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Alternative biological sources for extracellular vesicles production and purification strategies for process scale-up

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Abstract

Extracellular vesicles (EVs) are phospholipidic bi-layer enclosed nanoparticles secreted naturally by all cell types. They are attracting increasing attention in the fields of nanomedicine, nutraceutics and cosmetics as biocompatible carriers for drug delivery, with intrinsic properties beneficial to human health. Scientific work now focuses on developing techniques for isolating EVs that can translate into industrial-scale production and meet rigorous clinical requirements. The science of EVs is ongoing, and many pitfalls must be addressed, such as the requirement for standard, reproducible, inexpensive, and Good Manufacturing Practices (GMP) adherent EV processing techniques. Researchers are exploring the use of alternative sources to EVs derived from mammalian cultures, such as plant EVs, as well as the use of bacteria, algae and milk. Regarding the downstream processing of EVs, many alternative techniques to the ultracentrifugation (UC) protocols most commonly used in the laboratory are emerging. In the context of process scale-up, membrane-based processes for isolation and purification of EVs are the most promising, either as stand-alone processes or in combination with chromatographic techniques. This review discusses current trends on EVs source selection and EVs downstream processing techniques, with a focus on plant-derived EVs and membrane-based techniques for EVs enrichment.

Abbreviations

EVs	*Extracellular vesicles*
GMP	*Good Manufacturing Practices*
UC	*Ultracentrifugation*
ISEV	*International Society for Extracellular Vesicles*
TFF	*Tangential Flow Filtration*
OMV	*Outer Membrane Vesicles*
SEC	*Size Exclusion Chromatography*
FFF	*Flow Field Fractionation*
AEX	*Anion Exchange Chromatography*
AC	*Affinity Chromatography*
FFF	*Field Flow Fractionation*
MF	*Microfiltration*
UF	*Ultrafiltration*
dgUC	*Density Gradient Ultracentrifugation*
CQA	*Critical Quality Attributes*
mAbs	*monoclonal Antibodies*
UF/DF	*Ultrafiltration/Diafiltration*
MWCO	*Molecular weight cut off*
PES	*Polyether sulfone*
TMP	*Transmembrane pressure*
TFAC	*Tangential Flow for Analyte Capture*
dcTFF	*Dual cyclic filtration system*
Mf-F	*Microfluidic filtration*
AF4	*Asymmetric Flow Field Fractionation*
PDEVs	*Plant-Derived Extracellular Vesicles*

Keywords

Extracellular vesicles; Plant-derived Extracellular vesicles; Drug delivery system; Nanomedicine; Downstream Processing; Membrane-based separation processes; Process scale-up;

1. Introduction

EVs are a heterogeneous group of biological nanoparticles naturally released by cells - eukaryotes and prokaryotes. They are characterized by a bi-layer membrane made by phospholipids that encloses the cytosol of the deriving cell, rich in proteins, lipids and nucleic acids (mRNA, microRNA, tRNA, rRNA, DNA). The most popular way to classify EVs is according to their biogenesis mechanism (Figure 1), into exosomes, microvesicles and apoptotic bodies.

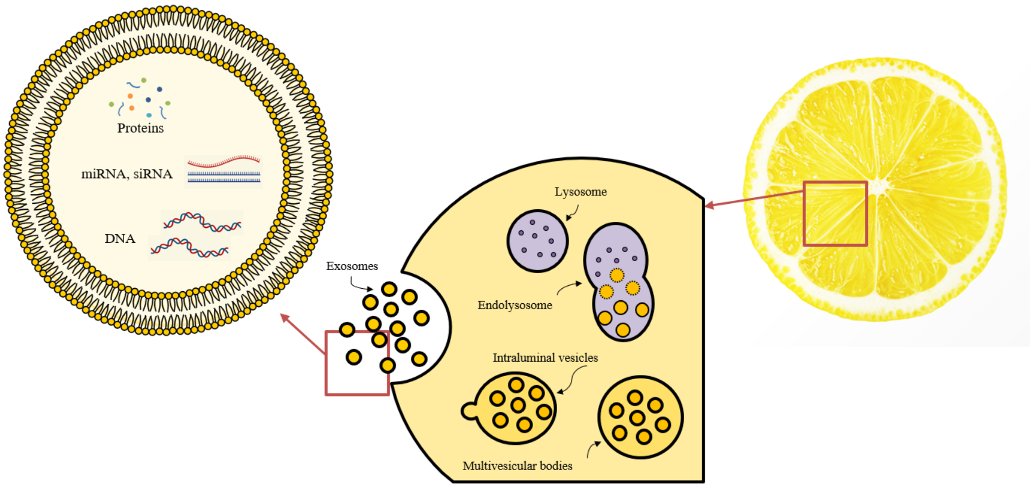


Figure 1: All types of cells release EVs, including plant cells. Fruits such as lemons can be exploited as biological source to isolate and purify EVs. These are released by cells through several biogenesis pathways – exosomes (30-150 nm) are produced during the formation of multivesicular bodies (MVB) of endosomal origin. Microvesicles (50–1000 nm) are formed by budding of the plasma membrane. The largest EVs, apoptotic bodies (800-5000 nm) are formed by blebbing of the membrane of apoptotic cells.

Nonetheless, a clear biological distinction between the different populations is missing and the International Society for Extracellular Vesicles (ISEV) recommends the use of “EVs” as blanket-term for “particles naturally released from the cell that is delimited by a lipid bilayer and cannot replicate” (Thery et al., 2018). EVs represent a “universal, evolutionary conserved mechanism for inter-kingdom and intra-kingdom communication” (Chronopoulos and Kalluri, 2020) and have been defined as “signalosomes, multifunctional signaling complexes for controlling fundamental cellular and biological functions” (Gandham et al., 2020). EV-mediated communication is involved in all the domains of life and in many cellular physiological and pathological processes. EVs contain bioactive cargos upon which they are able to deliver complex biological messages to target cells, leading to the induction and coordination of the immune response, maintenance of cellular integrity and homeostasis, cell development, cell differentiation and angiogenesis (Ramirez et al., 2018). A glaring example of EVs functionalities comes from human diet. The discovery that plants cells do secrete various types of vesicles spontaneously lead to the observation that, as we eat every day, these vesicles are continuously put in contact with our intestinal tract and microbiome (Halperin and Jensen, 1967; Marchant et al., 1967). Recent data suggest that EVs from food and their cargos might have relevant biological role on our digestive tract, contributing to the homeostasis of the whole body through gene regulation (Rome, 2019). Many studies have disclosed EVs role as cross-kingdom modulators, as EV-mediated interactions between mammals, plants, bacteria and parasites (Hou et al., 2019; Ionescu et al., 2014; Rutter and Innes, 2018;

Svennerholm et al., 2017; Szempruch et al., 2016). EVs have a promising potential in three main domains - nanomedicine, cosmetics and nutraceutics (Figure 2). In nanomedicine EVs can be used as drug-delivery systems, therapeutics and diagnostic tools. EVs are attractive candidates in clinical applications due to their *intrinsic* potential based on their specific bioactive cargo or exploiting their unique delivery properties. Concerning their use as drug delivery vectors evidence suggests a long-range action (e.g. ability to cross the epithelial endothelial barriers), cargo protection and engineering possibilities. In gene therapy, EVs can be modified for targeted delivery of nucleic acids-based drugs and viruses, as well as carriers for protein and small molecules to treat diseases and cancer (Gandham et al., 2020; Konoshenko et al., 2018). As stand-alone therapeutics, EVs produced by stem cells can be used to induce tissue regeneration, while EVs produced by dendritic cells and macrophages can regulate immune responses (Robbins et al., 2016). Besides, EVs have shown therapeutic effects against infectious diseases, diabetes, tumors, neurodegenerative and cardiovascular diseases (García-Manrique et al., 2018; Liu et al., 2020). The use of body-fluid-derived EVs (e.g. EVs from blood, urine, semen, and saliva) as non-invasive biomarkers for early diagnosis and prognosis of cancer, via liquid biopsies, has a revolutionary potential (Pang et al., 2020). EVs are also attractive candidates for the development of functional cosmetics for skin treatments as wound healing, rejuvenation, pigmentation and hair growth treatments (Carrasco et al., 2019; Peršurić and Pavelić, 2021). Furthermore, EVs from plants and animals are very promising to create alternative delivery options for nutraceuticals to enhance the bioavailability of poorly absorbed active food ingredients (Akuma et al., 2019).

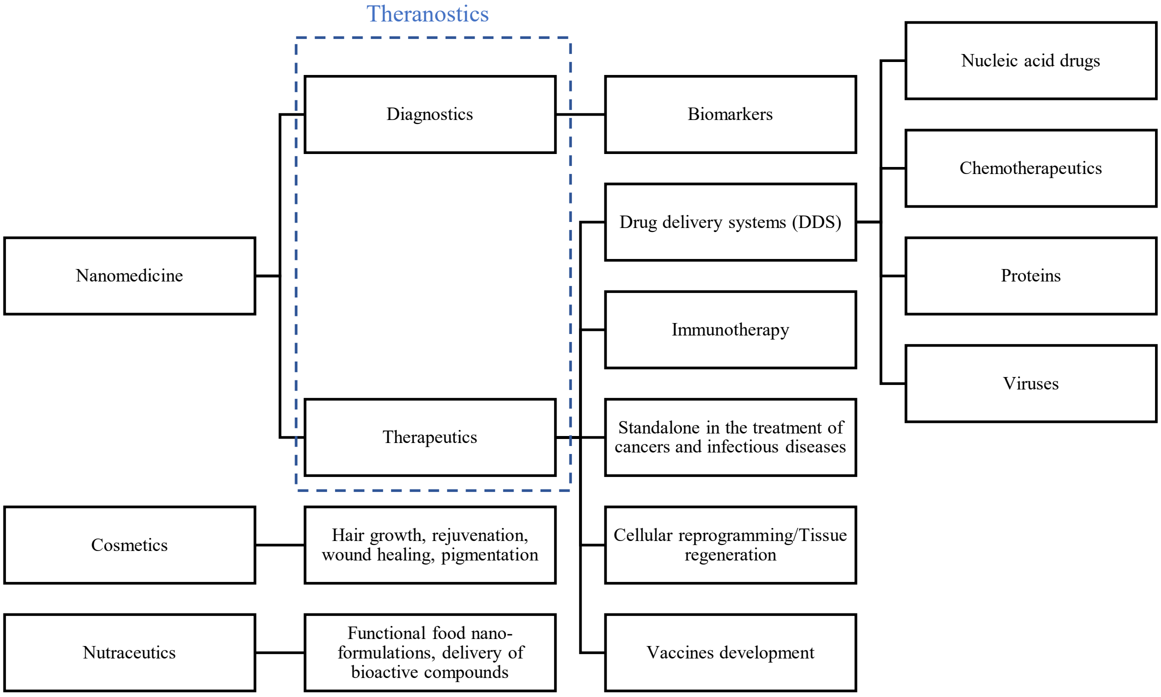


Figure 2: The three main application areas of EVs: nanomedicine, cosmetics and nutraceutics

The physical and biochemical properties of EVs mirror the mother cell phenotype. Thus, there are notable differences in the release rate, biochemical composition and size, depending on the state and characteristics of the cell of origin. Current EVs production is based on vesicles naturally released from a source or EVs obtained from cell culture conditioned media under a controlled environment. The use of a certain EVs source automatically implies a better suitability for a particular application. For example, EVs from physiological fluids are mainly used for diagnostic and prognostic applications. To date, studies of mammalian EVs produced by cell culture for clinical purposes are widespread. Mesenchymal stem cells (MSC), dendritic cells, tumor cells, red blood cells and macrophages are among the most frequently used sources of therapeutic EVs (García-Manrique et al., 2018; Liu et al., 2020). In recent years, interest has grown in the use of alternative sources to human cells for drug delivery applications, such as animal EVs, plant EVs, bacterial EVs and algal EVs. An introductory analysis of the current uses, advantages and disadvantages related to the employment of each different EV source is presented in this review. At present, most EVs have been isolated and purified by UC-based methods, but from a manufacturing perspective, UC has many limitations and lacks the potential for scalability. Its use has been reduced in favor of other methods such as filtration techniques, chromatographic separations, polymer precipitation, affinity-based processes and microfluidic technologies. Currently, the field of downstream processing of EVs is limited to laboratory-scale research, and there are many limitations that need to be overcome to move to clinical and industrial-scale research, such as typically low yields, lot-to-lot variability, lack of standardization, and development of cost-effective isolation protocols. Filtration techniques hold great promise as they are already being exploited industrially in the field of liposome and virus production, where tangential flow filtration (TFF) is considered the standard purification method. Membrane processes are flexible, scalable and adaptable to continuous operations, making them the optimal candidates as unit operations for large scale EVs production. Therefore, the second part of this review covers a detailed state-of-the-art of the most widely used membrane techniques for EVs isolation and purification to identify the crucial parameters that enable standardization and reproducibility of EV preparations.

2. EVs sources

Regarding biological source selection, EV production cannot rely on a single cell line, biofluid or tissue. Source selection is entirely driven by *the end user application,* as the properties of EVs are closely related to the functions and phenotype of the parent cell. Table 1 provides an overview of the most commonly used sources with the main processing characteristics. So far, most EVs are isolated from human body fluids or produced by different types of human cells, such as stem cells, dendritic cells, macrophages, epithelial cells, and tumor cells. Human cell cultivation requires optimization of several parameters, such as cell isolation and banking, composition of culture media and cell expansion to the desired density and amount. Some of the most crucial aspects in the framework of massive EV production for clinical trials are the low available volume, cost, safety and ethical compliance. In addition, the process of cell senescence and yield limitations resulting from the fact that human cells are generally adherent represent further complications (Paganini et al., 2019). Indeed, although some applications require specific human cell lines and their use cannot be avoided, these complications have encouraged researchers to explore alternative EV sources. Animal, plant, and bacterial sources are recently gaining attention in the field of EV production because they are cheap and highly available, allow EVs to be easily isolated from large volumes of fluid, and lead to better yields. Bacterial and algal cells cultivation has significant advantages over that of eukaryotic cells, especially in terms of proliferation ability and ease of gene editing strategies. Food-derived EVs, such as plant and milk EVs, do not require any cell cultivation, thus their use saves entirely on upstream costs and management. Besides, food-derived EVs are inherently biocompatible, safe and possess many beneficial effects on human health, by being part of our dietary regimen (Ly et al., 2023). Researchers around the world are trying to isolate EVs from many different natural sources in an effort to find the most economically viable and sustainable sources that could translate toward massive EV production. From the perspective of a circular bioeconomy, residues from animals, fruits and vegetables can be potentially employed as sources for EV production. In this context, EVs represent a promising valorization pathway, allowing the conversion of agro- and animal-waste into many EV-based added-value products (Sangiorgio et al., 2020). However, it is crucial to consider that there is still a substantial knowledge gap related to the biological role of EVs from plants and animals, and that the level of maturation of the field, compared to that of mammalian cells, is in its infancy.

2.1. Bacterial EVs

EVs are naturally released by both gram-negative and gram-positive bacteria. There are different kinds of bacterial vesicles, but Outer Membrane Vesicles (OMVs) from gram-negative bacteria are the most studied. They are generally smaller than eukaryotic EVs, having dimensions ranging from 20 to 300 nm, and are released through the blebbing of the cell wall. The presence of liposaccharides toxins on OMVs surface is a key molecular feature, besides the presence of outer membrane lipids and proteins, soluble periplasmic components and peptidoglycans (Schwechheimer and Kuehn, 2015). Bacterial EVs are much less studied than those of mammalian origin, but several studies have demonstrated their prominent physiological and pathological role as mediators, in bacteria–bacteria and bacteria–host interactions (Ñahui Palomino et al., 2021). Bacterial EVs are capable of triggering an innate immune response by presenting EV surface ligands – natural or engineered – to the immune cell pattern recognition receptors (Gilmore et al., 2021). Due to their potent immunomodulatory properties, the potential use of bacterial EVs as therapeutics is increasingly being studied, especially as immune adjuvants against infections, platforms for vaccine development and anticancer therapies (Chronopoulos and Kalluri, 2020; Jahromi and Fuhrmann, 2021). Bacterial EVs are extremely promising in vaccine design and development, as they can increase the antibody production by simultaneously carrying multiple viral antigens on their surface, (Cai et al., 2018; Gerritzen et al., 2017; L. Zhang et al., 2016). They are low cost, scalable, easy to manipulate, and their release can be spontaneous in a culture medium or even induced by the use of a chemical detergent (e.g., sodium deoxycholate), heat stress or antibiotics (Momen-Heravi et al., 2013). By genetically engineering donor cells, more efficient recombinant vaccines can be obtained, with further improvements to their safety profile, immunogenicity and yield (Jiang et al., 2019). Gerritzen et al. developed a vaccine platform based on OMVs produced by *Neisseria meningitidis* (Gerritzen et al., 2019). The vaccine's mechanism of action is based on the expression on heterologous antigens on the OMVs. The release of OMVs was powered by high concentration of oxygen in the culture media, and tangential flow microfiltration was used as a scalable purification strategy. The authors were able to obtain 90 mg of OMV proteins per liter of culture.

2.2. Algae EVs

Several studies have shown that microalgae are promising sources of EVs (Adamo et al., 2021; Kuruvinashetti et al., 2020; Picciotto et al., 2021). Microalgae are a natural, sustainable and renewable bioresource with attractive metabolic properties. Microalgal EVs are obtained under controlled environmental conditions from cultures of microalgal strains, characterized by high growth rates. Piciotto et al. performed microalgal selection and batch culture on seven different strains (Picciotto et al., 2021). After 30 days of incubation and a differential UC purification protocol, they were able to obtain 2 x 20^9 particles per mL of cultivation medium from *Cyanophora paradoxa*. According to Adamo et al. the production of microalgal EVs is scalable and could be performed in large scale photobioreactors and obtain EVs with comparable yield to other sources (Adamo et al., 2021). Algae EVs can be used to deliver biomolecules, drugs and high-value microalgal substances such as antioxidants, pigments, lipids and complex carbohydrates.

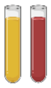
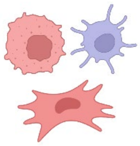
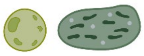
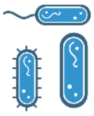
2.3. Bovine milk EVs

Over the years, milk has been adopted by researchers as the main alternative EV source to human cells. There is a massive amount of literature related to the use of EVs from bovine milk (Betker et al., 2019; Vashisht et al., 2017). Milk is one of the most promising scalable sources of EV for mass production, because it is easily accessible, inexpensive and it requires no cell culture. Several studies on the safety of milk-EVs have shown low toxicity levels and a good in vivo tolerability (Manca et al., 2018). Somiya et al. found that milk-EV administration in mice resulted in the induction of low cytokine levels and the absence of systemic toxicity (Somiya et al., 2018). Matsuda et al. observed developmental toxicity in zebrafish embryos following administration of milk-EVs loaded with RNA at high concentrations, while no acute toxicity was detected (Matsuda et al., 2020). Milk-derived EVs have been shown to increase the oral bioavailability of drugs and are optimal vectors to transport bioactive compounds for nutritional and therapeutics purposes (Carobolante et al., 2020). In cancer therapy, milk EVs can be functionalized with ligands such as folic acid to achieve tumor targeting (Munagala et al., 2016). In addition, milk-derived EVs have shown several therapeutic effects such as a selective interaction with macrophages and induction of intestinal stem cell proliferation (Maghraby et al., 2021). The three main steps involved in the isolation of milk EVs are milk defatting, establishing a method for casein depletion, and EVs enrichment. Somiya et al. concentrated 321 µg of milk-EVs from 1 mL of whey by performing casein removal through centrifugation and UC for EVs purification (Somiya et al., 2018). They also attempted casein removal by acid precipitation and obtained a 20-fold lower yield. Milk-EVs can be obtained from raw milk, commercial milk and dairy industry waste streams. Interestingly, others have found that industrial processing of commercial milk, such as pasteurization, homogenization, and ultra-heat-treated milk, impacts the integrity of milk-EVs, causing changes in their functionalities (Kleinjan et al., 2021). Sukreet et al. tested the enrichment of EVs from cheesemaking byproducts by TFF, resulting in low EV count (10^9 particles/mL of milk), but a high protein content (0.65 mg/mL of milk). They found heterogenous EV-enriched populations, which likely include components that escaped precipitation from the complex whey matrix, consisting of lipoproteins, fat globules and casein micelles (Sukreet et al., 2021). Therefore, heterogeneous preparations of milk EVs may be suitable for applications that do not require a high level of purity, given the excellent economic and environmental advantages of using EVs derived from milk waste.

2.4. Plant EVs

Plant EVs are released by vegetable cells and their structure resembles that of vesicles of mammalian origin (Pucci and Raimondo, 2020). To date, vesicles from ginger, grapes, grapefruit, orange, lemons, broccoli, apple, kiwi, tomato, ginseng, coconut, blueberry, and carrot, among many others, have been successfully isolated and observed by TEM microscopy. Over the past decade, the role of plant miRNAs as a functional component of food with therapeutic effects has been investigated by many studies (Díez-Sainz et al., 2021; Sanwlani et al., 2021; Teng et al., 2018). Due to their miRNA content, plant EVs are gaining attention as a new class of cross-kingdom modulators, capable of mediating animal-plant interactions at the molecular level, as well as playing crucial roles in plant physiology in terms of cell proliferation, differentiation and response to environmental stresses (Rome, 2019). Applications of plant EVs in nanomedicine and nutraceutics are based on their intrinsic biological properties, such as anti-cancer, anti-inflammatory, anti-aging, and anti-Alzheimer's, and on their use as nano-carriers to transport therapeutic biomolecules. Wang et al. demonstrated that grapefruit-derived vesicles can enhance the anti-inflammatory capability of intestinal macrophages, thus alleviating dextran sulfate sodium (DSS)-induced colitis in mice without any toxicity (Wang et al., 2014). Several studies have revealed the role of plant vesicles in inhibiting cancer cell proliferation. Ginger-derived EVs by Zhang et al. demonstrated their anti-tumor action in colitis-associated cancer. They were able to decrease the levels of cancer-associated pro-inflammatory cytokines and suppress the proliferation and apoptosis of intestinal epithelial cell (M. Zhang et al., 2016a). In addition, vesicles isolated from lemons by Raimondo et al. inhibit the growth of several cancer cell types through tumor targeting, reduction of oxidative stress, and activating of a TRAIL-mediated apoptotic cell death mechanism (Raimondo et al., 2015). Concerning the regenerative effects of EVs, Sahin et al. isolated vesicles from wheat grass and investigated their potential use in wound healing through *in-vitro* studies, demonstrating that they induce skin regeneration by triggering proliferation in a dose-dependent manner on epithelial, endothelial, and dermal fibroblasts (Şahin et al., 2019). Furthermore, Zhuang et al. studied the use of ginger-derived EVs to treat alcohol-induced liver damage in mice. These vesicles were seen to contribute to hepatoprotection by suppressing the generation of reactive oxygen species (Zhuang et al., 2015). In the context of industrial production, plant-EVs are extremely promising vectors for drug delivery. The large volumes availability and affordability may provide easier and faster industrial application than that of mammalian EVs. Like milk EVs, they are also potentially obtainable from agricultural wastes and residues. Plant EVs can be loaded, by both passive and active techniques, with therapeutics such as proteins, miRNAs, siRNAs and expression vectors to achieve superior effects against diseases, but also in nutraceuticals and cosmetics, enhancing the beneficial action of natural bioactive phytomolecules (Wang et al., 2014, 2013; M. Zhang et al., 2016). Furthermore, literature data show that plant-derived vesicles can be produced in higher yields (Chen et al., 2019; Lobb et al., 2015). Of course, these comparisons are merely qualitative and do not consider the variability of sources, the influence of upstream processing, the difficult reproducibility of isolation procedures, and the processing of complex and heterogeneous biological matrices. Importantly, it is crucial to fill the relevant knowledge gaps in the fields. More studies on plant EVs biological roles are needed, as well as the determination of specific plant EVs protein markers, *in-vivo* safety, stability and efficacy studies that could translate to clinical studies. There are currently five plant-EVs-based therapies in clinical trials (ClinicalTrials.gov Identifiers: NCT01294072, NCT04879810, NCT01668849, NCT05318898 NCT04698447). These studies are in their early stages and complete results of clinical trials using plant EVs are missing. Preliminary results have been published in only one study (ClinicalTrials.gov NCT04698447) on the use of dietary supplements containing nanovesicles derived from citrus lemon juice (CitraVes®), (Raimondo et al., 2021). The authors recruited 20 healthy volunteers who received 1000 mg/day EV CitraVes® spray-dried formulation for three months. After 4 weeks they observed a significant reduction of low-density lipoprotein cholesterol levels, an important risk factor for cardiovascular diseases. It is noticeable that in all the clinical studies cited above, guidance on EVs dosing strategies, a crucial factor in the establishing the safety and therapeutic profiles of plant EVs, was omitted.

Table 1: Classification of EVs according to sources and their main processing characteristics..

	EV classification			**Cell sources**	**Collection/ Upstream processing**	**Main applications**	**Cell culture platforms?**	**Scalability potential**
Eukaryotic	**Mammalian EVs**	**Animal EVs**	**Milk derived EVs**	Bovine milk 	Milk collection and pretreatments	Drug delivery, therapeutics, nutraceutics	No	High due to the large availabilit of cow's milk
		Human EVs	**EVs from body fluids**	Blood, saliva, semen, urine, cerebrospinal fluids, bronchoalveolar fluid, amniotic fluid 	Physiological fluids collected from the body	Diagnostics (e.g. liquid biopsies)	No	Low: need to find donors and ethical issues
			EVs from human cells	MSC, cancer cells, immune cells, dendritic cells, epithelial cells; cardiac, nerve, muscle, kidney, liver, intestinal cells 	Collection of conditioned culture medium from cell culture expansion	Therapeutics with specific targeted functions; drug delivery for cancer therapies	Yes	Medium: high cost of cell cultures
	Plant-derived EVs			From fruits, rhizomes, apoplastic fluids, seeds, roots 	Tissue disruption and juice collection, vacuum infiltration, hydroponic medium collection	Drug delivery, therapeutics, nutraceutics, cosmetics	No	High: Easy availability and low cost of sources
	Algae EVs			From microalgae (e.g. Cyanophora paradoza, Tetraselmis chuii) 	Microalgae strain selection and cultivation	Drug delivery, therapeutics, nutraceutics, cosmetics	Yes	Medium: requires cell culture, cost lower than human cells
Prokaryotic	**Bacterial EVs**			Gram-positive and gram-negative bacteria 	Spontaneous or induced release in a growth medium; possible genetic engineering strategies	Drug delivery, vaccines and cancer therapies	Yes	Medium: requires cell culture, cost lower than human cells

3. Downstream processing of EVs

3.1. State-of-the-art of EVs isolation methods

To date, researchers use several methods for isolating EVs on a laboratory scale. They can be classified according to the working principle on which they are based as reported in Table 2.

Table 2: Classification of methods used for the isolation of extracellular vesicles according to their working principle.

Methods based on size and buoyant density

- Ultracentrifugation-based techniques
- Size exclusion chromatography (SEC)
- Microfiltration/Ultrafiltration
- Flow field fractionation (FFF)

Methods based on solubility changes

- Precipitation with polyethylene glycol or protamine or sodium acetate

Methods based on charge

- Anion Exchange Chromatography (AEX)
- Electrophoresis

Methods based on highly specific surface interactions

- Immuno-affinity capture
- Affinity Chromatography (AC)

Microfluidic technologies

- Immuno-affinity based microfluidics
- Viscoelastic separation
- Microfluidic filtration
- Acoustic devices

The traditional methods used for isolating EVs are those based on vesicle size and density, namely UC, filtration techniques and size exclusion chromatography (SEC). Methods based on EVs solubility changes, such as chemical precipitation, have emerged later over the years. In addition, numerous methods for isolation of EVs populations based on highly specific interactions with molecules exposed on the surface of EVs or microfluidic technologies have recently appeared. The number of publications on the isolation of EVs has increased exponentially over the past decade, as shown in Figure 3a, where the number of publications found in PubMed with the search keyword “EVs isolation methods” for the years 2010-July 2022 is shown.

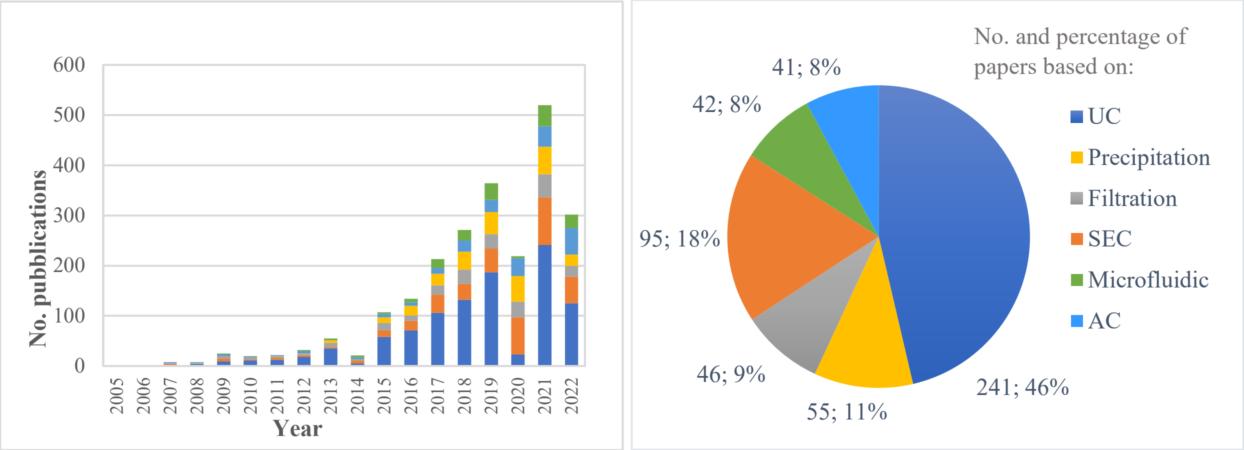


Figure 3: (a) Number of publications on the isolation of EVs in recent years. (b) In 2021, the total number of publications on the isolation of EVs was 520. Among them, 241 papers used UC as the primary method of EV isolation, 95 papers used SEC, 55 papers used precipitation techniques, 46 papers used filtration processes, 42 used microfluidics technologies and 41 affinity capture (source PubMed, July 2022).

Considering the year 2021, a pie chart that reports the worldwide distribution of different methods used for EVs primary isolation is shown (Figure 3b). From the figure it can be seen that UC remains the predominant isolation method (46%) adopted by researchers, while the other half of the pie is divided among SEC (18%), precipitation (11%), filtration techniques (9%), AC (8%) and microfluidic technologies (8%). It should be emphasized that the above statistics refer only to the "primary" isolation method, whereas usually researchers use a combination of different techniques to obtain EVs preparations. In fact, according to the 2019 worldwide survey on the methods for separation and characterization of EVs, more than half (60%) of the respondents use a combination of different isolation techniques in their protocols (Royo et al., 2020). Each separation process has resulted in unique characteristics of EVs and has advantages and disadvantages. Table 3 provides a comprehensive list of the advantages and disadvantages of the currently most widely used techniques for downstream processing of EVs, considering factors such as process time, potential for scalability, and cost-effectiveness. Clearly, it is not possible to entrust the entire production of EVs to a single isolation strategy, and downstream processing is strictly dependent on the desired application and the characteristics of the source material. The Minimal Information for Studies of Extracellular Vesicles (MISEV2018) conference outlined the key guidelines for EV research and standardization and proposed a very intuitive distinction between different EV isolation methods, to be placed on a *specificity vs. recovery* grid (Figure 4), (Thery et al., 2018). If EVs are to be used as diagnostics, the need for high EV yields is paramount, whereas high structural integrity may not be necessary. In contrast, for drug delivery applications, preserving the structure of EVs is a priority. In the case of highly complex samples such as biofluids, multiple purification steps may be necessary. UC is considered the golden standard in EV isolation. Ultracentrifuges are widely distributed in non-specialized laboratories, and the massive amount of literature available on differential UC protocols easily allows comparison with new separation processes. However, the technique has many limitations, such as the negative impact on EV integrity and aggregation, co-isolation of non-EV impurities, and low reproducibility. Standard commercial ultracentrifuges can process up to 400 mL of samples, thus the low sample throughput does not allow for scalability (Staubach et al., 2021).

Table 3: Comparison of the most commonly used techniques for EVs isolation.

	Principle	Time	Advantages	Disadvantages	Scalability[1]	Cost[1]
Ultracentrifugation (UC)	Sedimentation of biomolecules according to density using high g-force	140 - 600 min (Greening et al., 2015; Théry et al., 2006)	- Easy protocol - No additional chemicals - Most common method in the field for data comparison	- Low throughput - Efficiency affected by many factors - Low reproducibility - Possible damage of EVs - Long duration - Limited to small-scale	+	€€€
Density gradient ultracentrifugation (dg UC)	Separation according to density in a pre-constructed density gradient medium	250 min – 2 days (Greening et al., 2015)	- Higher EVs purity than UC - No additional chemicals	- Complex - Low throughput - Efficiency affected by many factors - Operator-dependent yields - Time consuming - Possible damage of EVs - Limited to small-scale	+	€€€
Size exclusion chromatography (SEC)	Separates by hydrodynamic volume	1 mL/min (Lobb et al., 2015)	- Reproducibility - Reduced contamination - Gentle method - Prevents EV aggregation - No additional chemicals	- Low resolution - Limitations on sample volume - Dilution of EV isolates - Co-isolation of same-size particles	++	€€

Filtration (MF/UF)	Uses membranes with specific pore sizes	130 min (Salih et al., 2014)	- Simple procedure - High throughput - Time efficient - Relatively gentle - No additional chemicals	- Membrane clogging - Loss of sample and aggregation - Low purity - Possible deformation of vesicles.	++++	€
Flow Field Fractionation (FFF)	Flow modulated by a normal force field	45-60 min (Liangsupree et al., 2021)	- Reproducible - Removal of lipoproteins - Non-invasive	- Low input volume	+	€€
Polymeric precipitation	Solubility changes by adding a crowding agent	8-12 h (Liangsupree et al., 2021)	- Inexpensive - Simple - Gentle method - High yield	- Need to remove the crowding agent - High contamination - Time-consuming	++++	€
Anion exchange chromatography (AEX)	Separation based on charge	180 min (Heath et al., 2018)	- Scalability - Short processing time - Structural and biological integrity or EVs	- Co-isolation of other negatively charged biomolecules - Need of a final concentration step.	+++	€€
Electrophoresis	Separation based on electrophoretic mobility in an electric field	60-120 min (Marczak et al., 2018)	- Easy control - Fast and efficient - Non-invasive	- Sample heating - Co-isolation of negatively charged biomolecules - Combination with other techniques may be required	++	€
Affinity capture (AC)	EVs capture using antibodies or other ligands	240 min (Greening et al., 2015)	- High purity - Target specific populations - Great potential in diagnostics	- Costly - Harsh elution - Limited knowledge of EVs markers - Isolation of a subset of EVs - Non-specific binding	+++	€€€
Microfluidics technologies	Flow manipulation in microscale	60-120 min (Meng et al., 2021)	- Specificity and selectivity - Low energy and material requirements - Quick	- Low sample loading - Possible blockage due to system clogging	++	€€€

[1] Qualitative criteria based on bioprocess engineering knowledge on unit operations and established processes

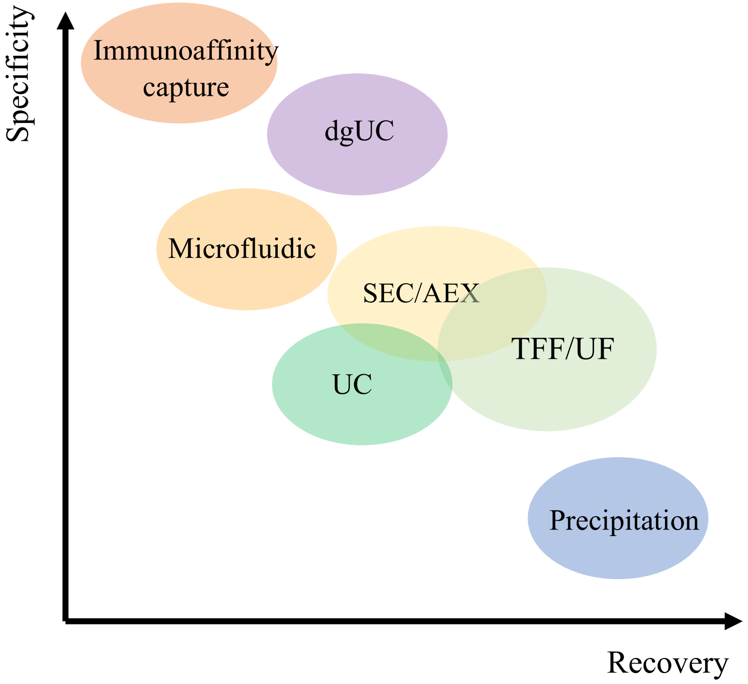


Figure 4: Specificity vs recovery grid; qualitative chart constructed according to ISEV recommendations on EV isolation techniques (Thery et al., 2018).

3.2 Main challenges in EV isolation techniques and process scalability

Many factors must be considered as essential requirements for the scalability of the EV process. Among them, the need for reproducible, cost-effective, and high-throughput isolation methods is critical. The methods chosen must comply to GMP standards in order to support large-scale manufacturing. The main challenge in GMP of EVs is quality control, and identification of the Critical Quality Attributes (CQAs) that affect the stability and efficacy over time of preparations, as well as standardization of sample collection, handling and storage (Chen et al., 2020; Herrmann et al., 2021). Standardization requirements must address several challenges associated with EV isolation (Table 4). First, the product of interest is present in complex biological fluids or matrices that contain a myriad of bioparticles. The biological samples contain protein assemblies or lipoproteins, with similar size and biological properties to EVs. Co-isolates may provide a synergistic effect to the actions of EVs. Often, when subjected to rigorous characterization, it is not necessary to consider them as "impurities", but rather to speak of an EV-enriched secretome as an end product (Wiklander et al., 2019). This strategy saves the high costs associated with achieving a high level of sample purity in downstream processing. In addition, a single EV sample contains heterogeneous populations, as EVs from the same source can be released from parent cells through various biogenesis pathways, leading to the simultaneous presence of various EVs subpopulations. Therefore, heterogeneity in EVs content can result in intra- and inter-batch variabilities, which must be taken into account in the isolation procedures.

Table 4: Overview of key process optimization strategies for EV separation to advance process scale-up.

Main limitations on EVs downstream processing	**Process optimization strategy**
There is no single best isolation method	- Sample and application-driven decisions; - Fit the process constraints to the sample type and the specific purpose.
EVs are heterogeneous in nature	- Define a method target;

	- Decide whether to focus on specific EVs properties or general physical/chemical characteristics.
Batch-to-batch variability	- Define and control the most important process parameters.
Regulatory requirements	- Define GMP compliant raw materials; - Define storage and administration strategies; - Identify CQAs; - Define a viral inactivation step.
Difficult characterization of the final product	- Define potency assays; - Establish the product *mode of action.*
Co-isolation/ impurities	- Characterization of co-isolates is a requirement; - A possible synergic effect between EVs and co-isolates needs to be evaluated; - Prioritize therapeutic efficacy over purity, depending on the application.
Low product yield	- Establish an optimal trade-off between yield and purity; - Switch to the EVs sources with a higher scalability potential; - Optimize the upstream processing technologies; - Switch from lab-scale techniques (e.g. UC) to large scale techniques already exploited in other industrial bioprocesses (e.g. TFF, SEC, AEX, AC).
Throughput limitations	- Use downstream processing technologies that can process several ten or hundred liters of conditioned media / starting material.

A common weakness of current isolation methods is the very low yield of vesicles. According to Haraszti et al. a dose of 10^9–10^{11} exosomes per mouse is typically required for a single test in mice models. This quantity is approximately obtained from one liter of conditioned culture medium, with current practices such as UC (Haraszti et al., 2018). The low EV yields severely limit the preclinical and clinical development of EV applications in medicine, as well as their industrial translation to other applications. In this context, considering upstream processing, yield improvements can be achieved by changing the EV source and/or bioreactor system, in case of EVs from cell culture supernatant. As for downstream processing, yield improvements can be achieved by changing and/or optimizing purification techniques. It is essential to take advantage of the knowledge previously gained in the fields of industrial production of liposomes, monoclonal antibodies (mAbs) and viral vectors, thus applying the same downstream processing strategies in the processing of EVs. Liposomes are the synthetic equivalent of EVs, having a comparable phospholipidic bilayer nanostructure. Since TFF is considered the golden standard in the field of industrial liposome production (Paganini et al., 2019), given the similarities between EVs and liposomes, TFF can be considered the most suitable unit operation for large-scale EV production. Viral vectors, in particular enveloped viruses, and EVs share similar properties, such as size, morphology and composition. At the industrial level, virus purification is mainly achieved through a combination of chromatography and membrane-based processes (Staubach et al., 2021). A common platform for downstream processing of viral vectors is based on AEX purification, UF concentration/diafiltration and polishing with SEC. Also in this field, the use of TFF as main capture/purification step for upscaling purposes is increasing (Geraerts et al., 2005). Industrial capture and purification of mAbs relies on the use of sequential chromatographic steps including AC and AEX, as well as the use of centrifugation, depth filtration, and/or microfiltration for clarification. Ultrafiltration/Diafiltration (UF/DF) performed in TFF mode is used as unit operation for concentrating and purifying mAbs solutions (Buyel et al., 2017; Tripathi and Shrivastava, 2019). Indeed, the use of TFF/chromatography multistep processes for EVs isolation is becoming increasingly adopted. Recently, Seo et al. proposed a large-scale purification protocol for EVs preparation using TFF and AEX: they isolated Cytotoxic T-lymphocyte EVs from 4 L of culture supernatant using hollow fiber TFF with 750 kDa polyether sulfone (PES) membranes and AEX. Two distinct subpopulations were observed, exosome-like particles that eluted at low NaCl concentration (2×10^{12} particles/mL), and microvesicle-like particles that eluted at high NaCl concentration (1.5×10^{12} particles/mL). Through AEX, they demonstrated to be able to distinguish between different functional EV subpopulations (Seo et al., 2022). A comparison between EVs, viral vectors and liposomes actual production systems is reported in Table 5.

Table 5: Comparison between EV, viral vectors and liposome production systems in terms of upstream and downstream processing techniques and product yields.

			Upstream	**Downstream**					
		Source	**Cell culture**	**Harvest/ clarification**	**Capture**	**Purification**	**Polishing**	**Yield**	**Ref.**
Small scale	**EVs**	**Human cells**	**T-flasks**	**C**	**UC**	**UC**			
		HEK293	T75	100 x g 10 min,1000 x g 10 min, 10000 x g 1 h	100,000 x g 3 h	200,000 x g 3 h		10^8-10^9 particles/mL of CM	(Lee et al., 2019)
Large scale	**EVs**	**Human cells**	**HF bioreactor**	**C + MF**	**UF/DF TFF**				
		HEK293 + miRNAs	Fibercell®	1000 x g 30 min + 0.85 µm	500 kDa PES HF module			10^{13} particles/mL of CM	(Yoo et al., 2018)
	Virus	**Human cells + plasmids**	**T-flasks**	**Chemical lysis, nuclease**	**UF/DF**	**UC**	**UF/DF, sterile filtration**		
		293T	2 x 10-layer cell factory		TFF 100 kDa cassette	76,000 x g, 2h		94 % recovery	(Geraerts et al., 2005)
	Liposome	**Lipids + cargo proteins**	**Ethanol injection**	**-**	**UF TFF**	**DF TFF**	**Sterile filtration**		
		DPPC + rh-Cu/Zn-SOD protein	Crossflow triple injection	-	PS 100 kDa TFF cassette			3.6 mg entrapped protein/mL	(Wagner et al., 2002)

List of abbreviations: C= Centrifugation; DF=Diafiltration; HF = Hollow fiber; CM = Conditioned media; UF = Ultrafiltration; MF = Microfiltration; PES = Polyethersulfone; PS = Polysulfone; DPPC = Dipalmitoyl-phosphatidylcholine.

There are several companies emerging in the production of EV-based therapeutics from human cell lines. Codiak Bioscience is a clinical-stage biopharmaceutical company focused on the development of engineered EVs-based therapeutics. Their production system is based on fed-batch or perfusion bioreactors, having volumes up to 2000 L and 500 L, respectively, to cultivate genetically engineered immortalized human cells. In downstream processing, clarification is performed through filtration steps, while purification is accomplished through filtration (UF/DF in TFF mode) and different chromatographic steps, such as cation and anion exchange chromatography (CEX, AEX) and mixed mode chromatography (MMC). All processes, CEX, AEX and MMC can be also performed with membrane chromatography. They claim to produce amounts of purified EVs 2000 times more than can be obtained with conventional centrifuges (Bourdeau et al., 2021). EVOX Therapeutics is a biotechnological company devoted to the development of protein and nucleic acid-based therapeutics via exosome engineering. Their proprietary exosome manufacturing processes are based on batch and perfusion bioreactors to cultivate genetically engineered human cell lines, downstream processes utilizing filtration processes and liquid chromatography (e.g., AEX, SEC). Recently the company patented an Affinity Chromatography (AC) purification method wherein EVs are engineered to achieve highly specific binding. In particular, the company invention involves the use of chromatography matrices comprising Fc domains and the development of engineered EVs presenting Fc binding polypeptides on their surface (Raymond et al., 2021). EVOX was recently able to scale its production up to 2000 L under GMP conditions. ExoCoBio is another exosome-based biomedicine company focusing on regenerative medicine and aesthetics. They developed a technological platform called ExoSCRT^{TM} for the large-scale production of EVs from MSC derived from adipose tissue entirely based on filtration processes. Briefly, it includes the use of 0.22 µm PES filters for clarification, concentration and subsequent diafiltration by TFF with a 500 kDa Molecular Weight Cut Off (MWCO) membrane (Lee et al., 2020).

3.3 Recent developments and challenges in affinity technology

TFF followed by AC and final polishing steps are the most promising approaches for clinical development of high-purity EVs (Colao et al., 2018). In this context affinity technology holds a remarkable potential for large scale EVs purification, as the technique allows for tunable specificity depending on the adopted ligand. Moreover, this field has recently seen important progress in the development of innovative stationary phases, such as magnetic microbeads, chromatographic membranes, monolithic columns and microfluidic devices. Recent advances in the manufacturing of human EVs (Ströhle et al., 2022), should be also considered in processing EVs originating from alternative sources. As biological knowledge advances, the exploitation of affinity techniques for large-scale purification of EVs from milk, plants, bacteria and algae will become increasingly likely. The use of antibodies that specifically target protein receptors on the surface of human EVs is perhaps the most traditional, with several studies dealing with antibodies targeting the protein markers CD9, CD63, and CD81 on the surface of EVs. However, as in all immunoaffinity techniques, the main drawback is the need for alkaline or acidic elution buffers, which can damage the integrity of EVs (Ströhle et al., 2022). The use of aptamers has emerged as a viable alternative to antibody-based AC. Like antibodies, aptamers have been developed to bind human EVs protein markers. Importantly, they provide for intact EVs, as they require milder elution conditions (e.g., saline solutions). Besides, they offer a greater chemical stability and a higher affinity for EVs, due to genetic modifications of the oligomer filaments (Ströhle et al., 2022). The use of antibody and aptamer ligands requires specific selection and modification strategies and their application on the field of non-human EVs is hindered by the lack of knowledge of EVs markers. To date, Alix, tetraspanins (CD9, CD63, CD81), heat shock proteins (HSP70, HSP90) and annexins are the most frequently used mammalian EVs protein markers (Deng and Miller, 2019). Interestingly, some proteins families are common to different EVs biological domains, such as heat shock proteins and annexins, that have been identified also in plant EVs (Pucci and Raimondo, 2020). To our knowledge, affinity purification strategies applied to plant and algae EVs have not been attempted yet. Concerning OMVs general protein markers have not yet been identified, but OmpA protein in *E.coli* has been explored as target receptor for affinity purification (Alves et al., 2017). Specifically, mutant OmpA-His6 OMVs were created through the incorporation of a non-native histidine amino acid repeat sequence (His-tag). These plasmids were spiked into a culture of native OMVs and purified utilizing immobilized metal affinity chromatography (IMAC). Affinity techniques based on pseudo-ligands, phospholipid membrane properties, and generic biochemical properties have also been developed for the purification of human EVs (Ströhle et al., 2022). These are more versatile approaches, compared to the use of antibodies and aptamers, as they do not require any specific knowledge on the EVs markers, thus they have a relevant potential for the purification of non-mammalian EVs. For example, as certain phospholipids are associated to the membranes of an entire EV population, their recognition allow to purify the whole EVs spectra of a sample rather than specific subpopulations, a matter that is commonly involved with the use of antibodies. Nakai et al. obtained highly purified EVs from conditioned culture media and biofluids by using Tim4, a transmembrane protein that works as a receptor for the phosphatidylserine present on the EVs surface (Nakai et al., 2016). EVs elution is simply achieved by adding a Ca^{2+} chelating buffer, given that Tim4-binding to phosphatidylserine is dependent on Ca^{2+} concentration. Recently, Morozumi et al. carried out a comparative study using membrane-affinity and phosphatidylserine-affinity isolation for cow milk EVs (Morozumi et al., 2021). Membrane affinity was conducted using an exoEasy Maxi Kit (Qiagen), based on a membrane affinity spin column. According to the producers, the method is based on a generic biochemical feature of EVs, to recover all the EV populations present in a sample. Phosphatidylserine-affinity isolation was performed using a MagCapture Exosome Isolation (Fujifilm Wako Pure Chemical Corp). A proprietary substance was applied to the EVs sample, fostering the binding to phosphatidylserine groups on EVs surface, in a calcium dependent manner. Streptavidin magnetic beads were used to immobilize EVs for capture. Overall, the EV preparations isolated with phosphatidylserine-affinity had a higher level of purity compared to those obtained with the membrane affinity isolation. Notably, in both cases, the particle concentration was lower than that obtained with SEC. Following another strategy, Kim et al. exploited the negatively-charged molecules present on plasma EVs surface by using poly-l-lysine coated on magnetic beads (Kim and Shin, 2021). To remove contaminating proteins, they used a buffer having a pH equal to their isoelectric point, which allowed the so-neutralized proteins to be released in solution. Final EVs elution was accomplished through 1 M NaCl, obtaining a 6.6-fold higher yield compared with that of UC. Another interesting affinity strategy is based on the use of heparin, that is a glycosaminoglycan ligand isolated from animal tissues. Heparin is widely used to purify a range of proteins and viruses. It is not dependent on an affinity-tag mechanism and it acts like a cation exchanger. A recent study evaluated the purification of stem cell-derived EVs through TFF and heparin affinity chromatography, the affinity step had a minimum recovery of 68.7% compared to a 39.8% recovery using SEC, based on particle counts, besides an average recovery of 98% and 99% of residual proteins and DNA, respectively (Barnes et al., 2022). Heparin AC was also used to separate EVs in distinct subpopulations. Overall, the study found a partial interaction between heparin and EVs, indicating that some populations can bind EVs and others cannot. These affinity differences may be used for fractionation between subpopulations of EVs once the mechanism of interaction between EVs and heparin is better elucidated.

4. Membrane based-techniques for EVs isolation

Membrane processes are the most versatile, as they can be exploited for clarification, concentration, and purification of fluids, and they can be used alone or in combination with chromatography. They are scalability-oriented. as modular systems allow the plant to be adapted to handle high volumes of fluids, offering different levels of functionalization and flexibility. This section provides an overview on the main membrane-based techniques used for EVs processing.

4.1. Microfiltration and Ultrafiltration

Filtration is a popular size separation technique used for both volume reduction and purification of EVs. Microfiltration (MF) membranes have pore sizes in the order of micrometers, and when clarifying EVs solutions by MF, filters with pore sizes of 3, 0.8, 0.45, 0.22, and 0.1 μm are typically used (Konoshenko et al., 2018). Ultrafiltration (UF) employs more selective membranes, with defined molecular MWCO ranging from 10 to 600 kDa for most applications. Recovery of EVs based on filtration techniques can be accomplished through different isolation protocols. MF and UF are often used in combination with other techniques, for example as a complement to UC protocols or as additional steps in SEC. However, MF and UF are also applicable as stand-alone techniques, as both UF and MF membranes can be exploited in sequential MF/UF isolation protocols: they rely on a series of filtration steps for EV enrichment. First, larger impurities (cells, cell debris, apoptotic bodies) are removed using MF filters, leaving a vesicle-rich permeate. Lower molecular weight impurities (free proteins, contaminants) are then eliminated by using UF membranes with smaller pores than the target EVs (0.22 μm, 0.1 μm, 600 kDa, 500 kDa, 100 kDa); they are able to retain vesicles and remove impurities into a waste permeate. In this way, the EV fraction of a given size is concentrated and purified (Konoshenko et al., 2018). For EVs concentration, their dimension should be larger than the MWCO of the membrane by a factor of 2 to 5 (Scott and Keith, 1995). The selection of a tighter membrane (5) will yield maximum EVs recovery with a lower flowrate. On the other hand, if processing time is a major concern the selection of a loose (2) membrane should be preferred. EVs have heterogeneous dimensions depending on their source, biogenesis and processing conditions. Following this rule of thumb, and assuming a correlation between the EVs diameter (D) and molecular weight (MW) like $\mathrm{D} \propto MW^{1/3}$ to isolate small EVs (e.g., exosomes) having average dimensions of 20 nm, an UF membrane having a MWCO between 200-500 kDa should be selected. This is only a rough estimate; it would be helpful if the average pore size of the membrane could be provided by membrane manufacturers, along with the MWCO. Merchant et al. proposed a MF protocol for urinary exosomes using a 0.1 μm hydrophilized polyvinylidene difluoride filter. They compared the EVs isolated from the membrane-based protocol with standard UC and obtained comparable EVs protein yields and reduced contamination by non-EVs proteins, (Merchant et al., 2010). Heinemann et al. developed an optimized sequential UF/MF protocol for the isolation of EVs from cell culture media or body fluids. The first step involves prefiltration in dead-end mode with a 0.11 μm modified PES membrane, to remove cells and cell debris. Microvesicles larger than 0.1 μm should also pass through the filter because of their flexibility. The second step is based on a 5-times TFF with a 500 kDa MWCO PES membrane to remove free proteins and contaminants and to concentrate the sample. In the final step a filtration with a 0.1 μm track-etched polycarbonate membrane for final enrichment of exosomes is performed at very low pressure to filter out microvesicles larger than 0.1 μm (Heinemann et al., 2014). Based on the sequential UF protocol, many companies have recently developed kits for the isolation of EVs. ExoMir^{TM} from Bio Scientific Corporation uses two membranes (200 nm and 20 nm) both placed in a syringe that allows rapid fractionation of exosomes and larger membrane-bound particles (Doyle and Wang, 2019). ExoTIC (Exosome total isolation chip) developed by Liu et al. is also based on the same principle: it is a solid device that houses a track-etched polycarbonate membrane (30 nm or 50 nm pore size) and a PES filter (200 nm pore size). It enables the purification of intact EVs in the 30–200 nm size range from various biological fluids (Liu et al., 2017). Both kits help make filtration-based exosome isolation a more reproducible and clinically simpler procedure. It is important to note that all the UF techniques mentioned in this section are small scale techniques, relative to the filtration of small sample volumes (< 250 mL). The development of large-scale UF techniques is mainly conducted in TFF mode, and it will be covered in Section 4.3.

4.2. Centrifugal UF

In centrifugal UF, the g-force applied on the centrifuge rotor provides the driving force to remove solvents and small molecules through an UF membrane. Centrifugal UF is usually carried out in centrifugal concentrators, centrifuge tubes packed with a membrane filter, usually suitable for small volumes, ranging from 100 µL to 200 mL. Cheruvanky et al. demonstrated rapid enrichment of urinary EVs using a centrifugal concentrator with 100 kDa PES membranes by centrifugation at 3000g, (Cheruvanky et al., 2007). Lobb et al. it have shown that centrifugation-based filters recover three times more particles from conditioned media than pressure-driven UF stirred cells. They found that centrifuge-based concentrators work well for small volumes (50-200 mL), while pressure-driven concentration is more appropriate with volumes greater than 400 mL, to reduce the gel layer formation by generating a convective crossflow motion across the membrane, (Lobb et al., 2015). The main challenge in UF processes is clogging and entrapment of vesicles on the membrane surface, which slows down the process and causes partial loss and aggregation of the target material. Membrane fouling is common and unavoidable in all filtration operations, but its formation can be limited and controlled through optimization of fluid dynamics, identification of an optimal membrane cut-off and materials, such as those with low non-specific protein adsorption.

4.3. Tangential flow filtration (TFF)

In conventional filtration systems, fluid flow is applied perpendicularly to the membrane, which causes particle accumulation, unpredictable change in the hydrodynamic resistance of the membrane, and membrane clogging. In TFF mode, on the other hand, the feed flows tangentially across the membrane, and membrane fouling is significantly limited compared with dead-end mode. It can be controlled by achieving steady conditions that ensure constant flux and cake thickness over time. Depending on the membrane MWCO, TFF can be applied to purify EVs from larger particles or from smaller impurities. In addition, it can be configured as buffer exchange in diafiltration mode or volume reduction to concentrate the product in the retentate stream. Busatto et al. applied TFF to isolate EVs from cell culture medium with a 500 kDa PES hollow fiber membrane. EVs can be concentrated and purified from a scalable sample volume with a high recovery rate in a rapid and sterile manner (Busatto et al., 2018). Comparative assessment of TFF and UC revealed that the former concentrates EVs with comparable physicochemical characteristics, but with 5-fold higher yield, improved batch-to-batch consistency, and less albumin contaminants in half the processing time (1 h). In contrast, the study by Heath et al. underlined that TFF provides EVs with lower purity than UC, detecting co-isolated lipids and proteins, despite having a higher yield (Heath et al., 2018). Moreover, one aspect that should be further evaluated is the potential deformation and lysis of EVs caused by shear forces. Overall, it can be observed that the high degree of flexibility offered by the TFF technique allow to preserve EV integrity through optimization of process conditions (e.g., transmembrane pressure (TMP), agitation speed, feed flowrate, feed concentration). Some authors demonstrated that, under optimal operating conditions, TFF is a gentler method than UC for liposome purification, (Dimov et al., 2017). In this context, the selection of an appropriate TMP appears crucial. The work done by Dehgani et al. offers an example of an optimized TFF isolation protocol for EV concentration from large volumes of fluid that involves standardization of the membrane cleaning step. The authors developed a filtration-based microfluidic system called tangential flow for analyte capture (TFAC), which is a modified version of TFF. In this three-step protocol: particles are first trapped on the surface of a membrane in tangential flow, then washed under the same flow conditions with a cleaning buffer to remove contaminants; finally, the TMP is reversed, releasing the particles from the membrane that are collected downstream (Dehghani et al., 2019). According to the authors, processing human plasma in TFAC mode enabled the capture of EVs with minimal contamination. Conventional TFF systems are single isolation units with only one type of membrane, which does not allow isolation of specific size ranges of EVs. Kim et al. proposed a dual cyclic filtration system (dcTFF) consisting of two TFF modules with 200 and 30 nm membranes, connected to two peristaltic pumps that provide continuous circulation while preventing clogging. The authors created a simultaneous dual flow condition that allowed them to isolate a specific size range of extracellular vesicles (30-200 nm) in a single step. The two modules were assembled to form three chambers: a sampling chamber, an isolation chamber and a waste chamber. They obtained active EVs with 1.3-fold more abundant CD63 exosome marker than a commercial filtration kit (K. Kim et al., 2021). TFF processes are modular and fully adaptable to continuous operations. They can be considered as a hybrid of concentration and purification strategies, which is highly suitable for large-scale EV isolation from diluted samples. In addition, industrial-scale input volumes can be used as crossflow filtration units, as they can hold volumes on the order of liters.

4.4. Microfluidic filtration

Recent advances in the science of microfabrication have led to the development of microfluidic devices, compact units composed of a network of microchannels that are intended to control fluid flow at the microscale. Microfluidic devices enable highly efficient and precise separation of micro- or nano-sized particles within a given volume of fluid. Indeed, at the micro- and nano- scale fluids possess distinctive properties, with frictional forces dominating kinetic forces. This offers the possibility of fine tuning and manipulating various process and material-related parameters. These devices are commonly referred to as *Lab-on-Chip*, i.e., capable of reproducing different laboratory processes on a single integrated micrometric platform, a *chip*. They thus offer high accuracy and specificity in the isolation of EVs and, compared to other conventional methods, allow a substantial reduction in the number of samples, reagents and time required for experiments, while increasing process automation. The most relevant microfluidic techniques recently developed for EV isolation are microfluidic filtration, immunoaffinity capture, chip centrifugation, acoustic separation, viscoelastic flow, and hydrodynamic flow. Microfluidic filtration (Mf-F) is a very promising tool for continuous separation and enrichment of EVs according to specific EV sizes. Davies et al. developed two types of pressure- and electrophoresis-driven Mf-F devices, that separate cells, debris and small EVs from blood through a nanoporous membrane with an adjustable pore size. The limitation of pressure-driven Mf-F is that the pores become blocked after obtaining approximately 4 µL of filtrate. Electrophoresis avoids this problem and increases the separation efficiency and purity (Davies et al., 2012). Double microfluidic filtration approaches have also been developed. Liang et al. constructed a Mf-F double-filtration system that includes a filter with a pore size of 200 nm to remove cells and large impurities, and a second filter with 30 nm pore size that allows proteins to pass through. This system achieves high yields, compared with UC, for isolation of 30–200 nm EVs, (Liang et al., 2017). Mf-F small scales are greatly advantageous in terms of reagent use and precise flow control. These features are particularly exploitable in bioprocess development, as they offer the ability to precisely direct process scale-up and scale-out, study and optimize fluid dynamic conditions, and perform quality control. To increase the throughput, microfluidic systems can either be scaled-out or scaled-up. Process scale-out is accomplished through parallelization. Many authors argue that by following this strategy, Mf technologies are indefinitely scalable (Webb et al., 2020). However, these designs are expensive, especially in terms of nanofabrication requirements, as well as requiring separate sets of pumps and controls.

In contrast, microfluidics scale-up involves increasing channel size in order to increase product throughput. The key to successful scale-up of a microfluidic process is the creation of a scale-independent process that maintains the optimal flow characteristics created at the microscale on larger scales, regardless of channel size. Webb et al. studied the use of microfluidic devices for continuous production of loaded liposomes, from bench scale (12 mL/min) to GMP volume production (200 mL/min), using different micromixer cartridge designs (Webb et al., 2020). With a particular design (toroidal mixer design) they achieved a scale-independent production process, ensuring homogeneous nanoparticle production over a range of flow rates and volumes using the same process production parameters.

4.5. Flow field fractionation (FFF)

Field-Flow Fractionation (FFF) is a size-based isolation technique that has been applied in the field of EVs isolation. Asymmetric Flow Field-Flow Fractionation (AF4) is the most widely used sub-technique of FFF. In AF4 separation is achieved by diffusion of particles flowing in a sub-millimetric thin film of laminar flow confined in a narrow chamber with a membrane at the bottom. A force field is applied perpendicular to the laminar flow and pushes the particles toward the UF membrane, which subsequently permeate according to their size. The feed flow has a parabolic profile because a constant laminar flow is employed (Zhang and Lyden, 2019). In addition, AF4 has a programmable crossflow intensity that can be optimized to increase the separation efficiency, making the process very flexible. Unlike elution in SEC, smaller particles elute first, followed by larger particles. This is because the smaller particles have a higher diffusion coefficient. The main disadvantage of the method is the low volume of sample input, as the field and membrane can be overloaded at high volumes. Usually, these devices are coupled with online detectors such as UV, dynamic light scattering (DLS) and multi-angle light scattering (MALS) for particle size distribution detection (Gandham et al., 2020; Liangsupree et al., 2021). AF4 can successfully separate EVs from lipoproteins and is becoming attractive for fractionation of EV subpopulations. Zhang and colleagues fractionated EVs into distinct subclasses: small exosomes (60–80 nm), large exosomes (90–120 nm) and discovered a new subpopulation of non-membranous nanoparticles that they called “exomeres” (35 nm) from various cell types. According to them, AF4 is a highly reproducible, rapid, simple, label-free and gentle process, (Zhang et al., 2018). Moreover, they isolated different subpopulations of exosomes in a single AF4 run with real-time measurements of various physical parameters of individual particles, showing that AF4 can also be an important additional analytical tool.

4.6. Membrane techniques combined with charge-based techniques

One potential isolation strategy could be to combine filtration techniques with charge-based separation methods, taking advantage of the negative surface charge that most EVs possess. Yang et al. recently developed a method for isolating lemon-derived EVs that combines an electrophoretic technique with a dialysis bag of 300 kDa MWCO for isolating plant EVs (Yang et al., 2020). With the application of an electric field, impurities and non-vesicular proteins were able to pass through the 300 kDa membrane, while lemon vesicles were retained and thus purified. The electrophoretic buffer was changed every 30 minutes, and the electrophoretic direction was reversed to prevent the membrane pores from being blocked by the vesicles. They obtained a preparation highly enriched in lemon vesicles in only 2.5 hours, demonstrating that the method is efficient for isolating lemon EVs, saving time and without the need for special equipment. The main drawback of electrophoretic separations is the heat generated during the process due to the huge amount of electric field required for efficient separation. This can be potentially detrimental to the vesicles. Marczak et al. addressed this problem by combining electrophoresis with an ion membrane process in a continuous configuration performed in a microfluidic chip. The applied electric field allows EVs to migrate to a cationic membrane. The pores of the agarose gel are in the order of 200-300 nm in size and prevent large particles, such as cell debris, from entering. These are washed away by the continuous flow provided by the pump, minimizing membrane clogging. EVs are concentrated and trapped on the membrane surface, as they do not enter it, as they are both negatively charged (Marczak et al., 2018). The cationic membrane allows the concentration and isolation of exosomes, while electrophoresis allows their purification. A comparison was made with UC and a commercial precipitation reagent kit. The authors found a recovery rate of 70-80%, while in comparison, from the same source, UC and precipitation achieved recoveries of 6% and 11%, respectively.

5. Isolation of Plant EVs

The isolation of plant-derived EVs (PDEVs) can be very challenging because plants, fruits, seeds, and roots are complex matrices consisting of different tissues with peculiar physical structures. UC has gained benchmark status in the isolation and purification of EVs from plant and mammalian sources. To date, the UC isolation protocol is mainly applied for the isolation of plant vesicles. The starting point is the extraction of plant juice, which is then subjected to a series of centrifugation steps with gradually increasing speed. At each step, the pellet is discarded and the supernatant is further processed. In the final step, the supernatant undergoes further higher speed UC of at least 100,000 g to obtain a pellet rich in EVs. The pellet containing EVs is subsequently resuspended and washed in a small amount of phosphate buffer. After this basic UC procedure, the resulting product is often contaminated with nucleic acids and protein aggregates (Dad et al., 2021). Therefore, for further purification, the homogenized suspension is subjected to ultracentrifugation in a sucrose gradient (dgUC) at a high speed of more than 150,000 g for 120 minutes.

To obtain ultra-pure EVs the high-speed UC cycle can be repeated several times. Although this is advantageous for achieving purity of EVs, it reduces the PDEVs concentration yield. In addition, repeated pelleting of EVs, under the high centrifugal force of differential UC, can compromise the structural integrity of vesicles and cause agglomeration (Dad et al., 2021). A comprehensive overview of the main results obtained so far in the isolation of plant EVs is presented in Table 6. So far, the vast majority of EVs have been isolated by UC methods, and the same drawbacks reported for purification of mammalian EVs also apply here. As an alternative to UC/dgUC for isolation of plant vesicles, Kalarikkal et al. developed a method for purification of ginger EVs based on polyethylene glycol-6000 (PEG6000). Using different concentrations of PEG6000, the authors were able to recover between 60 and 90% of EVs compared with the UC method. PEG-EVs exhibit almost identical composition, size and zeta potential to UC obtained vesicles, (Kalarikkal et al., 2020). PEG precipitation methods can provide a scalable and cost-effective alternative to purify plant EVs with high yields, although contamination by non-EV proteins and the need for additional cleaning steps to remove PEGs are limiting factors (Iravani and Varma, 2019). Bokka et al. explored the use of SEC to purify tomato-derived EVs (Bokka et al., 2020). The authors compared the performance of UC/SEC and UC/dgUC methods for the isolation of tomato EVs and found that while gUC allowed for the collection of distinct subpopulations of EVs, SEC provided a higher level of purity of EV products. You et al. used UF to reduce juice volume and SEC as the main purification step to isolate EVs from different types of cabbage. Interestingly, they compared the yield and purity of cabbage-EVs obtained by UC and precipitation with PEG (You et al., 2021). The authors concluded that the SEC/UF method was superior to the other methods, reporting similar yields (10×10^9 particles/μg of protein for SEC derived EVs) but consistently higher purity values. Of all the methods mentioned, filtration techniques are easy and fast and have a great potential in biomanufacturing of plant vesicles. So far, TFF for isolation of plant EVs has only been used in combination with other techniques such as UC. Kim et al isolated EVs from aloe vera peels by coupling UC and TFF. In particular, they used a standard UC protocol followed by UF using a 0.22 μm filter and a TFF concentration with a 300 kDa membrane. They recovered 5.35×10^9 particles/mL of aloe vera juice, (M. K. Kim et al., 2021). Further work should be directed toward the development of filtration techniques that can be suitable alternatives to UC, and not just additional purification steps.

Table 6: Review of the literature on nanovesicles (NVs) and microvesicles (MVs) of plant origin obtained, reporting the method of isolation, physical and biological properties, yield and particle number (when available).

Source	Part	Isolation method	Diameter [nm]	Yield	Particle Number	Cell uptake	Stability and biological activity	Ref.
Ginger	Rhizome	dUC/gUC	102 – 998 (mean ~386 and ~294)	NA	NA	Uptake by primary Hepatocytes	Very stable in stomach-like and small intestine-like solutions	(Zhuang et al., 2015)
Ginger	Rhizome	PEG precipitation	100-900 (mean ~400)	2-3.8 g/kg	NA	Uptake by the murine macrophages; protects cells from H_2O_2 induced oxidative stress.	/	(Kalarikkal et al., 2020)
Grape	Fruit	dUC/gUC	50-300 (mean 380.5 ± 37.47)	NA	NA	Uptake by mouse intestinal stem cells	/	(Ju et al., 2013)
Grapefruit	Fruit	dUC/gUC	105-390 (mean 210.8 ± 48.62)	NA	NA	Uptake by mouse intestinal macrophages	Very stable at 37 °C	(Wang et al., 2014)
Grapefruit	Fruit	dUC/gUC	180-200	2.21 ± 0.044 g/kg	NA	Uptake by splenic and liver cancer cells lines in mouse models	Very stable at 4 °C for more than one month and loaded with curcumin	(Wang et al., 2013b)
Tomatoes	Fruit	dUC/gUC/ SEC	50–500	MVs 35.6 ± 8.6 mg/kg (protein) NVs; 25.8 ± 11.4 mg/kg (protein)	MVs 2.7 x 10^{16} particles/ kg; NVs 3.8 x 10^{16} particles/ kg			(Bokka et al., 2020)
Broccoli	Flower	dUC/gUC	~18 and 118.	NA	NA		Broccoli NVs administration in mice protects from intestinal inflammation and prevent colitis	(Deng et al., 2017)
Apple	Fruit	dUC	100-400	NA	1.6 x 10^{13} particles/ L	Uptake by Caco.2 cells (intestinal epithelium)	NVs disappear when boiled or sonicated	Fujita et al.(Fujita et al., 2018)
Coconut	Fruit	dUC/MF	10-100 (Mean coconut water 59.72, milk 100)	NA	NA			(Zhao et al., 2018)
Citrus clementina	Fruit	dUC/gUC	75–345 (mean populations at 75, 120, 155)	1.67 x 10^{-3} g/L (protein)	1.16 x 10^{12} particles/ L juice		Significant presence of membrane transporters protein	(Stanly et al., 2019)
Citrus sinensis	Fruit	dUC	950, 480 (avg sizes)	0.178 g/L (protein)	NA			(Pocsfalvi et al., 2018)

(sweet orange)								
***Citrus paradisis* (grapefruit)**	Fruit	dUC	255, 350 (avg sizes)	0.134 g/L (protein)	NA			(Pocsfalvi et al., 2018)
***Citrus aurantium* (bitter orange)**	Fruit	dUC	5500, 700 (avg sizes)	0.161 g/L (protein)	NA			(Pocsfalvi et al., 2018)
Citrus limon	Fruit	dUC	820, 460 (avg sizes)	0.409 g/L (protein)	NA			Pocsfalvi et al.(Pocsfalvi et al., 2018)
Citrus limon	Fruit	dUC/MF/gUC	50-70	2.5 x 10^{-3} g/L	NA	Uptake by human lung carcinoma cell line and myeloid leukaemia cell line	*Citrus* NVs inhibit the growth of tumor cell lines inducing TRAIL-mediated cell death.	(Raimondo et al., 2015)
Carrot	Root	dUC/gUC	100-1000	NA	NA	Targeting properties to intestinal macrophages and stem cells	Data suggest that the vesicle size can be altered in a pH-dependent manner	(Mu et al., 2014)
Blueberry	Fruit	dUC/MF	100-900	NA	NA		* miRNA profiling of PDEVs of 11 different fruits and vegetables.	(Xiao et al., 2018)
Hami melon	Fruit	dUC/MF	100-800	NA	NA		*	(Xiao et al., 2018)
Pea	Seed	dUC/MF	100-800	NA	NA		*	(Xiao et al., 2018)
Pear	Fruit	dUC/MF	100-800	NA	NA		*	(Xiao et al., 2018)
Soybean	Seed	dUC/MF	100-700	NA	NA		*	(Xiao et al., 2018)
Orange	Fruit	dUC/MF	100-700	NA	NA		*	(Xiao et al., 2018)
Kiwifruit	Fruit	dUC/MF	10-700	NA	NA		*	(Xiao et al., 2018)
Sunflower	Seed	MF/ dUC	50-200	NA	NA			(Regente et al., 2009)
Strawberry *(Fragaria x ananassa)*	Fruit	dUC/MF	30-191	18 ± 3 µg/0.25 L juice	NA	Uptake by human MSCs preventing oxidative stress in a dose-dependent manner	Rich content of vitamin C and miRNAs cargo	(Perut et al., 2021)

6. Conclusions

EVs offer many therapeutic opportunities as natural nano-vectors for drug delivery applications. If they are to be exploited industrially, there are several challenges to overcome in moving from the current laboratory-scale research practices to reliable, GMP-compliant technologies for processing EVs on a large scale. The main hurdle facing the bioprocessing of EVs is the lack of analytics, that prevents the identification of specific EVs CQAs, thus hindering process development. There are many recent advances in EVs characterization techniques, and global efforts should be devoted to their implementation in EVs processing protocols. An example of advanced EV surface characterization technique to identify and quantify the expression of identity markers is given by the study of Skovronova et al.; they performed single vesicles imaging on MSC-EVs using super-resolution microscopy, allowing to characterize a large number of EVs at a single EV level. Besides, ExoView chip-based analysis allowed an easy quantification and comparison of MSC-EVs markers, through the evaluation of the number of particles captured on a chip coated with tetraspanins. The authors also performed semiquantitative bead-based flow cytometry using a MACSPlex exosome kit (Skovronova et al., 2021). Sanchez et al. developed Green Fluorescent Protein (GFP)-tagged EVs by engineering Chinese hamster ovary (CHO) cells to express CD81 fused to GFP through a flexible peptide linker. The GFP-tagged EVs can be identified through a fluorescence plate reader and GFP concentration can be estimated based on fluorescence intensity, (Carrillo Sanchez et al., 2022). This fluorescence approach allows to estimate EVs yields and track EVs recovery during purification processes, such as UF and SEC, greatly simplifying process development. There is growing interest in using alternative sources to human cells, as the latter require challenging and expensive cell culture and expansion. Cultivation of bacteria and algae cell is simpler and cheaper, and EVs derived from these sources possess distinctive characteristics that can interest a wide range of applications. EVs derived from foods, such as milk and vegetables, do not require any cell culture system, are widely available, inexpensive, and can be potentially isolated from the waste streams existing industrial plants. However, their use is limited by knowledge gaps and the need for extensive biological characterization (e.g., definition of specific protein markers) and CQAs. To date, food derived EVs are mostly isolated using UC-based protocols, achieving yields and product purity comparable to current mammalian EVs production systems. The use of chromatographic separations, such as gel filtration and ion exchange chromatography, as alternative isolation methods is increasing. They possess a good trade-off between recovery and product purity and they are already being exploited in the field of industrial bioprocessing of mAbs, liposomes and viral vectors. Affinity chromatographic techniques are particularly attractive for large-scale EVs production and their recent advances applied to the purification of human EVs could be exploited in processing EVs from alternative sources. For instance, the use of pseudoligands (e.g., heparin that exploits electrostatic interactions on the EVs surface) or receptors for the membrane's EVs phospholipids (e.g., Tim4 for cow milk vesicles), have good potential, as they guarantee high specificity and do not require knowledge of specific EVs markers. In this field, membrane processes are emerging for both product concentration and purification by diafiltration and have the greatest potential for scalability. They can be used as stand-alone techniques or coupled with others, such as liquid chromatography, UC or polymer precipitation. Filtration processes are flexible in that process parameters can be tuned and membranes can be selected to recover intact, well-defined EV populations. They are fast and inexpensive and offer many opportunities for functionalization (e.g., ionic membranes, affinity membranes). Here, the use of TFF for downstream processing of EVs to achieve high product yield is illustrated. Future efforts should be devoted to minimize membrane fouling through the development of novel filtration apparatuses aimed at optimizing fluid dynamic conditions. In this context, microfluidics techniques are particularly intriguing as emerging tools for understanding and optimizing membrane processes. They enable manipulation of fluid flow at the microscale, resulting in more predictable systems with improved flux and selectivity, exploiting shear-induced phenomena at the membrane surface to reduce particle aggregation and deposition (de Aguiar and Schroën, 2020). In the field of EVs production, *the process defines the product* (Rathore and Winkle, 2009) and the mentioned separation techniques should be designed in a product-specific context. Overall, to accelerate progress in the field, early actions are needed to define quality control matrices, as standard platforms for EVs characterization and product potency assays.

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