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# Review

# Recent advances and challenges in the recovery and purification of cellular exosomes

Exosomes are nanovesicles secreted by most cellular types that carry important biochemical compounds throughout the body with different purposes, playing a preponderant role in cellular communication. Because of their structure, physicochemical properties and stability, recent studies are focusing in their use as nanocarriers for different therapeutic compounds for the treatment of different diseases ranging from cancer to Parkinson's disease. However, current bioseparation protocols and methodologies are selected based on the final exosome application or intended use and present both advantages and disadvantages when compared among them. In this context, this review aims to present the most important technologies available for exosome isolation while discussing their advantages and disadvantages and the possibilities of being combined with other strategies. This is critical since the development of novel exosome-based therapeutic strategies will be constrained to the effectiveness and yield of the selected downstream purification methodologies for which a thorough understanding of the available technological resources is needed.

## 1 Introduction

Living organisms have evolved to use different highly-selective intercellular communication pathways that allow the transport of biological signals and materials, which guarantee the correct function of cells, tissues, organs, and systems [1]. In this context, a particular type of cell-derived vesicles, exosomes, were first observed in reticulocyte as internal bodies that were released from the cell as endosomal vesicles merged with the cell membrane. In this first observation, exosomes were found to play an important role in the removal of plasma membrane proteins [2]. However, since exosomes can be virtually obtained from all types of eukaryotic cells (but most commonly in those of the immune system) they have been associated with the transport of nucleic acids, proteic, and other signaling cargos in different models [3]. Recent evidence suggests a crucial role of exosomes in many aspects of disease from metastasis in cancer, to cardiomyocyte size in heart failure [4–6]. The study of these vesicles is paramount to fully understand the physiopathology of the leading causes of death worldwide. Moreover, previous studies have suggested the potential use of exosomes as biomarkers in cancer, cardiovascular disease, and neurodegenerative disorders [7–10].

As naturally occurring nanocarriers, the use of exosomes in therapeutic strategies have also been explored. Drugs and many other compounds may be loaded into them, harnessing highly specific delivery systems [8, 11]. Furthermore, functionalization techniques have investigated the use of exosomes as modulators of physiopathological processes [12,13]. For instance, these vesicles have been an important aid in the development of recent cancer immunization strategies [14] showing an enormous potential in different health-related applications. However, the use of exosomes as therapeutic agents is still far away from becoming a reality since their procurement is usually a difficult, time-consuming, and low-yield task.

Structurally, exosomes are composed by a lipid bilayer membrane with specific surface proteins, which differ between cell types although they possess a common set of conserved protein molecules [15]. Exosomes are usually smaller than other extracellular vesicles (between 15 and 75 nm) and thus require special considerations in their separation and analysis [16]. Although different approaches for the separation, purification, and analysis of exosomes have been explored, so far, there is no methodology providing enough robustness regarding purification yield, selectivity, and reproducibility. In fact, because of the inherent biochemical properties of these vesicles and the enormous differences

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**Abbreviation: HIC**, hydrophobic interaction chromatography

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between them that depend on the matrix from which they are obtained, usually a combination of techniques needs to be tailored to achieve the desired purification outcomes. In this sense, different physical and/or chemical methodologies have been studied and proposed to achieve a good exosome purification yield. This work aims to establish the current state of the art in exosome isolation strategies highlighting the advantages and disadvantages of each of the most commonly used techniques while presenting a perspective of the future of this important topic. Table 1 [17–54] presents an overview of the different types of exosomes regarding their biological source, their functions, applications and isolation procedures currently used to procure them.

## 2　Exosome recovery and purification

Regardless of their final intended use, exosomes need to be isolated from varied and complex biological samples. This is a critical procedure, since exosomes must preserve their physicochemical properties and biological function after isolation. Furthermore, many challenges arise when isolating these naturally-occurring particles. For example, exosomes belong to a large domain of extracellular vesicles, some of which present overlapping physicochemical properties [55]. Moreover, exosomes themselves exhibit high heterogeneity in size, cargo, and surface markers [15, 56, 57]. Therefore, exosome isolation methods must be efficient, specific, flexible, and have long-term perspective of clinical applications. In this context, current isolation methods include ultracentrifugation, filtration, precipitation, chromatography, immunoaffinity capture and microfluidics, which will be discussed in the following subsections. A description of the different and most commonly used exosome isolation techniques, as well as some of the recommended applications for each one is presented in Table 2.

### 2.1　Ultracentrifugation

The current golden standard for exosome isolation is ultracentrifugation [58]. As known, this technique exploits the particle movement principle due to gravitational acceleration in an inertial field [59]. Differential and density gradient ultracentrifugation are among the most commonly used ultracentrifugation methods for exosome isolation [60].

Differential ultracentrifugation is commonly known as the pelleting method, because it allows to obtain pellets containing exosomes. It is also referred to as the simple ultracentrifugation method since it only requires several ultracentrifugation steps [61]. To date, this is the most widely used method for isolating exosomes and has been successfully used in a variety of biological fluids and cell culture media [62,63]. During differential ultracentrifugation, exosomes are separated based on their density and size. Thus, contamination from other vesicles, molecules or particles that overlap in these parameters is expected. To reduce the presence of

cell debris and large vesicles, cleaning steps are needed before pelleting the exosomes [64–66]. In this context, differential centrifugation at low gravitational forces has shown to remove such contaminants, and it is widely recommended. In each cleaning step, contaminants are precipitated, and the final supernatant is finally ultracentrifuged to pellet the exosomes [67].

Exosomes are sedimented by ultracentrifugation using forces between 100 000–200 000 × *g* for 1 to 2 h [23, 66, 68]. The recovered pellet is then resuspended in a convenient buffer such as PBS. Sometimes further purification steps may be useful for certain types of downstream exosome analysis [69]. However, these need to be adjusted depending on the requirements of such analytical methods, since further purification steps will also decrease the final exosome recovery yield.

Multiple parameters can alter the consistency of a differential ultracentrifugation protocol [70]. Different rotor types require specific centrifugation parameters to achieve exosome sedimentation, not only fixed angle and swinging bucket rotors have different sedimentation pathlengths but also the *g*-force differs according to the distance from the rotational axis [68]. Thus, the centrifugation time and *g*-force should be properly adjusted to fit the particular rotor used. In this regard, the *k*-factor can be used to perform proper rotor conversion calculations. Cvjetkovic et al. compared, adjusted, and unadjusted protocols using fixed-angle and swinging-bucket rotors and found that an unadjusted protocol results in a lower exosomal RNA yield [70]. According to these results, a specific *g*-force does not pellet exosomes with the same efficiency in different types of rotors, and the centrifugation time needs to be properly considered. Otherwise, sample purity, protein content and exosomal yield will be compromised [68]. Nonetheless, differential ultracentrifugation has been used to isolate exosomes from cell culture medium, urine, plasma, cerebrospinal fluid, among other biological matrices [71–74].

It should be noted that the *g*-force used during ultracentrifugation protocols has a significant effect on the purity and yield of exosomes [75]. Moreover, exosome sedimentation efficiency has been found to vary among cell lines. Jeppesen et al. demonstrated a significant difference in the sedimentation profile of vesicles and proteins from FL3 cells compared to HEK293 cells [76].

Using the particle to protein ratio as a measure of sample purity, Jeppesen et al. showed that the samples with the highest purity are found after ultracentrifugation at 67 000 × *g* for KEK293 and after 100 000 × *g* for FL3. Remarkably, different expression patterns for exosomal markers TSG101 and syntenin were also found in the previous study, suggesting that TSG101 may be more expressed on larger or denser exosomes that sediment more easily, and that syntenin may be more expressed on smaller or less dense exosomes that sediment at higher *g*-forces. These observations highlight the importance of adapting ultracentrifugation protocols considering the cell line, since it is also possible that exosomes from different origins exhibit different sizes [76].

**Table 1.** Exosome classification based on the producer cell type

| Source | Function | Application | Isolation | References |
|---|---|---|---|---|
| **Nervous system** | | | | |
| Neuron derived exosomes | Reciprocal control of excitatory synapse, modulation of axonal branching, neuronal activity and plasticity, release and trans-synaptic transmission of proteins | Spinal cord and peripheral nerve regeneration, targeted therapy for neurological disorders, early detection of neurodevelopmental disorders, neurodegenerative disorders, and traumatic brain injury | Ultracentrifugation of neural cell culture medium, differential ultracentrifugation of brain tissue homogenates, and precipitation of cerebrospinal fluid | [17–21] |
| Schwan-Cell derived exosomes | | | | |
| Microglia-Cell derived exosomes | | | | |
| Astrocyte derived exosomes | | | | |
| Oligodendrocyte derived exosomes | | | | |
| **Cardiovascular system** | | | | |
| Cardiomyocyte derived exosomes | Induction of cardiomyocyte hypertrophy, regulation of oxidative stress and inflammation, inhibition of apoptosis, induction of cardiac endothelial cell proliferation, and modulation of cardiac fibrosis | Biomarker of myocardial injury, targeted therapy for myocardial infarction and heart failure, and prognostic marker of cardiovascular diseases | Differential ultracentrifugation of cardiomyocyte homogenates, multi-step centrifugation of cardiac fibroblast culture medium, and precipitation of pericardial fluid | [6, 22–24] |
| Cardiac Telocyte-derived exosomes | | | | |
| Cardiac Progenitor-Cell derived exosomes | | | | |
| Endothelial-Cell derived exosomes | | | | |
| Cardiosphere-derived exosomes | | | | |
| **Liver** | | | | |
| Hepatocyte derived exosomes | Induction of hepatocyte survival, growth, migration and proliferation, trans-differentiation of myofibroblast hepatic stellate cells, regulation of fibrosis, and the inflammatory response in the liver | Targeted therapy for liver regeneration, biomarkers for assessing the safety of liver transplantation, diagnostic and prognostic markers of nonalcoholic fatty liver disease, and alcoholic liver disease | Density-gradient ultracentrifugation of liver tissue homogenates, differential ultracentrifugation, and precipitation of primary hepatocyte culture medium | [25–29] |
| Cholangiocyte derived exosomes | | | | |
| Kupffer Cell-derived exosomes | | | | |
| Liver Endothelial Cell-derived exosomes | | | | |
| Hepatic Stellate Cell-derived exosomes | | | | |
| **Stem cells** | | | | |
| Mesenchymal Stem Cell-derived exosomes | Development and growth of the embryo, adult tissue regeneration, differentiation and transformation, immunomodulation, and stromal remodeling | Induction of tissue remodeling, targeted regenerative therapy for neoplasms, ischemic myocardium, graft-versus-host disease, memory dysfunction, autoimmune disorders, and other degenerative disorders | Differential ultracentrifugation and one-step sucrose cushion ultracentrifugation of stem cell culture medium, and precipitation from stem cell culture medium | [30–33] |
| Neural Stem Cell-derived exosomes | | | | |
| Induced Pluripotent Stem Cell-derived exosomes | | | | |
| Embryonic Stem Cell-derived exosomes | | | | |
| Endothelial Progenitor Cell-derived exosomes | | | | |
| **Skeletal muscle** | | | | |
| Myotube derived exosomes | Control of myoblast differentiation and proliferation, regulation of skeletal muscle metabolic homeostasis, oxidative stress and inflammation | Targeted therapy for muscular dystrophy, insulin resistance and other metabolic disorders affecting muscle physiology | Differential ultracentrifugation of myoblast and myotube cell culture medium | [34, 35] |
| Muscle-derived Fibroblasts exosomes | | | | |
| **Pancreas** | | | | |
| Pancreatic stellate cell-derived exosomes | Regulation of cell proliferation, migration and modulation of immune responses (regulation of chemokines expression in pancreatic cells, antigen deliver and activation of dendritic cells) | Proposed as biomarkers for pancreatic cancer and as therapeutic target to control autoimmune responses in type-1 diabetes | Differential ultracentrifugation of cell culture medium | [36–38] |
| β-cell-derived exosomes | | | | |

(*Continued*)

**Table 1.** Continued

| Source | Function | Application | Isolation | References |
|---|---|---|---|---|
| ***Urinary exosomes*** | | | | |
| Bladder-derived exosomes<br>Kidney-derived exosomes<br>Prostate-derived exosomes | Induction of cell migration, modulation of angiogenesis, control of biological processes associated to the progression of advanced fibrotic disease, and regulation of inflammatory processes | The exosome concentration and several exosomal miRNAs and proteins are proposed as biomarkers for bladder cancer, diabetic nephropathy, lupus nephritis, Parkinson's disease, IgA nephropathy, and prostate cancer | Differential ultracentrifugation and precipitation of human urine | [39–43] |
| ***Immune system*** | | | | |
| Dendritic cell-derived exosomes<br>B-cell-derived exosomes<br>Macrophage-derived exosomes<br>T-cell derived exosomes | Antigens distribution and delivery for activation of immune response, elimination of immune cells for suppression of immune response, and regulation of the expression of pro-inflammatory molecules | Potential use as nanocarriers for the delivery of immunomodulatory molecules, and vaccines for immune therapy | Differential ultracentrifugation and ultracentrifugation followed by sucrose gradient purification of culture medium | [44–48] |
| ***Breast*** | | | | |
| Breast milk-derived exosomes | Regulation of immune response, protective role against vertical transmission of HIV-1, and stimulation of intestinal stem cell activity | Prevention of necrotizing enterocolitis in intolerant breastfeeding infants and immune therapy | Differential centrifugation of human breast milk of healthy mothers and precipitation of human and rat breast milk | [49–52] |
| ***Tumor-derived exosomes*** | | | | |
| Glioblastoma cell-derived exosomes<br>Colon cancer cell-derived exosomes<br>Melanoma cell-derived exosomes<br>Nasopharyngeal carcinoma cell-derived exosomes<br>Prostate cancer cell-derived exosomes<br>Cervical cancer cell-derived exosomes<br>Diffuse large B-cell lymphoma side population cell-derived exosomes<br>Lung cancer cell-derived exosomes<br>Bladder cancer cell-derived exosomes<br>Breast cancer-cell derived exosomes<br>Mesothelioma cancer cell-derived exosomes<br>Pancreatic cancer cell-derived exosomes<br>Renal cancer cell-derived exosomes<br>Chronic myeloid leukemia cell-derived exosomes<br>Hepatocellular carcinoma cell-derived exosomes | Promotion of tumor proliferation, invasion and metastasis, enhance angiogenesis, impair immune response and increase cancer resistance to therapies | Proposed as biomarkers for the detection of several types of cancer and nanocarriers for targeted drug delivery against cancer proliferation | Differential centrifugation and precipitation of human fluids and cell culture medium | [53, 54] |

**Table 2.** Proposed classification of exosome isolation methods based on their main principle. Advantages and disadvantages are highlighted after each method. Grouped rows indicate the most commonly associated techniques. Feasibility of the different potential applications depending on the isolation method are also identified

| Method | Type | Advantages | Disadvantages | Nucleic acid quantification and sequencing | Biomarker screening | Protein quantification and identification | Drug delivery systems |
|---|---|---|---|---|---|---|---|
| Centrifugation | Physical | Simple protocols<br>Preservation of physicochemical properties | Low purity<br>Specialized equipment required | ✓ | ✓ | ✗ | ✓ |
| Ultrafiltration | Physical | Fast protocols<br>Cheap materials<br>High protein and RNA yield | Low purity<br>Exosome deformation and extrusion | ✓ | ✓ | ✗ | ✗ |
| Precipitation | Physicochemical | Highly reproducible<br>High vesicle and RNA yields<br>Requires only lab-bench equipment | Low purity<br>Exosome aggregation | ✓ | ✓ | ✗ | ✗ |
| Chromatography | Physicochemical | High exosome purity yields<br>Preservation of physicochemical properties | Medium to high processing times<br>Exosome degradation due to selected operation buffers | ✓ | ✓ | ✓ | ✓ |
| Microfluidics | Physical | Miniaturization<br>Device functionalization<br>Short analysis times | Low reproducibility<br>Low exosomal yields<br>Exosome aggregation | ✓ | ✓ | ✓ | ✓ |
| Immunocapture | Chemical | High specificity<br>High purity<br>Potential scalability | Expensive materials<br>Low exosomal and RNA yields | ✓ | ✗ | ✓ | ✗ |

The exosomes isolated with this method have been successfully identified by immunoblotting, electron microscopy, and quantified with nanoparticle tracking [77–79]. For instance, exosomes have been purified from culture medium of cardiac fibroblasts by a simple differential ultracentrifugation protocol. These fibroblast-derived exosomes were confirmed by the presence of CD63 using Western Blot and flow cytometry. Moreover, exosome uptake experiments were performed with the PKH26 fluorescent dye and RNA sequencing was performed, showing that cardiac fibroblasts secrete exosomes to mediate cardiomyocyte hypertrophy [23].

In general terms, differential centrifugation methods are considered easy to perform, moderately time-consuming and do not require sample pretreatments [58]. However, as it has been mentioned, this method is not specific and contamination with other extracellular vesicles is unavoidable. If the protocol is not well standardized and adapted (in terms of time and gravitational force) to the characteristics of the equipment being used, exosome isolation will not be consistent, and losses will occur inevitably [59].

On the other hand, density gradient centrifugation is a well-known technique for the purification of cellular organelles [80–82]. If exosomes are considered as such, this ultracentrifugation variation is well within the capabilities of performing successful exosome isolations. In this technique, a continuous or stepwise density gradient is formed in the medium during (or before) the centrifugation procedure [58, 68]. Thus, once submitted to the centrifugal force, exosomes will move through the gradient according to their density [83].

Density gradient ultracentrifugation results in a density region of interest, where exosomes are concentrated [84]. Density gradient ultracentrifugation can be performed by the bottom- or the top-loading method. In the case of bottom-loading method, also called isopycnic ultracentrifugation the major effector is density [85]. On the other hand, in the top-loading method, also called moving-zone ultracentrifugation, the major effector is particle size [84]. Exosomes may move to a zone where they have the same density as the medium, as is the case for isopycnic ultracentrifugation in which a density gradient medium embracing the entire range of solute densities is used [85,86]. Exosomal density has been reported between 1.10 and 1.21 g/mL in cesium chloride density gradients [55]. On its part, moving-zone ultracentrifugation uses a gradient density medium with a lower density than the sample [84]. In this particular variation, exosomes and all

the other solutes will eventually precipitate, thus the centrifugation time dictates the position of the vesicles according to their size. A single or double sucrose cushion is often used in both techniques to avoid losses and excessive contamination [87,88]. Regardless of the density gradient ultracentrifugation procedure being used, the fraction of interest is subjected to another round of simple ultracentrifugation at $>100\,000 \times g$ for further purification [83]. It should be noted that this particular technique may be considered complex, time-consuming and inefficient, due to considerable exosome loss [68]. However, density gradient centrifugation may be more specific than differential ultracentrifugation to certain exosome types, especially when using moving-zone ultracentrifugation.

As an example, density gradient ultracentrifugation has been successfully used to isolate exosomes from culture supernatants of N2a cells for the study of Alzheimer's disease [89]. In this case, differential ultracentrifugation was first used to pellet the exosomes. Then, the exosomes were resuspended in a 2.5 M sucrose solution, and a step gradient of sucrose was layered over it. The gradient was further spun, and fractions were collected for immunoblotting and electron microscopy. This study showed exosomal proteins bound to Alzheimer's disease signature plaques, suggesting a role in the pathogenesis of such disease.

Another approach to avoid contamination and excessive losses is to use an iodixanol cushion or gradient. A recent study showed that differential centrifugation, ultracentrifugation with an iodixanol cushion, and ultracentrifugation on an iodixanol density gradient yielded the same number of particles [90]. This was the same result as for clearance from an *in vivo* model. Nonetheless, according to electron microscopy, the exosomes of the gradient method were more dispersed.

It should be noted that another advantage of ultracentrifugation protocols is their capability to clearly yield vesicles with diameters of approximately 100 nm, which are observable by electron microscopy [66]. Other methods, such as precipitation, usually result in large aggregates and non-spherical lumps with diameters larger than 1 µm [58]. Moreover, exosome size distribution and zeta potential appear to be unchanged by ultracentrifugation [90], which is of great importance for the different exosome applications that are being proposed and studied.

## 2.2 Filtration-based strategies

Ultrafiltration is another available technique to obtain exosome enriched samples from a variety of sources including cell culture medium and biological fluids [91]. In this process, extracellular vesicles suspended in a solution can be separated by size or molecular weight. Usually, different forces are applied to make them pass through (or be retained on) a selective membrane. Centrifugal force, pressure or vacuum are usually applied for ultrafiltration through a membrane that is commonly built from low protein affinity materials. In this context, nanomembranes have been used to enrich exo-

some samples as effectively as ultracentrifugation, in smaller volumes and shorter times [58, 68].

Ultrafiltration is usually a complement to other exosome isolation techniques [92]. Several protocols use this method as a cleaning step, especially after ultracentrifugation and precipitation techniques [93–95]. Such protocols have demonstrated to selectively separate exosomes as confirmed by cryo-electron microscopy, dynamic light scattering, and Western Blot analysis for CD63 and CD81 [96]. However, ultrafiltration with selective filtration membranes can also be used to isolate exosomes by itself. Several protocols, which are faster, easier and less expensive than ultracentrifugation, are available [68]. Filtration by size and molecular weight are usually coupled in the same protocol [97]. Filters with pore sizes of 0.8, 0.45, and 0.22 µm and membranes with a molecular weight cut-off ranging from 10 to 100 kDa are commonly used [14, 61, 98–101]. Furthermore, exosome-derived RNA and protein concentrations are considerably larger than in most methods, as the final sample will be highly concentrated [102]. For instance, this method has been used to obtain highly-concentrated urinary exosomes that may serve as biomarkers of renal diseases [87, 103]. First, raw urine samples were clarified using conventional centrifugation. Then, the clarified urine was microfiltered through a 0.1 µm pore size filter and further concentrated using a 10 kDa molecular weight cut-off membrane. The presence of exosomes was validated by electron microscopy.

Nevertheless, ultrafiltration, a simple protocol, is incapable of isolating only exosomes, as microvesicles and apoptotic bodies will also be present in the resulting product. Moreover, large amounts of highly abundant proteins, that mimic exosome size or molecular weight, will also be found in the resulting solution. Such contamination arises from the physical limits of the procedure and the overlapping properties of the particles in the matrix being processed. Furthermore, the effects of the applied force and the contact with the membrane on the exosomes need to be further studied. Potential deformation and exosome losses due to extrusion and membrane binding are expected [104]. However, it has also been reported that exosomes isolated by this method have physical properties comparable to those obtained by ultracentrifugation or precipitation [90].

Other filtration techniques include hydrostatic filtration dialysis and size-exclusion chromatography. In hydrostatic filtration dialysis, the sample moves through a dialysis membrane and exosomes are retained as the solvent and other solutes equilibrate [105]. This method is particularly useful for enriching and concentrating the sample. It shows one of the highest exosomal yields of all methods, and does not require ultracentrifugation [92, 106]. It is also scalable and applicable to a large volume [107].

Size-exclusion chromatography, on its part, is a filtration procedure in which a stationary phase sorts dissolved particles according to their size distribution. Depending on their hydrodynamic radii, these particles will be differentially eluted from the column [108]. This technique is hyphenated with other methods like ultracentrifugation, immunoaffinity

capture, and even precipitation since these combinations usually enrich the sample and produce better quality exosomes (based on morphology evaluation by electron microscopy) [109, 110]. A recent study compared exosome isolation by size-exclusion chromatography to polymer-based precipitation and protein organic solvent precipitation [111]. Although the three methods successfully isolated exosomes (confirmed by flow cytometry and Western Blot of CD9, CD63, and CD81) only size-exclusion chromatography did not alter vesicular size and produced the highest purity as observed by cryo-electron microscopy. This procedure preserved biological function but more importantly, it was found that size-exclusion chromatography usually yields the highest representation of exosomal biomarkers [112]. Limitations found include the sample volume (as it depends on the column volume) and its yield (as it is lower than other techniques when used on its own) [113]. It is important to mention that other chromatographic modes, especially hydrophobic interaction chromatography (HIC), have also been used. For instance, novel PET capillary-channeled polymer fibers have been used for HIC with similar results regarding exosome size distribution and number density as those obtained by centrifugation [114]. However, reports on other chromatographic modes being employed for this particular application are scarce. In this regard, it should be mentioned that most reports using chromatography as a tool to separate exosomes use different prepacked gravity-based commercial columns (i.e., in most reviewed cases the columns are not connected to chromatographic equipment). This suggests possible vesicle damage due to pressure changes that chromatographic equipment may exert over them. The buffers needed to operate other chromatographic modes besides SEC, like HIC or ion-exchange chromatography, may also have implications on the viability of the exosomes once they are processed. Therefore, although chromatography represents an interesting strategy to obtain pure exosome samples from different biological sources, more research is needed, especially if high-throughput strategies are needed to acquire sufficient amounts of these vesicles for all the possible applications that are arising.

## 2.3 Precipitation

Exosome precipitation is a recently developed technique that is now the second most used isolation method after ultracentrifugation, most likely because it is fairly easy to perform and does not require specialized or expensive equipment [68]. This technique was originally developed to isolate viruses and other macromolecules, but exosomes can also be settled from biological fluids using the same principles [115]. Most precipitation methods consist on mixing the sample, which can be either a biological fluid or cell culture medium, with a hydrophilic polymer. After mixing, the sample is incubated overnight at 4°C and afterwards low speed centrifugation is used to precipitate the exosomes which are later resuspended in the preferred buffer for further analysis [116]. Protamine,

sodium acetate, and organic solvents can also be used for precipitation procedures [117, 118].

Nowadays, exosome precipitation can be achieved with several commercially available reagents like ExoQuick® (System Biosciences), Total Exosome Isolation Reagent® (Invitrogen) and Exosome Purification Kit® (Norgen Biotek), among others. Most of the kits are also available for urine, plasma, serum, cerebrospinal fluid, and cell culture medium matrixes. Precipitation by these reagents is achieved by forcing the exosomes out of solution and trapping them in a porous mesh that facilitates their pelleting at low speed centrifugation. Most available kits contain polyethylene glycol as the hydrophilic compound that excludes water molecules and forms the network in which the exosomes sediment [111, 119].

For instance, this technique was used to isolate exosomes from serum samples to study non-coding RNAs as biomarkers for human hepatocellular carcinoma. Serum was centrifuged at 3000 rpm for 10 min and then the ExoQuick® isolation solution was added to the supernatant in an appropriate volume. The sample was incubated overnight and centrifuged at 1500 $\times$ $g$ for 30 min. The pellet was then resuspended in 100–500 µL of PBS. The procedure successfully yielded vesicles for further RNA isolation [120]. It was also shown that precipitation with ExoQuick® was a highly reproducible and efficient method. When compared to ultracentrifugation, precipitation resulted in a higher number of particles [121]. Ultracentrifugation showed higher levels of protein amount than precipitation. However, ultracentrifuged samples were contaminated with albumin and IgG. Both methods showed equivalent expression of CD9, IAMP2, and Grp94.

In another example, exosomes were isolated from serum using the Total Exosome Isolation Reagent® kit for the study of prostate cancer [122]. The reagent was added to the serum in an appropriate volume, incubated for 1 h and centrifuged at 10 000 $\times$ $g$ for 10 min at room temperature. The pellet was re-suspended in PBS. Exosomes were identified using transmission electron microscopy and quantified by nanoparticle tracking analysis. CD81 and CD63 were identified by Western Blot and flow cytometry analysis. RNA was extracted and quantified by qRT-PCR showing a differential expression profile before and after radiation treatment, suggesting a promising new biomarker that needs to be further studied.

It has also been shown that precipitation-based methods resulted in the highest exosome, miRNA, and mRNA yields from urinary samples when compared to ultracentrifugation and filtration, making precipitation ideal for RNA and proteomic analysis from this matrix [123]. Exosomes, precipitated from urine, were quantified using a CD9 ELISA, while miRNAs and mRNAs were assessed by qPCR.

There appears to be a consensus that precipitation-based methods yield the highest number of extracellular vesicles but with low purity, as it was found when compared to column-based methods [116]. Thus, another approach

consists on the incorporation of these methods after precipitation to further purify the sample.

Precipitation is easy to perform, fast, and it does not require an ultracentrifuge or any other expensive equipment. Moreover, several studies support the fact that this method yields the highest number of exosomes, total protein, and RNA [116, 123, 124]. Thus, it is considered a cost-effective technique.

It is important to mention that it has been shown that exosomes isolated by precipitation preserve their biological function, as demonstrated by in vivo particle tracking analysis and miRNA transference assessment [66, 125]. Moreover, in these studies, precipitation outperformed ultracentrifugation regarding the number of particles, while showing the same size distribution when compared to ultrafiltration and ultracentrifugation. Furthermore, since precipitation yields the highest total protein and RNA amount, it is also considered the ideal method for RNA analysis. Electron microscopy has demonstrated the preservation of exosome size and morphology after isolation by this technique. Also, precipitation-based exosome isolation kits are compatible with physiological pH range.

This method is very promising in the clinical context since it does not require sophisticated equipment and processing times are much shorter when compared to other methods, making it ideal for bedside or point-of-care diagnostics. Nonetheless, low purity is a key disadvantage. Coisolation of non-vesicular contaminants such as lipoproteins and ribonucleoprotein complexes, albumin, immunoglobulins and other soluble proteins is unavoidable [126]. Contamination with other vesicles is also expected. Unfortunately, this contamination may interfere with further biochemical and immunological analysis. In this context, the need for further purification has produced modified protocols that include pre- and post-isolation cleaning steps, lengthening originally fast procedures [127], and increasing workload and costs as well. Modified precipitation-based protocols are usually coupled to centrifugation or ultracentrifugation also reducing exosome yields [61].

In addition to coisolation of nonvesicular contaminants and other vesicles, recent studies have found exosome aggregation after precipitation [128, 129]. Exosome aggregates may also interfere with downstream analysis. Such findings have led to recommend precipitation for RNA analysis but the alternative use of other methods, such as ultracentrifugation, for protein and biochemical assessment.

New precipitation-based approaches consist of coating magnetic $Fe_3O_4$ nanoparticles with polyethylene glycol [130]. Such strategy has resulted in the removal of albumin and various immunoglobulins, while preserving the number of particles, particle size distribution and CD63 and CD9 expression in the sample. Novel strategies, that address the main disadvantages of precipitation, are required to improve the capabilities of this technique. This is crucial to fulfil the potential of precipitation as a method with serious clinical implications.

## 2.4 Immunoaffinity procedures

So far, we have mentioned immunoblotting as a mean to characterize or to identify exosomes after isolation. However, immunoaffinity techniques can also be used to selectively isolate exosomes from complex biological fluids. Immunoaffinity approaches take advantage of the many proteins, receptors, lipids, and polysaccharides that are present in the outer surface of exosomes. This technique exploits the highly specific affinity interactions that occur between an antigen and an antibody. All the molecules in the surface of exosomes are potential ligands. Nonetheless, exosome biomarkers ideally are highly concentrated or only present on the exosome membrane and lack free counterparts. Proposed exosome biomarkers include tetraspanins, heat shock proteins and MHC antigens, for example, CD9, CD10, CD24, CD63, CD81, EpCAM, Alix, AQP2, FLT1, TSG101, and HSP70, to name a few [131, 132]. Also, heparin has been proposed to bind exosomes [133]. Even lectins have been used to selectively identify and isolate specific subpopulations of these types of vesicles [134].

Immunoaffinity techniques are usually coupled to other strategies that integrate the specific selection of exosomes with a physical separation or isolation procedure [135, 136]. Such strategies allow the enrichment of the sample with exosomes or the depletion of unwanted vesicles. For instance, it was shown that tumor-derived exosomes can be captured using mAb 763.74 specific for the CDPG4 epitope unique of melanoma cells [137]. After monoclonal antibody production, characterization, and biotinylation, the mAb 763.74 was incubated with previously extracted exosomes for 12 h at 4°C. Tumor-derived exosomes were captured using streptavidin-coated magnetic beads. The bead-exosome complexes were then collected using a magnet. Such complex was further used for the fluorescent detection of the antibody with flow cytometry. The data showed that the capture of tumor-derived exosomes with the anti-CSPG4 mAb was highly reproducible, with an intra-class correlation coefficient of 0.98 with the 95% prediction interval at ±5.8.

Commercially available exosome isolation kits have been compared to immunoaffinity purification for the isolation of prostate derived vesicles from plasma, using atomic force microscopy and nanoscale flow cytometry. Immunoaffinity approaches resulted in the elimination of a significant portion of plasma proteins from the sample and a higher yield of prostate-derived exosomes [138].

Novel methods include modifications of the exosome membrane. A method combining immunoaffinity and lipid membrane surface modification was developed for the isolation and quantification of exosomes. Exosomes are captured, as previously mentioned, with immunomagnetic beads, and then a B-Chol-labeled DNA was anchored to the exosome membrane. The sticky end of the anchor initiated the HRP-linked hybridization chain reaction for signal amplification, easily measured with UV-Vis spectrometry. This method detected $2.2 \times 10^3$ exosomes per microliter, with 100%

higher sensitivity compared to ELISA [139]. It is believed that assembled assays, which can exploit several proteins and antibodies for plate and bead functionalization, may allow faster quantification and validation of exosomes over current techniques.

Several studies have also been performed to compare immunoaffinity procedures with other isolation methodologies. For instance, a study to compare ultracentrifugation, density-gradient isolation, and immunoaffinity capture methods was performed. Using the colorectal cancer cell line LIM1863, the methods were evaluated by electron microscopy and immunoblotting for Alix, TSG101 and HSP70. Although, all protocols contained 40–150 nm vesicles that were positive for exosome markers, the proteomic analysis based on the MS/MS spectra identified the immunoaffinity capture methods as the most effective [60]. This method has also been shown to yield good quality exosomes for further analysis, as it was shown after isolation of exosomes from serum using magnetic beads. Exosomal miRNAs were extracted after mixing the beads with a solution of a nonionic detergent and salt, and heating. qRT-PCR was then used to analyze the sample. In another example, a comparison between ultracentrifugation, polymer-based precipitation and this technique was performed. Findings showed that immunomagnetic capture isolated eight times more exosomes than ultracentrifugation and two times more than polymer-based precipitation. Moreover, cellular uptake experiments showed that captured exosomes retained biological activity [140].

The main advantage of the immunoaffinity procedures used with this purpose is the better quality and purity of the resulting isolated exosomes [141]. Since antigen-antibody interactions are highly specific, this technique is very useful to selectively isolate a subpopulation of extracellular vesicles without contaminants. Also, this technique is compatible with routine lab-bench equipment. Nonetheless, preparation of antibodies and magnetic beads among other immunoaffinity strategies requires expertise, not to mention expensive materials and reagents. It should also be noted that immunoaffinity methods are almost invariably hyphenated to other techniques [126]. Initially, exosomal content and RNA yields appeared to be the main issue with this strategy. However, the latest reports show a comparable or even greater yield when compared to ultracentrifugation and precipitation-based methods [140]. Besides higher capture efficiency and sensitivity, this approach can also be easily scaled due to the lack of limitations regarding the initial sample volume.

## 2.5 Microfluidics

The advent of microfabrication technology has increased the interest in microscale processes capable of performing biological analysis and purification procedures with high accuracy and specificity. Microfluidic devices, based on the manipulation of fluids at the micrometer scale, are an innovative strategy to efficiently isolate exosomes minimizing time, equipment and costs [142]. Nowadays, lab-on-a-chip devices that involve the integration of functional components explore the behavior of exosomes at the microscale. Exosome manipulation techniques based on microdevices include approaches like electrophoresis, dielectrophoresis, acoustics, magnetism, and immunoaffinity [97, 143, 144]. Furthermore, devices that combine two or more separation techniques have been designed.

For instance, a two-stage magnetic-based microdevice, which integrates isolation and *in situ* electrochemical analysis of exosomes from blood samples has been developed [145]. The device integrates an array of Y-shaped posts, to enhance Tim4-modified magnetic beads-exosome interaction and a cascading ITO electrode. The capture method involves magnetic enrichment on the surface of the electrode. In this device, the electrode integrated a signal transduction strategy based on a sensor containing CD63 and a mimicking DNAzyme sequence. CD63-positive exosomes enhance the production of $H_2O_2$ by NADH oxidation accompanied by signal enhancement. This device captured tumor-derived exosomes within 3.5 h in 30 μL samples and results were confirmed by ELISA and Western Blot [145].

Size-exclusion methods can also be integrated in microdevices. Nanoporous membranes or nanoarrays can be built inside the channel. In this case, exosomes are isolated by a pressure-driven filtration process. One of the main advantages of this type of devices is their capability to remove over 95% of the contaminants. Results are good enough to be confirmed by Western Blot and RT-PCR [144].

Some authors classify the exosome isolation mechanism within the microdevice as passive (static sorting) and active (dynamic sorting) [68, 142]. Passive sorting techniques include immunoaffinity, size-exclusion, and flow-induced methods. On the other hand, active sorting includes electroactive separation, immunomagnetic isolation, and acoustofluidics. Both active and passive sorting may also be combined in a single device.

Electroactive strategies are somewhat simpler than other techniques since no antibody affinity or other biochemical methods are needed. Moreover, no pumps or moving parts are required since the separation occurs based on charge-to-mass ratios. Shi et al. used a dielectrophoretic approach to design a low voltage nanopipette for entrapment of exosomes from plasma [146]. This study showed exosome entrapment at a glass pipette tip using direct current at a minimum 10 V/cm. Maximum enrichment was achieved after 1 h of applied voltage, but particles were detected after 100 seconds. Results were confirmed by detected fluorescence intensity measurements [146]. Such results opened the door to the further development of dielectrophoretic entrapment of exosomes and other electroactive strategies.

As known, microfluidic devices have the potential to replace expensive and spacious laboratory equipment. Also, in contrast to other methods the required volume of the sample and reagents is considerably lower, and the process may be automated and performed in a very short period of time [147]. These isolation techniques also have the potential of

high throughput and customization [148]. However, it should be noted that the device design is key for scalability and will impact process standardization. Nonetheless, no microfluidic device is free of shortcomings. For instance, exosome yield in immunoaffinity-based devices or microporous systems is lower when compared to ultracentrifugation or precipitation [149]. Moreover, although trained personal is not required to operate microdevices, reproducibility remains a challenge [150]. Analysis of the sample after isolation by microfluidics also needs to be standardized since results are usually not consistent [58, 146, 148, 149, 151].

The physical properties of exosomes are of interest when discussing the disadvantages of microdevices. Exosomes tend to aggregate, so device blocking is common [152, 153]. Furthermore, the effect of microfluidic forces on exosome integrity needs to be studied. Also, functionalization of such cellular nanoparticles within the device may challenge post-isolation analysis [151].

Finally, the movement from basic research to the clinical or application setting is a difficult quest. Although the potential for clinical or other types of applications is limitless, micro-devices need to be more robust and effective than the available standard methods. The full potential of this technology will be seen when the collaboration between engineers and clinicians or other experts results in overcoming the challenges posed by different application settings.

## 3 The future of exosome isolation procedures

Exosome isolation remains a challenge for biomedical research. There is still no consensus over which purification technique produces the best results and there is intense competition within the field. Moreover, an accurate comparison between methods cannot be easily made because of the inherent exosome complexity [12, 154]. However, exosome isolation has evolved over the last decade. Current methods have made progress in elucidating the role of exosomes in health and disease [67, 155]; and several characterization techniques have been employed to successfully prove the presence of exosomes in enriched samples from a variety of complex biological fluids [16, 156]. Yet, the gap between basic research and clinical applications remains wide. Current methods are not scalable for the clinical setting. Moreover, reasonable throughput and validation are required for bedside technology. In addition to avoid the use of specialized equipment, an ideal isolation method should be fast, reproducible, easy to perform, and flexible (i.e., must work with several biological matrices). Moreover, coisolation of contaminants should be minimum since contamination is the most common complication of current isolation techniques [58]. Almost invariably, coisolation of other vesicles and non-vesicular molecules occurs, interfering with data comparison between research laboratories [123].

Although, ultracentrifugation is currently the gold standard for exosome isolation. There is no ideal method that fits all purposes. The selection of the procedure usually depends on the capabilities and resources of each research team and sacrifices must be made in terms of recovery, purity or work load. Moreover, downstream analysis may be compromised by the isolation technique that is chosen [90]. In this sense, the final selection of the most suitable technique for exosome isolation and purification needs to consider the effects that the methodology may exert over the sample integrity particularly for the intended final use. For instance, recovery techniques such as ultracentrifugation and filtration tend to render a population of "saucer-like" or "deflated-football" shaped vesicles that might no longer be useful [157]. Furthermore, the integrity of the exosomal cargo to unravel exosome-specific functions and biomarkers should also be considered even when no apparent degradation is present [158]. This is especially true for microfluidic techniques or after isolation when exosomes are stored under freezing or other harsh conditions [159].

In this work, we have discussed the advantages and disadvantages of the most commonly used exosome extraction methods and some comparisons have been made. In general terms, the exosome isolation method selection should depend on the downstream analysis requirements, but it should be noted that technique combinations can improve isolation efficiency [72, 96, 107]. Nonetheless, additional time, reagents, and cost must be considered. Furthermore, additional separation procedures raise the error rate and may reduce exosome recovery yields. Thus, a proposed solution consists of integrating isolation and analysis procedures. In this sense, since immunoaffinity techniques have proven that isolation and characterization can be done in a few easy steps [126] it is believed that these research lines will be considerably important in the following years. A one-size-fits-all exosome recovery and purification method is still something difficult to develop, but the standardization of existing protocols that combine techniques for the resolution of a particular problem is the key. It is clear that the future of exosome isolation is a translational technology and that the development of robust and high-throughput protocols is required for the upcoming applications in all types of settings.

## 4 Concluding remarks

There are currently different strategies being used in the isolation and purification of exosomes whose selection depends on the intended application for the exosomal extract. To date, it is difficult to identify a strategy that yields the highest quality and properties of the isolated exosomes and usually a combination of the different methodologies is required to achieve the best results. Ultracentrifugation, to our belief, will continue to be one of the most used methodologies in exosome procurement. However, novel emerging strategies involving immunoaffinity and microdevices are appearing with interesting results and as a viable option to obtain these important nanoparticles.

As it has been mentioned, exosome-related applications are gaining attention by the scientific community since these vesicles can be used as carriers for different formulations with low side-effects and high specificity. In this line, one of the most promising applications refers to the use of these nanovesicles as carriers for gene therapies in tissue restoration and cancer applications. However, in order for these approaches to find a potential market and profitable application, exosome isolation and purification methods need to be further studied and scaled. This work presented some of the most used techniques for this purpose to date. Nonetheless, it is believed that in the following years new advances will be incorporated in these procedures with improvements to the current strategies or with completely new approaches. Furthermore, the development of exosome purification technologies will be closely related to the design of novel applications for these interesting and versatile nanoparticles.

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