissue derived mesenchymal stromal cells
HDMEC: human dermal microvascular endothelial cells
HUVEC: human umbilical vein endothelial cells
CPC: cardiac progenitor cells
HEK293: human embryonic kidney 293 cells
PBMC: peripheral blood mononuclear cells
RBC: red blood cells
MWCO: molecular weight cut off
NTA: Nanoparticle Tracking Analysis
BCA: bicinchoninic acid
ELISA: enzyme-linked immunosorbent assay
TEM: transmission electron microscopy
UC : ultracentrifugation
TFF: tangential flow filtration
SEC: Size exclusion chromatography
GMP: Good Manufacturing Practices
hetIL-15: interleukin 15 in heterodimeric form
TNF- α : tumor necrosis factor alpha
IL8: interleukin 8
LTB4: leukotriene B4
PEG: polyethylene glycol
PLGA-PEI: poly(lactic-co-glycolic acid) - polyethylenimine
PFA: paraformaldehyde
DTT: dithiothreitol
mTHPC: meta(tetrahydroxyphenyl)chlorin
sTRAIL: soluble form of tumor necrosis factor-related apoptosis-inducing ligand
PTGFRN : Prostaglandin F2 receptor negative regulator
BASP-1 : Brain acid soluble protein 1

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