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Review

Scalable production and isolation of extracellular vesicles: available sources and lessons from current industrial bioprocesses

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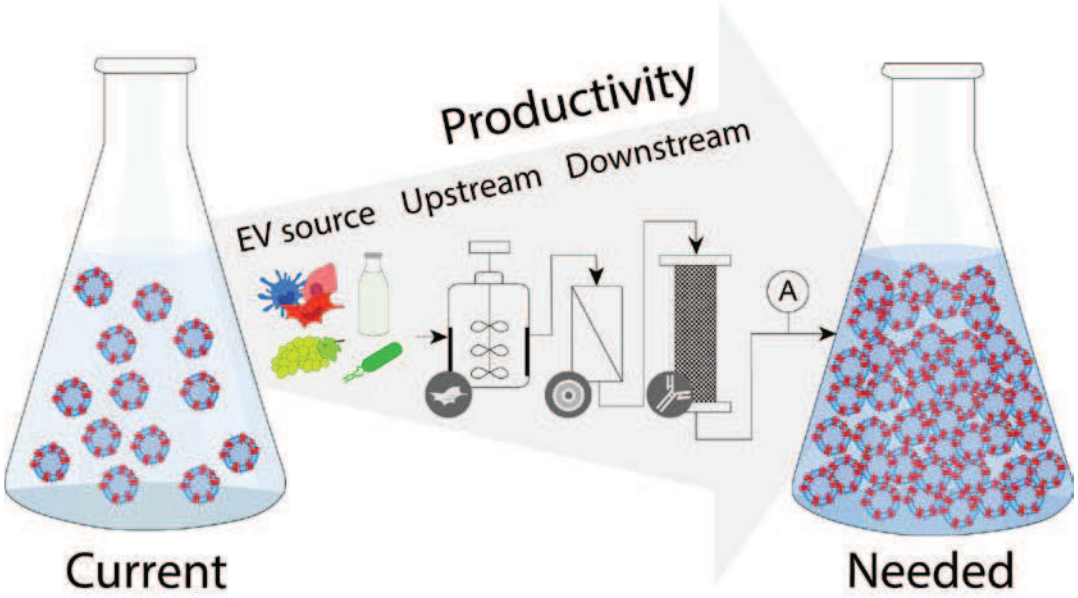
Abbreviations: **EV**, extracellular vesicle; **UC**, ultracentrifugation; **UF**, ultrafiltration; **SEC**, size-exclusion chromatography; **GMP**, good manufacturing practices; **CHO**, Chinese hamster ovary; **HFBR**, hollow fiber bioreactor; **FBS**, fetal bovine serum; **TFF**, tangential flow filtration; **PEG**, polyethylene glycol; **AIEX**, anion exchange chromatography; **BSA**, bovine serum albumin; **AC**, affinity chromatography

Abstract

Potential applications of extracellular vesicles (EVs) are attracting increasing interest in the fields of medicine, cosmetics and nutrition. However, manufacturing of EVs is currently characterized by low yields. This limitation severely hampers progress in research at the laboratory and clinical scale, as well as the realization of successful and cost-effective EV-based products. Moreover, the high level of heterogeneity of EVs further complicates reproducible manufacturing at large scale. In this review, we discuss possible directions towards the scalable production of EVs. In particular, we consider two strategies: (i) the optimization of upstream unit operations and (ii) the exploitation of well-established and mature technologies already in use in other industrial bioprocesses.

Graphical Abstract

Extracellular vesicles have great potential for applications in medicine, cosmetics and nutrition. Yet, severe limitations in their production on large scale considerably hamper advancements in the field. In this review, the authors discuss the state-of-the-art technologies for scalable production of EVs and provide the basis for further process optimisation.



1 Introduction

Extracellular vesicles (EVs) have been shown to transfer biomolecules such as lipids, proteins and RNAs to other cells, distal organs and even to other organisms[1–3]. Flourishing EV research can potentially open many new possibilities in the fields of medicine, cosmetics and nutrition[4]. For instance, EVs have the potential to naturally perform cell-specific drug release[5]. This feature would allow to overcome the limitations of existing commercial liposome-based formulations[6] which still do not exhibit this specificity[7]. The specificity and selectivity of EVs arise largely from the incredibly rich EV biocargo which include different biomolecules such as surface proteins, RNAs and lipids, which cooperate to target and deliver biomolecules to specific cells in a selective way[4]. The type of encoded message depends on the cell releasing the EV. For instance, stem cells release EVs to stimulate tissue regeneration[2]. Similarly, dendritic cells produce EVs to regulate immune responses[8]. In addition to functional biology, release of EVs can also be associated to pathology. For instance, EVs containing amyloid-β-derived-peptides can contribute to the progress of Alzheimer's disease[9] and, in the case of certain cancer cells, EVs can even induce metastasis[10]. This feature makes EVs key players in several physiological and pathological processes and attractive candidates for many therapeutic applications[2,8,11]. Being naturally present in every

living organism, endogenous EVs at physiological concentration are also intrinsically characterized by low toxicity, high stability, biocompatibility and permeability to biological barriers[5,12,13]. Hence, increasing research efforts have been devoted to evaluate the outstanding potentials of EVs as therapeutic agents[2,14], diagnostic tools for liquid biopsy[11,15], delivery systems for drugs[5,12,13,16], cosmetics[13] or nutraceuticals[17].

Despite the vast interest in EV-based technologies[18,19], the clinical translation of EVs is still in its infancy[20] and the knowledge on the underlying mechanisms of EV biogenesis is still very limited[19,21]. It is emerging that cells can produce vesicles through different mechanisms that still require full elucidation and release a heterogeneous mixture of EVs whose composition is highly sensitive to operating parameters[2,4,22,23]. Yet, the accurate control of EV properties and composition is essential for their final function[24].

One of the most severe bottlenecks of the progress in the field is the typical low EV yield[25,26]. Indeed, from a liter of conditioned culturing media approximately 10^9-10^{11} EVs can be obtained, an amount which is typically sufficient only for one single test in mice models[25]. These low EV yields severely limit lab scale experiments and clinical trials, making the transfer of EV-based treatment to humans even more difficult.

The goal of this review is to investigate the directions in which research in the field of scalable EV production is moving and to ascertain whether approaches previously followed in other bioprocesses (e.g. stem cells, liposomes and therapeutic protein production) can be exploited to advance the production of these novel therapeutics. After a brief summary of the state of the art, we will discuss progresses in both upstream and downstream processing.

2 State-of-the-art of EV production

To date, most EVs have been produced from human cells cultured in T flasks and purified by ultracentrifugation (UC)-based methods[19,27]. These processes have severe limitations in both the upstream and downstream: in the former the current culturing conditions considerably limit the EV production[13,25,28–30], while in the latter the laborious procedures complicate the large scale production[27,29,31,32]. Ultracentrifugation-based workflows are the most common isolation methods since they enable the recovery of relatively high EV yields at lab scale, especially when compared to alternative methods, such as ultrafiltration (UF), size-exclusion chromatography (SEC), immunoaffinity capture and polymer precipitation[24,33–35]. However, its implementation at large scale is challenging because of several reasons: (i) it requires large and heavy rotors which demand a consistent amount of electrical power to be operated[26]; (ii) being a batch process, it has several dead times that reduce the overall productivity[32,35,36]; (iii) it often copurifies contaminants; (iv) it involves high shear forces, causing aggregation and rupture of EVs[35,37]; (v) the composition of the isolated EV mixture is highly sensitive to a variety of experimental settings such as tube type and rotor type, leading to low consistency of EV mixtures obtained with different ultracentrifuges[19,26,36,38].

To increase the production yield of reproducible EV mixtures, the current production process requires extensive optimization. **Table 1** summarizes the recent progress regarding the development of a scalable process to produce EV with compliance to good manufacturing practices (GMP)[25,39–42], and the yield improvements achieved by changing EV source, bioreactor systems, or purification techniques[28,43–49]. These advances are discussed in detail in the following sections of this review.

3 Methods for improvement of upstream EV yield

3.1 Choice of the EV biological source

The functions of EVs are strictly correlated to the cell phenotype[2,14,50]. As a consequence, the cells used for EVs production depend on the EVs final application and, therefore, the field of EVs cannot rely on a single cell line. This is an important difference compared to the production of monoclonal antibodies (mAbs) for instance, where Chinese Hamster Ovary (CHO) cells have been chosen as standard protein expression system thanks to their immortality, their ease of handling and their rapid growth under standard culture conditions[51,52].

To date, EVs have been recovered from various sources, as depicted in **Figure 1**. Most EVs are produced from different types of human cells, including stem cells, dendritic cells, mast cells, macrophages, epithelial cells and cancer cells[2,4,14]. However, cultivation of human cells can be challenging to upscale for several reasons. Firstly, these cells stop dividing after repeated subculturing as they undergo the process of senescence. To overcome this issue, either new cells are obtained from the donor to prepare a new culture, which makes the process time-consuming and susceptible to variability, or the cells must be immortalized[53]. Secondly, many human cells are adherent, meaning that they grow as monolayers on a substratum and they stop dividing once they reach confluence[30]. As a result, the maximum number of cells per culture is limited by the surface area available for their growth. Thirdly, cultivation is even more problematic for stem cells which have the inherent potential to differentiate into various cell types during expansion, hence potentially releasing a mixture of EVs with unpredictable properties[54]. This additional hindrance is reflected in the number of studies on human EVs that reached clinical trials. In fact, while mesenchymal stem cells (MSCs) are currently the most prolific cell source of EVs[55] and the applications of MSC EVs are among the most studied and promising, only four clinical trials out of a hundred involve MSCs[56,57].

These issues motivated researchers to explore alternative EV sources, in particular food such as bovine milk[43] and plants[58–61], which have the main advantage of being easily accessible, cost-effective and scalable[43,62]. Since they are common components of our diet, milk and plants are also considered biocompatible and safe sources of vesicles [43,62–65]. Due to their nature, food-derived vesicles could be advantageous to deliver bioactive compounds for nutritional benefits upon ingestion[66] and promise to open new opportunities for research in food

nanotechnology[63,65]. Indeed, their potentials as selective drug delivery vehicles is being investigated[43,62,67,68] and tested in two different clinical trials (NCT01294072 and NCT01668849) related to grape-derived nanovesicles. Up to now, milk-derived EVs have been shown to selectively interact with macrophages[66], while plant-derived vesicles interact with intestinal stem cells[65] and are able to cross the blood-brain barrier[69]. It is important to note, however, that the safety of these materials still requires validation for each individual case. For instance, in a recent study Maji and colleagues performed *in vitro* toxicological experiments and observed that EVs derived from bovine milk caused adverse effects, such as enhancement of platelet aggregation, reduction of the macrophage phagocytosis and transfer of bacterial endotoxins derived from the exogenous source[70].

In parallel to food-derived vesicles, bacterial EVs are explored as promising tools for novel vaccine designs, given their cost-effective and scalable production and their ability to activate the innate immune response by presenting their natural surface ligands to the pathogen recognition receptors of immune cells[71–73]. In particular, Zhang *et al.* showed that these vesicles can increase the production of antibodies with respect to individual antigens, thus demonstrating the convenience of such vaccine formulation[74]. Moreover, bacteria can be easily genetically modified allowing to refine the functionality of EVs and to produce novel recombinant vaccines to tackle the issue of antibiotic resistance[71,75]. However, despite these potentials, bovine and bacterial EVs and plant-derived vesicles cannot substitute human EVs in all applications. Hence, they cannot solve completely the problem of low EV productivity.

3.2 Optimization of upstream processing conditions

To increase EV productivity, in parallel with the selection of the most suitable biological source, it is crucial to optimize the upstream conditions, such as the composition of the cell culturing medium and the bioreactor setup. These improvements can lead to dramatic increases in the bioproduct yields, as demonstrated in the context of mAbs, in which the improvements of the upstream processing led to a 10-100-fold increase in titer[51].

Different bioreactor systems have been tested to scale up the EV production, directly transferring the advancements developed in the field of stem cell expansion[76–79]. The simplest scale up approach relies on the substitution of single layer T-flasks with multi-layered cell culture flasks[40,41] to provide larger surface area for cell expansion. Despite its easy application, the homogeneity of the culturing conditions is difficult to monitor and at large scales the batch-operations increase processing times and promote batch-to-batch variability[79].

Hollow-fiber bioreactors (HFBRs) have increasingly been implemented for EV production[29,39,45,46]. In these dynamic setups, cells are expanded on cylindrical hollow fibers which can host 100-fold more cells than common T flasks[29] and are constantly supplied with nutrients and deprived of waste material by circulating fresh medium in the fibers. This bioreactor avoids contamination of the produced EVs with exogenous EVs present in fetal bovine serum (FBS), a common

component of culturing media, by keeping the two mixtures constantly separated with a filter[29]. Two applications of this system yielded 10-fold[29] and 40-fold[46] more EVs than conventional flask-based culturing methods.

Harastzi *et al*. explored the possibility to use a stirred tank bioreactor, the current system of choice for MSC cultivation[25]. In this setup, cells are cultivated on microcarriers, typically of spherical shape, which provide a high surface area to volume ratio for cell growth[78]. Impellers are used to enhance mixing and maintain homogeneous culture conditions that can be easily monitored and controlled[77]. This configuration led to a 140-fold increase of produced EVs[25].

Lastly, Cha and colleagues investigated the benefits of growing MSCs as spheroidal aggregates rather than as sheets on supporting surfaces[28]. In MSC-aggregates the innate properties of MSC are highly preserved thanks to the creation of an *in vivo*-like microenvironment. Hence, these cell culturing methods could significantly enhance MSC expansion and consequent EV production[28]. A 100-fold EV-yield increase was observed compared to that obtained in a common 2D static culture[28].

These mature technologies represent an ideal starting point for cell-based EV production[76]. However, it has been observed that cells respond to alterations of culturing conditions such as culturing time, cell confluence, passage number and cell adherence, as well as to mechanical and physical stresses linked to the bioreactor design[80] and to chemical stimuli. Cultivation parameters and their effects on EV production are reported in **Table 2**. It has been observed that cells produce more EVs upon stimulation, but the biological mechanisms underlying this response are yet unknown. The hypothesis that cells release EVs with different properties and functions under different culturing conditions still needs deeper investigation[29]. Hence, research in this direction is crucial to provide useful tools to better understand EV biogenesis mechanisms and optimize upstream processes.

4 Development of scalable EV purification processes

In addition to upstream units, also downstream processes in EV production require drastic improvements. Ideally, the downstream process should consist of unit operations that are able to isolate EVs with high yield and purity while preserving the EV quality, i.e. its structure and activity. Additionally, the unit operations should be simple, easy to use, reproducible and adaptable to the purification of EVs with different properties. Lastly, to be applied at a larger scale, the EV isolation method should be scalable, cost-effective and enable high-throughput processing.

In this context mature technologies are readily available, since they have been previously optimized for other industrial bioproducts that share structural similarities with EVs. For instance, the field of EVs could benefit from orthogonal techniques from both the liposome and the therapeutic proteins field to achieve the target purity, as shown in **Figure 2**. This approach could yield a purification protocol similar to the one used for viral vectors, which exhibit similar attributes of EVs in terms of size, biomembrane and presence of surface proteins including

glycoproteins. In this process, the mandatory purity is achieved by combining chromatography with filtration techniques. Specifically, after cell lysis, clarification, DNA digestion and virus inactivation, viral vectors are purified by ion exchange chromatography, concentrated by UF, dialyzed, further purified by SEC, newly concentrated by UF and finally dialyzed before storage[81].

To date, several techniques have been tested to replace UC for EV isolation and to optimize the recovery of intact EVs with constant purity both in small and large scale processes. One important example is tangential flow filtration (TFF) which has been increasingly applied in the field[25,39–42]. In this technique, two streams flow tangentially to a tubular filter membrane which allows the passage of particles smaller than the pore size from the feed stream into the permeate stream, while it retains larger objects in the retentate stream. Depending on the choice of the pore size, this strategy can be applied to isolate the desired product from larger particles by allowing it to diffuse into the permeate stream or to purify the target product from smaller impurities when it is maintained in the retentate stream. Moreover, the same configuration can be applied for buffer exchange or for product concentration in the retentate stream[82]. This flexibility, together with the short processing times, the scalability and the adaptability to continuous operation, established TFF as the standard purification method for liposome production. These strengths make TFF also an advantageous unit operation for large-scale production of EVs, considering their comparable lipid bilayer membranes and structures[82–85]. Moreover, the results obtained by Dimov and coworkers demonstrated that the shear stress on the filter does not alter the integrity of liposomes at optimal operational conditions, thus offering a gentler purification method in comparison with UC[84,86]. However, despite the high purification yield of intact vesicles, TFF provides EVs with lower purity than UC[31]. The large amount of co-isolated proteins and lipid impurities demand a further purification step which would negatively impact the processing time and overall yield[31]. Nevertheless, filtration processes were identified as the most versatile and cost-effective EV isolation methods for scale-up[26].

To achieve a greater EV purity compared to UC and TFF, Watson *et al.* coupled TFF with SEC[39]. The additional chromatographic step enabled more efficient removal of several protein contaminants and yielded a similar amount of EVs compared to UC. Additionally, the protocol isolated bioactive EVs without altering their size, morphology and protein content. However, SEC throughput is intrinsically limited by the column volume. Thus, a pre-concentration step is usually necessary to purify large volumes of conditioned media. Moreover, SEC tends to dilute samples, which then need to be subsequently concentrated[83,86,87]. This additional step further increases EV losses and the possibility of introducing environmental contaminants[31], thus possibly limiting the use of SEC for EV purification.

Both TFF and SEC are scalable and GMP-compatible techniques[39], but they need to be coupled with additional purification steps since they cannot separate EVs from contaminants with overlapping sizes, such as bovine serum-EVs, protein aggregates and lipid particles[27,88]. In particular, bovine serum-EVs are undistinguishable from the produced EVs and have unspecified properties.

Therefore, some efforts are currently focusing on using EV-free serum in media preparations or serum-free media to avoid unexpected functional variations of the EV mixtures induced by the presence of exogenous EVs[89]. In contrast, protein aggregates and lipid particles differ from EVs in terms of surface charge and chemical properties which can be exploited for their removal through other purification techniques.

Among methods based on affinity interactions, polyethylene glycol (PEG) precipitation was applied by Jong and colleagues for large-scale isolation of EVs[48]. They managed to process volumes of conditioned media up to 5 L and to isolate amounts of EVs comparable to UC. However, the team of Gámez-Valero showed that this method interfered with the structure, composition and functionality of EVs and it yielded EVs samples richer in plasmatic proteins with respect to SEC due to the co-precipitation of many impurities[90]. Ghosh *et al.* developed synthetic peptides to specifically precipitate EVs through their interaction with heat-shock proteins[91]. However, independently of the employed precipitation additive, the scalability of this method is intrinsically hampered by the need to remove the additive after EV isolation with further purification steps, reducing significantly yields and cost-effectiveness[26].

Anion exchange chromatography (AIEX) is currently attracting increasing attention in the field as an alternative at both large[91] and small scales[31,92,93], as it is already a well-established technique for the isolation of proteins[94] and viral vectors[81,95]. The interactions between the negative charges on the EV membrane and the positively charged stationary phase retain the vesicles inside the chromatographic column. Elution can be easily induced by increasing the ionic strength of the fed buffer. Heath and colleagues reported that this technique purified EVs more quickly and easily than UC and with greater purity and quality than TFF. It was also found that FBS derived proteins, such as bovine serum albumin (BSA) and apolipoprotein A, were efficiently removed with this method[31]. Additionally, flow rates up to 10 mL/min using a macroporous monolithic stationary phase have been reported as suitable, demonstrating the potential to overcome the throughput limitations of chromatography resins previously documented for other biopharmaceutical processes[31,96,97].

Affinity chromatography (AC) has only recently been applied for purifying EVs[98–100] and only preliminary results have been achieved until now. This method relies on specific reversible interactions between an immobilized ligand and a surface molecule of EVs. To date, different affinity approaches have been exploited for EV isolation[98,101–105], most of them in combination to small scale substrates, such as magnetic beads, microfluidic chips, plastic plates, cellulose filters, membrane affinity filters or porous monolithic silica microtips[32,35,98,101,103,104,106,107]. Hung *et al.* recently attempted to purify EVs with a commercial anti-FLAG affinity gel, but they obtained a low EV recovery, possibly because of the small portion of functionalized surface area that could be accessed by EVs within the nanoporous beads[99]. Even though AC is commonly used to selectively isolate products in a variety of bioprocesses[94], none of these approaches have yielded pure EVs due to the knowledge gaps regarding specific surface biomarkers of EVs. Thus, in the current state of the art, this technique can only yield enriched and concentrated EV

mixtures rather than pure preparations[19]. Efforts should be directed towards the identification of specific EV biomarkers, the development of protocols with elution conditions that do not deteriorate the EV quality, and the design of macroporous stationary phases and membranes that are both compatible with EVs and easy to functionalize.

Currently, none of the methods documented in the present review can be used alone to efficiently isolate purified EVs. Even though multi-step downstream processes are industrially applied, a purification protocol that relies on a single operation would be clearly preferable since every step affects the yield, the processing time and the costs[31]. To pursue this goal, Corso and colleagues combined bind-elution and SEC, thus moving towards the direction of mixed mode chromatography[36]. In their setup, proteins and impurities smaller than 700 kDa were captured in the pores of the stationary phase by positively charged octylamine ligands[100], whereas larger particles like EVs flowed through the column without interacting[36]. Despite improved purification, this approach still requires a concentration step prior to the SEC column loading[36].

5 Storage and stability

Storage conditions represent an additional factor that can impact the amount and the quality of EVs both for research and industrialization. The use of siliconized vessels throughout purification and storage is recommended to prevent adherence and loss of EVs to surfaces[33]. Typically, EVs are stored in phosphate buffered saline[33]. Storage at -80°C is currently the most commonly adopted method[33], since freezing does not impact EV characteristics, while storage at 4°C causes EV damage and aggregation[108–110]. However, Lőrincz and colleagues have shown that EVs can lose some functional properties at -80°C, even if they do not change in number and morphology[109]. Freeze/thaw cycles should be minimized[33], although it has been reported that EVs are relatively stable after several cycles[33,111]. In this context, cryoprotectants such as trehalose, already used for labile proteins, vaccines and liposomes, appear to have a positive impact on EVs[112]. Additionally, Frank *et al.* reported that freeze-drying does not have a significant impact on the size and particle number of MSC-derived EVs and that, upon addition of cryoprotecting sugars, the enzymes in EVs stored in lyophilized form or at -80°C have comparable activity[113].

However, in analogy with other aspects of EV bioprocessing, also the optimization of storage conditions and their impact on EV characteristics still requires further research which, in turn, demands larger amounts of available EVs[114].

6 Conclusions

The inherent nature of EVs as vehicles for intercellular communication has huge potential for exploitation in numerous applications, ranging from therapeutics and drug delivery to cosmetics[2,8,11,13,14]. However, the advancements in this emerging field are severely hampered by low production yields, which currently represent

one of the most crucial bottlenecks in the EV field and emphasize the need for more efficient upstream and downstream operations.

One promising direction is the exploitation of accessible and scalable sources of EVs such as bovine milk, plants or bacteria which avoid issues related to human cell cultivation. A second attractive route is the implementation of mature technologies that have been developed for other industrial bioprocesses to accelerate the development of a standardized EV production. In particular, bioreactors for stem cell expansion have been applied for upstream operations, while filtration techniques for liposome isolation and chromatographic methods have been successfully utilized for downstream processes.

The implementation of such approaches is already leading to significant yield improvements. However, the numerous uncertainties regarding EV biology, i.e. their biogenesis, their specific composition and their susceptibility to environmental conditions, complicate the possibility of controlling the final properties of EVs.

To improve sample reproducibility, we believe that it is crucial to increase our fundamental understanding of how the physico-chemical properties and functions of EVs change as a function of (i) cell growth conditions, (ii) type of isolation method, (iii) scale of the purification method. A first urgent step in this direction is the establishment of standardized characterization techniques for EVs, capable to simultaneously monitor both physical and biochemical properties with high throughput. In this context, in analogy to the analytics for therapeutic proteins[115,116], microfluidic technology is emerging as an attractive platform[117,118].

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8 Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Tables

Table 1. Current processes for scalable EV production and for improved EV yield.

[a)] most common EV production process consisting of cultivation of human cells and EV release in culturing T flasks and EV separation from the clarified medium by ultracentrifugation

[b)] compared to 2D flask culturing

[c)] compared to 2D flask culturing combined with isolation by ultracentrifugation

EV source	Application	Upstream reactor system	Downstream unit operations	Yield	Ref.
Human cells[a]	-	**Flasks**[a]	**Sequential centrifugation, ultracentrifugation**[a]	-	[23]
Bovine milk	Drug delivery	-	Sequential centrifugation; Ultracentrifugation	335 ± 48 mg particles/L_{milk}	[39]

Grapes juice	Protection from colitis	-	Sequential centrifugation; Sucrose gradient centrifugation	-	[40]
ECs	Therapeutic vascularization	**Tubular perfusion bioreactor**	Sequential centrifugation; Ultracentrifugation; Sterile filtration	14-fold increase [b]	[45]
HEK293	Drug delivery	**Hollow-fiber bioreactor**	Sequential centrifugation; Centrifugal filtration; Dialfiltration; Ultracentrifugation	5-fold increase [b]	[46]
MSCs	Regenerative medicine	**3D cell culture with shaking**	Sequential centrifugation	100-fold increase [b]	[28]
Umbelical cord-MSCs	RNA delivery to neurons	**Stirred tank bioreactor**	**TFF**	140-fold increase [c]	[25]
HEK293	Targeted cancer immunotherapy	**Hollow-fiber bioreactor**	**Sequential centrifugation;** **Filtration;** **TFF;** **SEC**	0.6 mg/L_{CC}	[39]
MSCs	Therapeutics for acute spinal cord injury	**Large-scale culturing flasks**	**Pre-filtration;** **TFF**	-	[119]
CPCs	Cardiac repair	**High yield culturing flasks**	**Pre-filtration;** **TFF;** **Diafiltration;** **Sterilizing filtration**	$2.9x10^{13}$ particles/ 5.9×10^{8}cells	[120]
MSCs	Regenerative medicine	Flasks	**Ultrafiltration**	-	[42]

DCs	Immunotherapy	Flasks	**Pre-filtration;** **Ultrafiltration;** **Diafiltration;** **Sterilizing filtration**	-	[47]
NKs	Immunotherapy	Flasks	**Filtration;** **Precipitation with PEG;** **Dialysis**	$2x10^{11}$ particles/L_{CC}	[121]
MSCs	Regenerative medicine	Plates	**Centrifugation;** **Filtration;** **AIEX**	-	[49,93]

Table 2. Impact of cell culture parameters, mechanical stresses, physical stresses and media composition on EV production.

Type	Parameter	EV source	Effect	Ref
Cell culture	Cell confluence	MSCs	Altered expression of various genes	[27,80,122]
		Cancer cells	Decrease in EV production with higher cell confluence	[30]
	Culturing time	Dendritic cells	Increase in EV production in time until a plateau at day 7	[29,47]
	Cell passage	MSCs	Increase in EV production at high cell passages, but decrease in EV bioactivity	[80]

	Cell detachment	Adherent cancer cells	Increase in EV production upon cell detachment	[30,123]
Mechanical and physical stresses	Shear stress	MSCs	Cell phenotypic alterations; changes of EV characteristics	[27,54,80]
	Microbubbles-assisted ultrasound	FaDu cells	Increase in EV production upon exposure to microbubbles-assisted ultrasound	[124]
	Aeration	Cancer cells	Increase in EV production in hypoxic conditions	[29,30,125]
	Light exposure	Cancer cells	Increase in EV production upon incubation with a photosensitizer and exposure to light	[29,126]
Media composition	FBS content	Neuroblastoma	Increase in EV production in serum-free conditions	[27,30,89]
	pH	HEK293	Increase in EV production in acidic conditions; no EV production in alkaline conditions	[29,127]
	Liposomes	Cancer cells	Increase in EV production upon addition of cationic liposomes; inhibition of EV production upon release of pegylated EVs; effect dependent on dose, surface charge, membrane fluidity, PEG modification and cancer cell type.	[128]
	Calcium	Neurons	Increase in EV production in presence of ionomycin	[29,128,129]

Oxidative stress	Mouse mast cells	Increase in EV production upon exposure to hydrogen peroxide	[29,130]
Chemotherapy treatment	Cancer cells	Increase in EV production after treatment with Doxorubucin	[29,126]

Figures

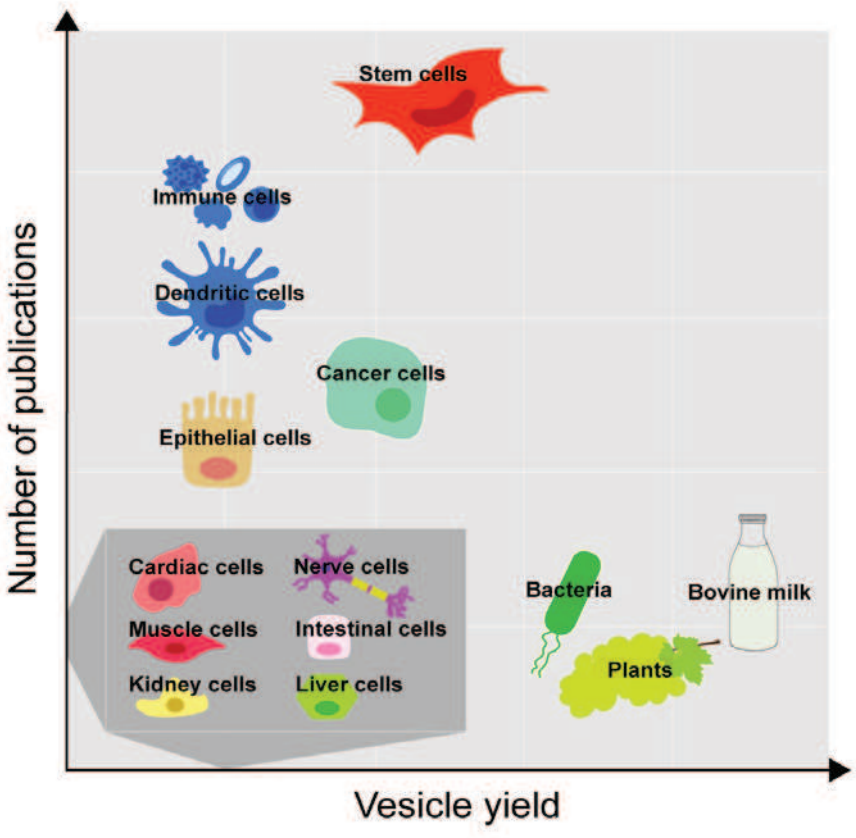

Figure 1. Most common sources of therapeutic vesicles and corresponding productivity.

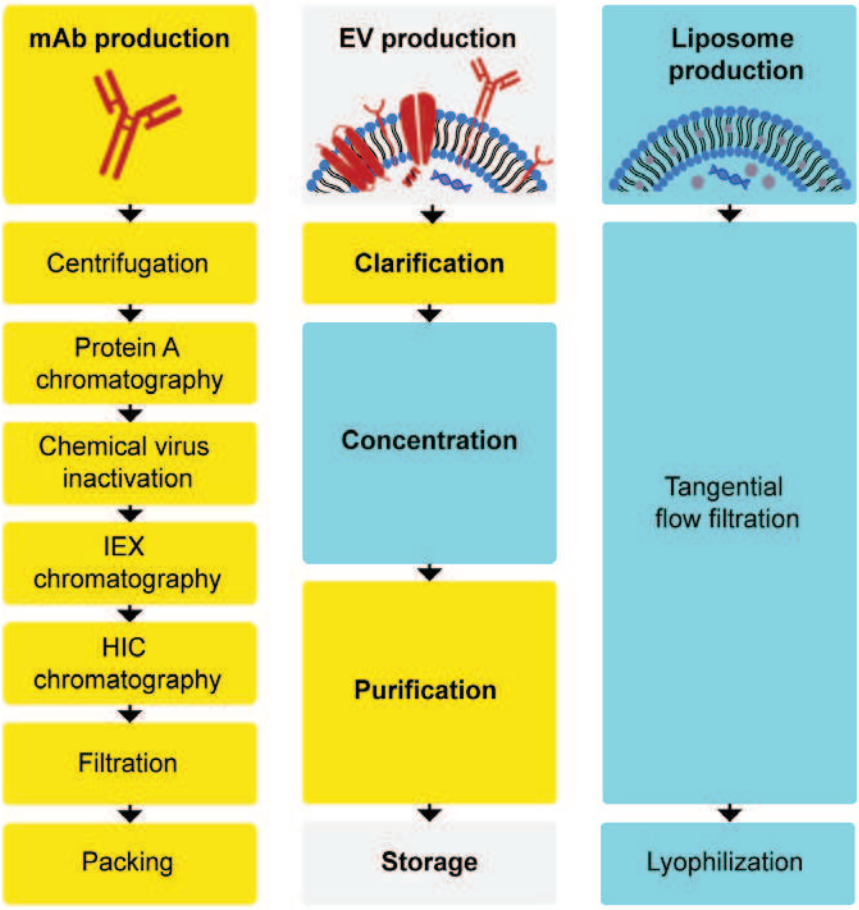

Figure 2. Flow diagrams of the typical platform purification process for mAbs (left column) and liposomes (right column) and diagram of the main steps used for purification of EVs from culture supernatant and other highly diluted media (central column). The unit operations used for mAbs and liposomes can be potentially applied in the downstream processing of EVs as indicated by the color code (yellow for mAbs and blue for liposomes).

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