UNRAVELING THE COMPLEXITY OF THE EXTRACELLULAR VESICLE LANDSCAPE WITH ADVANCED PROTEOMICS

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1. ABSTRACT

Introduction: The field of extracellular vesicles (EVs) is rapidly advancing. This progress is fueled by the applications of these agents as biomarkers and also as an attractive source to encapsulate therapeutics.

Areas covered: Different types of EVs, including exosomes, and other nanoparticles have been identified with key regulatory functions in cell-cell communication. However, the techniques used for their purification possess inherent limitations, resulting in heterogeneous preparations contaminated by other EVs subtypes and nano-size structures. It is therefore urgent to deconvolute the molecular constituents present in each type of EVs in order to accurately ascribe their specific functions. In this context, proteomics can profile, not only the lumen proteins and surface markers, but also their post-translational modifications, which will inform on the mechanisms of cargo selection and sorting.

Expert opinion: Mass spectrometry-based proteomics is now a mature technique and has started to deliver new insights in the EV field. Here, we review recent developments in sample preparation, mass spectrometry (MS) and computational analysis and discuss how these advances, in conjunction with improved purification protocols, could impact the characterization of the complex landscape of EVs and other secreted nanoparticles.

2. KEYWORDS

Exosomes; Extracellular Vesicles; Purification techniques; Proteomics, Mass Spectrometry, Biomarkers

3. ARTICLE HIGHLIGHTS

- Cells secrete proteins, lipids and nucleic acids within extracellular vesicles (EVs). This cargo can be efficiently and safely transferred to target cells, playing a crucial role in cell-cell communication. Because the packaged content seems to reflect their cell of origin, EVs have awaken widespread interest among clinicians as a potential source of biomarkers. Further, EVs can be engineered to encapsulate diverse therapeutical payloads.
- The landscape of EVs types reported in literature is constantly increasing, including exosomes, microvesicles, large oncosomes, apoptotic bodies, exomeres and other nanoparticles. However, there is not a universal isolation method for each type of EVs, as none of them can simultaneously achieve high yield, high purity and high specificity. In fact, the choice of protocol often depends on the sample type and the goal of the downstream analysis.
- Purification procedures possess inherent limitations and technical biases that introduce significant amounts of contamination. This is especially problematic when specific regulatory functions are attributed to EVs subtypes. Therefore, big efforts are being made in the field for identifying universal and highly selective molecular markers of EV types. In addition, carefully re-assessing the molecular constituents of each EV type will be important to study the functional roles, and potential applications of these nanoparticles.Several technological advances have been introduced in the last-decade in the field of mass spectrometry-based proteomics. On the one hand, novel methodologies that minimize manipulation and sample loss, which can be easily automatized in liquid handling systems. On the other hand, novel MS acquisition modes such as DIA and BoxCar that improve reproducibility and dynamic range. Finally, sophisticated computational tools that facilitate the analysis of complex MS data. These improvements are being incorporated in the proteomic toolbox for studying EVs and will enable analyses of limited sample amounts and comprehensive data sets in large clinical longitudinal studies.
- Post-translational modifications (PTMs) play key roles in regulating the mechanisms of cargo selection. However, systems-level analyses of PTMs in EVs remained challenging due to technical limitations and the low stoichiometry of PTMs. However, several studies have now identified thousands of phosphorylated, glycosylated and ubiquitinated sites among other PTMs in vesicular proteins. These data sets provide rich resources for exploring the roles of these modifications in EVs biology.

4. THE COMPLEX LANDSCAPE OF EXTRACELLULAR VESICLES

Virtually all eukaryotic cells release biomolecular components to the extracellular *milleu*. The function of these secretory processes is diverse. For instance, in response to bacterial infection, macrophages secrete a large number of factors that regulate innate immune response. Besides the secretion of such soluble proteins via classical secretory pathways, cells also release large amounts of cellular material encapsulated within membranous particles. Although the physiological function of these extracellular vesicles (EVs) has been typically attributed to few specific processes, such as neurotransmission and as a waste-disposal mechanism, it is now widely accepted that EVs are important players of intercellular communication. EVs are subject of a rapidly-growing and intense research field aiming to understand EV biogenesis and their inherent biological functions [1,2].

Different types of extracellular vesicles have been identified and these are often classified on the basis of their size, composition and biological origin. Advances on this regard have been mainly driven by the development of new approaches for the isolation and characterization of EVs. However, the lack of defined guidelines and criteria for the unambiguous assignment of EVs, as well as issues with the purity and heterogeneity of EVs preparations, have caused a confusing nomenclature in current literature. Indeed, classification of the EVs landscape is continuously evolving[3]. One of the major types of EVs are microvesicles (MVs), sometimes referred as ectosomes and micro-particles. MVs are lipid-bilayered particles of 100-1000 nm that originate via shedding of the plasma membrane (Figure 1A). Their biogenesis is controlled by intracellular Ca2+ levels and a set of proteins that include flippases, translocases, scramblase, actin cytoskeleton and members of the Ras family of GTPase [4], ultimately leading to the outward budding of the plasma membrane, trapping inside the intra-cellular material.

Unlike MVs, exosomes are cup-shaped vesicles of 40-150 nm that are originated within the endo-lysosomal pathway (Figure 1B). The biogenesis of exosomes involves the invagination of the plasma membrane via endocytosis in membranous structures known as early sorting endosomes (ESE) containing different surface proteins and soluble factors from the extracellular space (Figure 1B). Likewise, organelles such as mitochondria, trans-Golgi network (TGN) and endoplasmic reticulum (ER) can fuse with ESE and incorporate their content in the biogenesis process (Figure 1B). Then, the ESE matures in the so-called late-sorting endosome (LSE), ultimately evolving in the multi-vesicular body (MVB) (Figure 1B). A second invagination process in the MVBs generates the intraluminal vesicles (ILVs) which are the precursors of the exosomes. At this stage, MVBs can be either degraded and recycle their content in the autophagic pathway or release it to the extracellular space via fusion with the plasma membrane (Figure 1B). Different regulatory elements involved in all this process have been identified including tetraspanins (CD9, CD63, CD81), the ESCRT and SNARE complexes, SDCBP (Syntenin-1), TSG101 and ALIX[2]. However, their precise roles remain sometimes unclear because the existing cross-talk with other vesicular-dependent pathways.

Exosomes encapsulate a wide range of active biomolecular material that includes proteins, RNA, DNA, metabolites, as well as a unique lipidic composition (Figure 1C). This content is protected by the lipid bilayer and is very stable in biofluids such as plasma, cerebrospinal fluid and urine. Owing to their capacity to transfer this cargo to target cells, exosomes have important autocrine and paracrine functions and regulate several physiological responses such as immunomodulation, coagulation and stem cell maintenance. Moreover, exosomes play key roles in diseases such as neurodegeneration, viral infection and cancer. For instance, exosomes secreted by cells from primary pancreatic ductal adenocarcinomas have been found to condition the pre-metastatic niche in the liver[5]. Most importantly, because the diverse constituents encapsulated in EVs reflect the state of the cell of origin, exosomes represent an attractive source of biomarkers in liquid biopsies with both prognostic and diagnostic value [6]. Besides, exosomes can be engineered to incorporate different therapeutic payloads. These include a wide range of molecules of both hydrophilic and lipophilic nature, proteins, lipids, shRNAs, chemotherapeutic agents and immunomodulators. In addition, by modifying their surface composition, their tropism to specific organs and cell types can also be tuned. In fact, exosomes do not induce toxicity and are highly efficient at entering target cells, delivering the desired payload with minimal immune clearance.

Very diverse biological effects have been attributed to exosomes, indicating that the complex content of exosomes exert different functions or, alternatively, cells secrete a mixture of exosomes with unique functions. In the last years, it is becoming clear however that exosomal preparations are highly heterogeneous and other types of EVs and non-vesicular nanoparticles are frequently co-purified. The diversity of EVs is continuously expanding and includes apopotic bodies, oncosomes, large oncosomes, enveloped viruses, exomeres, exophers, secretory autophagosomes, migrasomes, supramolecular attack particles and elongated particles[7] (Figure 1A). The heterogeneity in these preparations confound analyses and difficult our understanding of the constituents and functional properties of each secreted particle. Therefore, precise assignment of the protein content to their correct extracellular compartment is crucial for the identification of biomarkers and the design of novel exosomal-based therapeutics. Here, we discuss how different proteomic approaches can be coupled to specific purification methods for the exploration of the EVs landscape. We introduce novel developments in mass spectrometry (MS) and discuss how they could be applied for the study of these agents. In addition, we highlight recent proteomic studies that have provided significant contributions regarding the identification of universal markers and the re-assessment of genuine vesicular cargo.

5. APPROACHES FOR THE ENRICHMENT OF EXTRACELLULAR VESICLES

Over the years, many different approaches have been developed for the isolation and purification of EVs from diverse biological samples and biofluids (Figure 2). There is a large body of literature with detailed protocols for EVs isolation[8–11]. Yet, there is no agreement for a universal isolation method. . In fact, each protocol has advantages and disadvantages (Table 1) and should be carefully selected

based on the type of sample and the goal of the downstream analysis. The use of combined isolation methods has demonstrated to outperform individual methods; however, this results in an increase in cost and time. Here, we give an overview of most commonly used isolation methods and briefly comment on some strategies of recent development, with a focus on those that can be coupled with downstream MS analysis.

1. Differential ultracentrifugation

Differential ultracentrifugation (dUC) was the first method applied for EVs isolation and remains the most widely used method[12]. dUC is based on the different sedimentation rates of particles with different sizes and densities under a certain centrifugation force. In most cases, successive centrifugation steps with increasing *g* forces and duration are applied to separate particles based on their density and size[13]: 10 min at 300g-2,000g to remove cells and apoptotic fragments, 20-30 min at 10,000-16,000g to isolate MVs and 1-2h at 100,000g to pellet exosomes and other small EVs (sEVs). The efficiency of dUC depends on four main factors: acceleration (*g*), type of rotor, viscosity of the sample and time of centrifugation [14]. For example, sEVs isolation from blood plasma samples requires higher-speed and/or longer time as dUC efficiency decreases with an increase in sample viscosity [15]. However, very long centrifugation cycles might add high levels of contamination of soluble proteins [14] and induce aggregation or rupture [16]. In general, dUC provides EVs samples that are easily profiled by MS but that, on the other hand, are prone to contamination from soluble proteins, which is particularly problematic in EVs purified from biofluids. An additional step of purification, such as density gradient (see below) can be combined to increase the purity of the isolated vesicles [17].

2. Density Gradient Ultracentrifugation

EVs can be separated from soluble proteins and proteins aggregates by density gradient ultracentrifugation (DG), since vesicles have lower densities (1.13-1.19 g/mL) than proteins (1.35-1.41 g/mL). In this strategy, a continuous or discontinuous pre-constructed density gradient, which increases in density from top to bottom, is used. Different mediums are available, being sucrose and iodixanol (Optiprep) most commonly used. Upon ultracentrifugation for several hours, sEVs (including exosomes) migrate to the point where their density is the same as the medium density. DG is very effective in separating sEVs from proteins and non-membranous contaminations, avoiding vesicle aggregation, and can even separate EVs subtypes. However, these benefits are accompanied by increased costs, time and workload. In addition, this strategy requires an extra step, such as ultracentrifugation or ultrafiltration, to remove the density medium prior to MS analysis. In combination with dUC, DG was one of the first attempts to address the heterogeneity of EVs [18].

3. Size-exclusion chromatography

Size-exclusion chromatography (SEC) separates the components of a sample based on their hydrodynamic volume. SEC is typically performed with a porous stationary phase in which small molecules, such as proteins, diffuse into the pores slowing down their travel time through the column. Hence, EVs, which are largerin size,, elute earlier from the column, as they cannot enter the polymer´s pores. There are different stationary phases, being Sepharose CL-2B mostly employed [19], and some commercial kits available. This technique was first applied for sEVs isolation from plasma samples in 2014 [20]. Although SEC is not as popular as dUC, its use has increased widespread in the last years [12]. SEC is simple, fast, can be scaled up, does not require expensive equipment and does not affect vesicular integrity (structure and biological activity) [21,22]. In many cases, SEC is used in combination with other methods to overcome some of its limitations. Diluted samples, as urine or culture medium, often need to be pre-concentrated, by filtration or dUC, as the sample volume should not exceed 10% of the resin SEC volume. In the case of plasma samples, lipoprotein particles (LPPs) cannot be completely depleted using SEC. Van Deun *et al.*[23] reported a new strategy where they combine two orthogonal separations: 1) SEC to remove high-density lipoproteins (HDLs) and 2) cation exchange to clear positively charged LPPs from negatively charged sEVs. Several studies have also demonstrated the benefits in coupling SEC with dUC [24,25] and DG [26]. Wei *et al.*[24] analysed proteomic differences in isolated plasma EVs by three strategies: dUC, SEC and SEC followed by dUC. By coupling SEC and dUC they were able to identify a higher number of proteins compared to one step SEC or dUC and the EV- associated proteins showed an increase in the abundance. In addition, they observed a reduction in protein contamination from highly abundant proteins, such as albumin or immunoglobulins, demonstrating a higher degree of purity. These results demonstrated that the combination of SEC and dUC greatly improved the proteomics profiling of plasma EVs. A similar result was observed by Alameldin *et al.*[25].

4. Ultrafiltration

Ultrafiltration (UF) is another popular size-based separation technique used for EVs isolation [12]. UF utilizes commercially available porous membranes with defined molecular weight cut-off (MWCO) and the filtration is often performed using low-speed centrifugation [27,28]. In UF, solvent and particles smaller than the MWCO, such as proteins, will pass through the filter and the larger particles, *i.e.* EVs, will be retained. Hence, this technique is ideal for large volume samples, such as cell culture media and urine, and to concentrate EVs isolated using other strategies. In fact, UF is frequently used in combination with other isolation methods, such as SEC [29–31]. UF is a relatively simple, quick and easy to implement and does not require special instrumentation. However, it suffers from protein contaminants, EVs losses due to unspecific membrane abortion and potential damages on vesicule

structure. An improved version of UF is tangential flow filtration (TFF) [32,33]. In this case, the fluid flows tangentially across the membrane surface, avoiding filter cake formation.

5. Precipitation

Polymer-based precipitation is based on the use of a water excluding polymer, such as polyethylene glycol (PEG), which changes the hydrophobicity of the media altering the interactions between EVs and leading to pellet formation. Its popularity is mainly due to the existence of several commercially available kits [34]. The whole procedure involves mixing equal volumes of a polymer solution and the sample of interest which are incubated overnight. Precipitated EVs can be then recovered by low-speed centrifugation. This strategy has many advantages: low cost, simple, fast, and does not require any technical expertise or expensive equipment. One of the major drawbacks of this strategy is however that PEG not only causes the precipitation of vesicular particles, but also extracellular proteins, proteins aggregates and other contaminants, including PEG itself, which is incompatible with MS analysis [35]. Hence, this method is often combined with others to increase the quality of the isolation [36]. An alternative strategy is the PRotein Organic Solvent PRecipitation (PROSPR) method [37] in which the soluble proteins from plasma are removed by precipitation in cold acetone, leaving purer EVs in the supernatant.

6. Affinity-based isolation methods

Affinity-based strategies exploit specific interactions between EVs surface markers with antibodies, molecules and functional groups immobilized onto a variety of solid supports (e.g. magnetic or agarose beads, plates, monolithic columns). Antibodies targeting tetraspanin family proteins, such as CD9, CD63 and CD81, are commonly used for the immune-purification of sEVs, as there are several ready-to-use kits commercially available. Additionally, other antibodies have been used for the isolation of sEVs, e.g. annexin, EpCAM or A33 [38]. Besides immunoaffinity methods, other strategies have been used for isolating different EV populations. Ghosh *et al.*[39] described the potential of Veneceremin (Vn), a synthetic peptide that specifically binds to heat shock proteins (HSPs), for isolation of sEVs from cell culture media and other body fluids. The affinity of lectins for glycoproteins, present on the vesicle surface, has been also exploited for sEVs isolation [40]. One of the main advantages of immunoaffinity is that it can provide high sEVs purity compared to other approaches [41]. This enables purifying specific sEVs subpopulations, which is essential for understanding EVs biology [42]. Nonetheless, this selectivity can be an issue when the aim of the study is the general sEVs population. It should be highlighted that, in many cases, this strategy requires pre-enrichment step to remove contaminants that bind the resin/antibody in an unspecific manner or to reduce the sample volume.

7. Asymmetric Flow Field Flow Fractionation

A relatively new sized-based EVs isolation method is asymmetric flow field flow fractionation (AF4). This technique does not require a stationary phase as the isolation takes place in a thin, flat and narrow chamber with a semi-permeable ultra-filtration membrane at the bottom. EVs are resolved by the use of two perpendicular flows: a laminar flow that carries the sample through the separation chamber and a variable crossflow which is responsible for the vesicular separation. One of the greatest advantages of this technique is its high-resolution with a large dynamic size separation range, which allows the fractionation of EV subpopulations. Using an optimized AF4, Zhang *et al.*[43] managed to identify two small exosomal subpopulations with distinct sizes and molecular properties (i.e. large exosome vesicles, Exo-L, 90–120 nm and small exosome vesicles, Exo-S, 60–80 nm) and discovered a novel and abundant population of non-membranous nanoparticles, named exomers (~35 nm). Despite being a powerful tool, AF4 also has its limitations. Due to the small working volumes, the yield is low and, therefore, it may not be practical for all studies, and some samples may need a pre-concentration step. In addition, it is not easy to implement, requiring specific instrumentation and trained personnel to customize and optimize protocols.

8. Microfluidics

Microfluidics devices are innovative and emerging platforms for EVs isolation. The main interest lies in the development of EVs-based diagnostic devices to bring their diagnostic and prognostic potential to the clinical setting [44]. Microfluidics isolates EVs based on different principles including markers (immunoaffinity), size (nanowire trapping, microporous filtering) and other sorting mechanisms (such as thermophoresis or acoustofluidics). There are several commercially available microfluidics devices for sEVs isolation. One of them is ExoChip whose surface is functionalized with anti-CD63 antibodies [45]. These devices require low sample volumes, as little as 10 µL, and reagents amount. In addition, they are fast and efficient and provide a high level of EVs purity. Despite their potential, their application in downstream proteomics analysis is limited due to their minimal sample volume and therefore, low protein amount. However, this limitation can be overcome by concentrating the sample using other strategies such as UF [46].

9. Other methods

The potential of EVs continuously fosters the development of highly innovative methodologies for their purification. EVtrap [47] is a novel isolation method based on functionalized magnetic beads modified with a combination of hydrophilic and lipophilic groups, which display a unique affinity towards lipid-coated EVs. Wan *et al.*[48] exploited the non-covalent interactions between hydrophobic fatty acid tails with the lipid bilayer membrane of sEVs. They developed a lipid-nanoprobe system that enables sEVs labelling and their subsequent enrichment with Neutravidin magnetic beads. They showed that isolation

efficiency and protein cargo composition was similar to that provided by dUC, but in a much shorter time. Recently, *Gao et al.*[49] described a strategy that exploited the specific interactions between titanium dioxide and the phosphate groups on the lipid bilayer of EVs. They showed that sEVs can be eluted from TiO_2 microspheres in basic conditions, without disturbing the vesicle structure, or directly lysed them for MS analysis. Fang *et al.*[50] proposed a magnetic beads-mediated strategy (MagExo) for a simple and easy-to-operate sEVs isolation. This method is based on the phenomenon that sEVs are selectively absorbed on the surface of magnetic beads in the presence of PEG or other hydrophilic polymers. Although sEVs purity improved greatly compared to the one achieved by commercial polymer precipitation kits, the protein contamination was higher than the obtained by DG. A big advantage of MagExo is that it could be automated with robotic workstations, significantly improving isolation throughput.

6. MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF EVs

Once EVs have been isolated, it is necessary to evaluate their quantity and purity. The international Society of Extracellular Vesicles (ISEV) proposed a panel of minimal experimental analysis of EVs, which include: quantification of EVs, morphology and characterization of protein markers[3]. Nanoparticles tracking analysis (NTA) and dynamic light scattering (DLS) are typically used to determine both particle size distribution and concentration. NTA can detect particles with diameters from 30 to 1000 nm and DLS from 1 nm to 1 µm. However, none of these techniques can differentiate EVs from particles of similar size such as lipoproteins or proteins aggregates and therefore other orthogonal assays have to be carried out. The morphology of EVs can be assessed by electron microscopy, with transmission electron microscopy (TEM) and scanning electron microscopy (SEM) being the most common EV imagine techniques. However, these techniques have several drawbacks associated to sample preparation and imaging acquisition conditions that can alter the EV morphology. Cryo-TEM could be a promising alternative as it preserves the integrity of EVs. Howevere, none of these methods inform on the purity of EVs. It is therefore recommended complementing these methods with the evaluation of positive and negative protein markers to demonstrate the vesicular nature of EV preparations and to track the presence of non-EV components. At least one protein of the following categories is often assessed: 1) A transmembrane or GPI-anchored extracellular proteins (e.g. CD9, CD63, CD81); 2) Cytosolic proteins with lipid or membrane protein-binding capacity (e.g. TSG101, annexins, Rabs). 3) Proteins found in most common co-isolated contaminants of EVs (e.g. albumin as negative marker for plasma/serum EVs).

7. PROTEOMIC APPROACHES FOR CHARACTERIZIGNG THE PROTEIN COMPOSITION OF EVs

In principle, the relatively low complexity as well as the low dynamic range of proteins in EVs makes them ideal for direct MS analysis (Figure 2). In reality, however, EVs preparations are prone to contamination with other EVs structures as well as many other non-vesicular components that notably increase their complexity. Additionally, the extreme high dynamic range of soluble proteins present in biological fluids contaminates preparations with highly abundant soluble proteins that difficult the identification of vesicular proteins of low abundance. Given the protein composition of EVs and the comparative nature of most studies, arguably, the most suited approach for profiling these samples is single-shot label-free proteomics. In the last years, the field of proteomics has matured enormously owing to developments in sample preparation, mass spectrometry and computational approaches. These advances enable the analysis of EVs at unprecedented sensitivity and high-throughput. In this section, we review these developments and discuss their applicability for the analysis of EVs.

1. Sample preparation

The most popular approach for digesting EVs-enriched preparations is in-solution digestion in MS-compatible buffers using urea [51], rapigest [52] and even trifluoroethanol [17] (Figure 2). Alternatively, FASP has been also widely adopted for the analysis of EVs [53]. Unfortunately, detergents and chaotropic agents are often incompatible with MS and require removal. This inevitably increases handling and subsequent peptide/protein loss, might introduce biases and are time-consuming. These issues have fostered the development of novel methodologies including the integrated Stage-Tip (iST) protocol [54] and the single-pot solid-phase-enhanced sample preparation (SP3) [55]. These approaches are simple and minimize handling while maintaining a high sample recovery and therefore are well-suited for the analysis of EVs preparations [56,57]. As discussed above, one of the main potential of EVs is in the field of biomarkers. However, the robustness, reproducibility, sensitivity and, especially, high-throughput needed to analyze large cohorts of samples with minute amounts have sometimes preclude large-scale clinical studies. Recently, technological developments in robotic liquid handling stations have provided an end-to-end automated method that is ideal for these studies. These platforms can exploit the benefits of paramagnetic SP3 beads [58] and deliver peptides ready for MS analysis in 96-well plate format with minimal human intervention. Therefore, it is probably a matter of time that these robotic systems will be adopted and introduced in biomarker proteomic studies of EV-related particles.

2. Mass spectrometry

Given the moderate-low complexity of the EVs protein content compared to unfractionated whole cell extracts, single-shot LC-MS/MS enables to comprehensively detect most of the proteins present in these

samples [59,60] (Figure 2). However, the presence of contaminants and the high dynamic range of EVs preparations obtained from biological fluids might demand multi-dimensional approaches using high pH in an offline system [61] or in StageTips [62]. Although pre-fractionation certainly increases the depth of the analysis, its application in large biomarker studies is rather unpractical as this will dramatically increase the analysis time and introduce variability for protein quantification. For proteomic applications, nano-flow LC has been the mainstay. However, this implies multiple drawbacks including unstable spray conditions, column overloading and long overhead times. Very recently, Kuster and colleagues have shown that micro-flow LC-MS/MS allows the analysis of several thousands of samples with great reproducibility even for plasma and urine samples [63]. Another recent development is a novel LC system that embeds analytes in a pre-formed gradient for rapid and ultra-fast proteomic analyses, reducing the overhead time to a few minutes and enabling the analysis of more than 200 samples per day [64]. These chromatographic improvements would certainly be an interesting implementation for clinical studies of EVs, facilitating large-cohort analyses.

The standard method for collecting MS spectra is data-dependent acquisition (DDA). Despite its simplicity, DDA presents some caveats, mainly related to the stochastic nature of peptide fragmentation which results in high rates of missing values. This is particularly problematic in large longitudinal studies and when sample amount is limited, a typical scenario in clinical studies. The recent development of Data-Independent Acquisition (DIA) modes overcomes the inherent irreproducibility and under-sampling of DDA. DIA acquires MS/MS spectra for, theoretically, all peptide precursor ions and it does so without bias to precursor ion selection. Not surprisingly, several authors have begun to exploit the potential of DIA approaches for the analysis of sEVs [65–68]. A crucial parameter in proteomics is the dynamic range, which is the ability to detect low abundance proteins in the presence of highly abundant ones. A way of improving the dynamic range is by means of gas phase separation using high-field asymmetric waveform ion mobility spectrometry (FAIMS) in Orbitrap-based instruments[69] or even the combination of trapped ion mobility with time-of-flight MS[70][71], both of which have shown much promise for proteomic applications.

3. Computational approaches

Noticeably, proteomic software has become increasingly powerful and user-friendly, allowing processing massive amounts of MS raw data in relatively short time [72,73] (Figure 2). A key limitation in DIA approaches is the need of spectral libraries for peptide identification. A major change in this area has been made recently through deep learning[74] and machine learning[75] which have solved the long-standing issue of peptide fragmentation prediction even for post-translationally modified peptides[76]. These advances go hand in hand with algorithms that predict peptide chromatographic behaviour and accurate retention times[77]. Although some authors have reported the generation of custom, deep spectral libraries for sEVs samples[66], these novel computational improvements have

now paved the way for library-free DIA approaches[78,79] that will certainly boost their wide implementation in routine EVs proteomic workflows. Due to its simplicity, single-shot label-free LC-MS/MS analysis using DDA is the standard approach for the analysis of EVs. However, as mentioned above, the presence of missing values in DDA hampers accurate and precise quantification. Recently, Krijgsveld and colleagues presented a new computational workflow named IceR (Ion current extraction Re-quantification) [80] that improves data completeness, similar to DIA. Therefore, it is very likely that IceR and other similar solutions will be soon incorporated in the proteomic toolbox for EVs studies.

4. Data repositories

A fundamental aspect to advance in our understanding of EVs biology is data sharing and dissemination (Figure 2). There are several repositories dedicated for the annotation of the molecular cargo of EVs, such as Exocarta (http://www.exocarta.org/), EV-TRACK (https://evtrack.org/) and Vesiclepedia (http://microvesicles.org/). Intriguingly, the number of proteins that are annotated in these databases as present in EVs is suspiciously large (*i.e.* more than half of the human proteome) which somehow questions the true identity of many of these proteins as genuine vesicular proteins. In addition to these databases, it is important to make raw MS data publicly accessible via repositories such as the ProteomeXchange consortium (http://www.proteomexchange.org). Data re-analysis through these platforms could be important for the reassessment of EV proteins in published data sets. Of note, the number of EVs data sets in ProteomeXchange has nearly doubled each year, which reflects the interest of data deposition for the EV field.

8. POST-TRANSLATIONAL MODIFICATIONS IN EVs

Several post-translational modifications (PTMs) have been shown to play roles in sEVs biology as they seem to regulate the mechanisms of cargo selection and sorting among other functions [81]. However, PTMs are present in low abundance and at low stoichiometry levels and therefore their analysis requires large input material, which in the case of EVs is often difficult to obtain. Here, we review some of the studies that have performed a mass spectrometry-based characterization of PTMs in EVs cargo [82].

Protein phosphorylation plays key roles in cell signaling and regulates almost all aspect of cell function. Indeed, the production and release of sEVs seems to be also controlled by phosphorylation events. There are several large-scale phosphoproteomics studies of EVs from different biological origins including conditioned media [51,83,84], serum [85], plasma [86,87] and urine [47,88–90]. The first phosphoproteomic study in sEVs was carried out by Gonzales *et al.* [88] in urine samples, who merely identified 19 phosphorylations. However, with recent advances in MS technology, the number of phosphosites that can be identified from EV samples have increased dramatically. Guo *et al.* [83] identified 1,019 phosphosites from 313 phosphoproteins in sEVs isolated from colorectal cancer cells and found a remarkable high level of tyrosine phosphorylation (6.4% in sEVs vs 0.6% in whole cell

extracts). In other study, Chen *et al.* [86] profiled sEVs derived from plasma of breast cancer patients and found 144 phospho-sites upregulated in cancer patients compared to healthy controls, demonstrating that the phosphorylation status of vesicular proteins can also be used as biomarkers. Interestingly, some plasma samples used in this work were collected 5 years earlier, proving also the high stability of vesicles and their phosphoproteins cargo. Recently, Rontogianni *et al.* [51] performed a comprehensive phosphoproteome analysis of sEVs isolated from supernatants of 10 breast cancer cell lines. They identified 25,800 phosphosites of which 4,602 were quantified across all samples. Among the phosphoproteins identified, it was evident the presence of activated kinases in sEVs, indicating their potential to alter signalling in recipient cells.

Glycosylation is also known to play an important role in cellular uptake of sEVs and seems to have an impact on their biogenesis. Further, several studies have shown that sEVs are preferentially enriched in high-mannose and sialic acid structures compared with parenteral cells [91]. The first systematic analysis of the N-glycoproteome of sEVs was conducted by Saraswat *et al*. [92]. N-glycopeptides were enriched by both lectin affinity chromatography and SEC. In total, they characterized 126 N-glycopeptides from 37 glycoproteins. By using hydrazine chemistry enrichment, the group of Tao identified 1,453 glycopeptides from 1 mL of plasma [93]. These data revealed several differences in glycopeptides from breast cancer patients and healthy donors EVs, highlighting the value of glycoproteins as potential biomarkers.

Ubiquitination targets proteins for degradation and is involved in protein turnover. However, in EV biology, ubiquitin and ubiquitin-like modifiers (ULBs) have a crucial role in the sorting of proteins [94]. Protein ubiquitination mediates ESCRT-0 complex recognition and the subsequent deubiquitination seems to be a crucial step for their loading into ILVs. A few studies have attempted to systematically identify ubiquitinated proteins, mainly using di-glycine (K-ε-GG) immunoaffinity [95]. Huebner *et al.*[96] identified 619 ubiquitinated proteins in EVs purified from urine samples. Interestingly, these results imply that proteins can elude deubiquitination or be sorted into sEVs through ESCRT-independent pathways.

9. DECODING THE COMPLEXITY AND FUNCTIONAL RELAVANCE OF EVs

The number of publications reporting proteomic analyses of EVs is constantly growing. However, several issues concerning the genuine identity of vesicles and their constituents might question some of the reported findings. EVs types are normally distinguished based on their morphology, size, biogenesis and composition; criteria which are, to a certain extent, ambiguous and interpretable. In addition, technical biases inherent to isolation protocols might introduce artifacts in the results. Indeed, it is becoming apparent that different types of vesicles are in fact overlapping populations. Consequently, the field of EVs is making efforts in improving the guidelines for reporting data derived from EVs as well as unifying the nomenclature of the complex family of secreted vesicles. Prompted by these issues,

the group of D. Lyden undertook a massive proteomic endeavour for identifying universal molecular markers of extracellular nanoparticles (EVPs) which include small exosomes (Exo-S), large exosomes (Exo-L), and exomeres [59]. Using dUC and single-shot DDA, the authors profiled a disparate set of 497 human and murine samples and identified HSPA8, HSP90AB1, CD9, ALIX as the most prominent markers in human-derived EVPs. In addition, they reported the identification of 13 additional proteins shared by >50% of human samples, which significantly expands the available panel of pan human EVP markers. A similar effort has been made recently by Kugeratski *et al*. [60]. To identify ubiquitous sEVs proteins, the authors compared sEVs-enriched preparations by dUC with whole cell extracts using super-stable isotope labelling with amino acids in cell culture (super-SILAC). They identified a cohort of 22 proteins highly increased in sEVs, which included biogenesis-related proteins, GTPases and membrane proteins. On the basis of their data, the authors proposed Syntenin-1 (SDCBP) as a putative universal sEVs marker. Although the value of these studies is undeniable, it is somehow surprising that syntenin-1 was not present among the panel of markers proposed by Lyden's group. This discrepancy might be attributable to technical differences in MS and database search but also indicates the need for refining and unifying proteomic criteria in this type of studies. It is also worth mentioning that both studies analysed sEVs-enriched samples by dUC, a procedure that is prone to contamination with proteins from other sources. The presence of contamination is a serious problem when ascribing specific regulatory functions to subtypes of EVs, particularly exosomes. Bearing this in mind, Jeppesen *et al.* [17] analysed exosomes using a strict isolation protocol and challenged several of the accepted properties of these vesicles in cell-cell communication. The authors first used dUC to isolate small EVs, and the resulting pellet was further separated using high-resolution DG. This enabled the separation of low density vesicular structures from highly abundant and more dense non-vesicular components. Then, using immunoaffinity, exosomes were further separated from other sEVs. MS analyses revealed that classical exosomes did not contain luminal proteins commonly assigned to exosomes, such as metabolic enzymes (GAPDH, ENO1), HSP90 and cytoskeletal proteins. Therefore, these results questioned models of non-selective encapsulation of the parent cell's cytosol and instead implied the existence of regulated sorting mechanisms. Further, the absence of Argonaute proteins and other miRNA-related enzymes indicated that, in contrast to previous reports [97], exosomes lack the necessary components to facilitate cell-independent miRNA biogenesis. Therefore, this work is a proof that, in order to attribute functionality to specific extracellular entities, a careful assessment of their molecular components is needed.

10. CONCLUSION

The analysis of EVs can reveal important information to understand the mechanisms of cellular communication and their roles in organ homeostasis. The landscape of EVs types and subtypes is complex, challenging purification methods which suffer from cross-contamination. This is an important

issue which confounds results and limits our knowledge of the constituents and functional properties of each secreted agent. However, recent methodological, technological and computational developments in the field of mass spectrometry-based proteomics make possible the analysis of EVs with exceptional sensitivity levels throughput. In combination with refined purification procedures, proteomics will be able to deconvolute the unambiguous identity of EVs protein cargo. This information will be crucial for defining their functional roles and to exploit their potential as molecular readouts of human diseases.

11. EXPERT OPINION

The implementation of advanced MS-based approaches in EVs biology is expected to enable a systems-level view of these entities, providing critical information on their biogenesis and exploiting their potential as biomarkers. However, there are still multiple challenges and opportunities for future developments. We have previously discussed the issues related with the heterogeneity of different EVs during purification and how this can confound the functional results. In a similar context, a big challenge will be addressing the heterogeneity of specific EV subtypes, *i.e.* within EV populations. It is becoming clear that, based on the levels of surface markers, different sub-populations of sEVs exist. The mechanisms that explain such heterogeneity are not understood and could be multi-factorial. For instance, it has been shown that ESCRT-dependent and -independent mechanisms result in ILVs that greatly differ in size and luminal cargo. Therefore, future analytical methods should go beyond current bulk analyses and aim to study EVs at the level of subtypes and, ideally, single-particle analyses. This will need highly selective immunoaffinity methods and FACS sorting approaches which should be coupled to ultrasensitive analytical tools. Although MS technology is still far from reaching the sensitivity levels required for such analyses, recent advances in single-cell proteomics have paved the way for future applications in the field of EVs. One of them is the Single Cell ProtEomics by Mass Spectrometry [98] (SCoPE-MS) in which one isobaric channel is reserved to a carrier proteome to improve peptide identification, while the remaining channels are used for quantifying peptide levels across multiplexed samples. However, owing to limitations in dynamic range inherent to isobaric tagging and MS technology, caution should be taken to increase the number of sampled ions accordingly to the increase in carrier proteome [99].

Most of our current knowledge of EVs is largely based on cell cultures. These cellular systems can be easily scaled-up, providing high yields of EV material that facilitate subsequent proteomic analysis. However, EV properties and composition might be largely determined by long-term culture conditions. Further, transformed cell lines may no longer be representative of the tumor of origin as they lack other cell types that influence the tumor microenvironment. Therefore, it remains to be proven whether some of the reported functions of EVs are relevant in more physiological contexts, such as tissues and organisms. However, studies reporting analyses of EVs obtained directly from tissues are scarce in the literature, as these have proven more challenging than isolating EVs from conditioned cell media. A

critical point seems to find a balance between the efficient release of EVs from the extracellular matrix where they are entrapped and avoiding cell disruption that could cause contamination by intracellular vesicles [10]. Therefore, optimizing protocols for isolating EVs directly from tissues, organs and other explants material should be an area of major research. Great progress has been made in this regard by the group of Lötvall, who established a method for isolating different EV populations directly from melanoma metastases, although it can be easily applied to other cancer and non-cancer tissues [10]. In this protocol, tissue pieces are first dissociated by enzymatic digestion and EVs are subsequently purified by dUC. Interestingly, these approaches also open the possibility of exploring the relationships between the protein content of EVs and the intracellular expression of proteins in the parental cells. These integrative analyses can inform, for instance, on the potential mechanisms of cargo selection, and in the case if biomarker studies, validate potential candidates. However, few studies have analysed such dependencies. Rontogianni *et al.* found that EVs signatures better differentiate breast cancer sub-types than whole cell lysates and concluded that EVs have higher potential diagnostic power than tumor biopsies [51].

The proteomic analysis of EVs derived from biological fluids also demands some attention. The extreme high abundance of soluble proteins in plasma, urine and CSF introduces significant amounts of contamination in EVs preparations, which difficult the identification of low abundant vesicular proteins. Indeed, the number of proteins identified in EVs purified from these biofluids is substantially smaller than cell culture supernatants. This has important implications in biomarker studies as it largely limits our ability to detect potential biomarkers within the isolated vesicles. For instance, plasma data sets often report a few hundred proteins [100,101] while conditioned media from cell lines enables the identification of several thousand proteins [102]. Indeed, some authors have documented this issue and found that 35% of the identified proteins in EVs from serum are in fact serum components[103]. Likewise, a recent meta-analysis showed that more than 50% of the proteins present in urine are also represented in urinary EVs studies [104], and surprisingly, only 7-22% of urinary proteins are identified in common in four independent studies [104], indicating that the analysis of EVs from biofluids still remains challenging. Therefore, there is a need to find alternative sources of EVs that could better reflect the condition under study, such as CSF for neurological disorders [105] and nasal lavage for respiratory conditions [106]. Recently, García *et al.*[53] analysed sEVs from lymphatic drainage, also known as exudative seroma, of melanoma patients obtained after lymphadenectomy and found that they are enriched in proteins resembling melanoma progression. Interestingly, the authors showed that proteins detected in exudative seroma-derived sEVs outnumbered proteins in plasma-derived sEVs in matched samples, highlighting the potential of this biofluid as liquid biopsies for cancer. On the other hand, the high dynamic range of biofluids-derived EVs could be tackled by innovative MS methods as BoxCar [107]. In BoxCar, multiple narrow mass-to-charge segments are filled to increase the mean ion injection time more than 10-fold compared to that of a standard full MS1 scan. This method has shown

unprecedent proteome coverage in single-shot runs and enabled the identification of more than 10,000 proteins from brain tissues in 100 min. Thus, it will be interesting to evaluate the applicability of such strategy for the analysis of EVs isolated from biofluids as this might uncover biomarkers that remained unidentified using standard MS approaches.

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13. HIGHLIGHTED REFERENCES

Ref 3. (**): The ISEV guideline: a list of minimal information and experimental requirement for the analysis of EVs.

Ref 5. (*): Analysis of the role of exosomes secreted by cells from primary pancreatic ductal adenocarcinomas in metastasis, revealing that they trigger the formation of the liver pre-metastatic niche.

Ref 10. (*): Detailed protocol that combines dUC and DG to isolate different EV subpopulations from tissues, which can be applied to other biofluids and conditioned cell media.

Ref 17. (**): Demonstration of the need of improved EV isolation methods for a more accurate characterization of exosome molecular cargo.

Ref 18. (**): Pioneering work in the field of EVs that revealed the presence of exosomal and non-exosomal subpopulations within EVs preparations. They also demonstrated that classical exosomal markers are present in other EVs.

Ref 20. (*): First work showing the potential of SEC for the isolation of EVs from biofluids.

Ref 43. (*): Optimized AF4 technology enabled the identification of two exosome subpopulations and discover a novel small (35nm) non-membranous nanoparticle, termed "exomeres". Their proteomics profiles suggest distinct biological functions.

Ref 59. (**): A deep proteomics characterization of 497 human and murine samples for the identification of universal EV markers in health and disease (cancer).

Ref 60. (**): Comprehensive characterization of the exosomal proteome in multiple cancer cell lines using metabolic labelling. They proposed syntenin-1 as a candidate for universal biomarker.

Ref 86. (*): Phosphoproteomics study that reveals the potential of exosomal PTMs as biomarkers, a higher occurrence of tyrosine phosphorylation in exosomes and the possibility of using plasma samples from biobanks in biomarkers studies.

14. FIGURE LEGENDS

Figure 1. The heterogeneity of EVs and the biogenesis of exosomes. Multiple EVs have been identified in the last years. These particles are typically classified on the basis of their size, composition and biogenesis. A) An overview of EVs and other nano-particles secreted by cells, indicating their biological origin and size as proposed recently by Jeppesen *et al.* [17]. B) Schematic of the biogenesis of exosomes, showing some of the organelles and steps involved in the pathway. ILVs, intra-luminal vesicles. C) Representation of the complex cargo of exosomes in terms of biomolecules that are present in the lumen as well as their membrane surface.

Figure 2. Recent technical developments in proteomics and their implementation in the analysis of exosomes and other EVs. Schematic of the main steps in a typical proteomic workflow for the analysis of EVs. Some recent technical developments that are mentioned in the text are shown in grey colour. How these advances could benefit research in exosomes and other EVs it is indicated in orange.

Table 1. Pros, cons and characteristics of EV purifications strategies.

15. FUNDING

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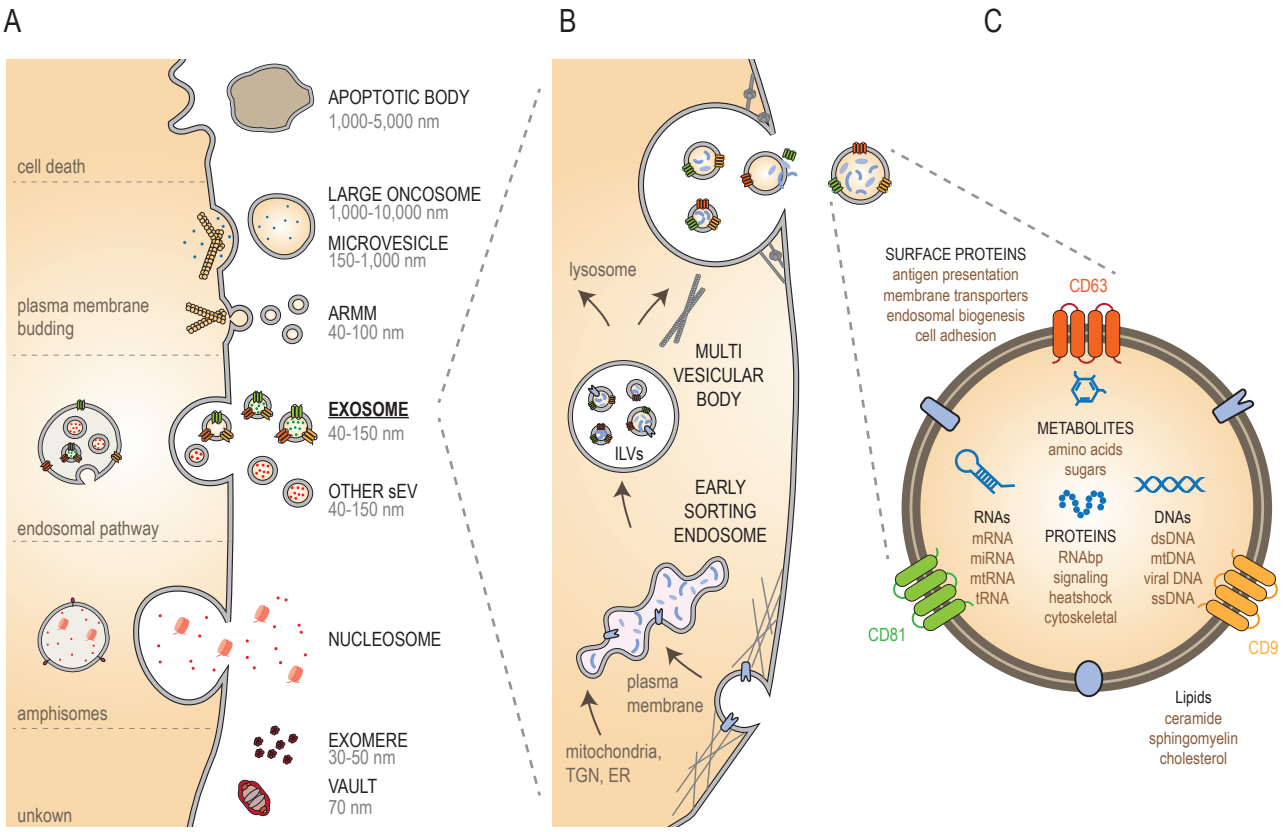
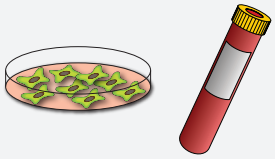
16. DECLARATION OF INTERESTS

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.


A
B
C
APOPTOTIC BODY
1,000-5,000 nm
cell death
LARGE ONCOSOME
1,000-10,000 nm
MICROVESICLE
150-1,000 nm
plasma membrane
budding
ARMM
40-100 nm
EXOSOME
40-150 nm
OTHER sEV
40-150 nm
endosomal pathway
NUCLEOSOME
amphisomes
EXOMERE
30-50 nm
VAULT
70 nm
unkown
lysosome
MULTI
VESICULAR
BODY
ILVs
EARLY
SORTING
ENDOSOME
plasma
membrane
mitochondria,
TGN, ER
SURFACE PROTEINS
antigen presentation
membrane transporters
endosomal biogenesis
cell adhesion
CD63
METABOLITES
amino acids
sugars
RNAs
mRNA
miRNA
mtRNA
tRNA
PROTEINS
RNAbp
signaling
heatshock
cytoskeletal
DNAs
dsDNA
mtDNA
viral DNA
ssDNA
CD81
CD9
Lipids
ceramide
sphingomyelin
cholesterol

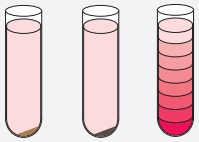
1. BIOLOGICAL SAMPLES



- cell lines
- biofluids
- tissues
- FACS
- organoids

- EVs biology and biogenesis
- clinical proteomics studies

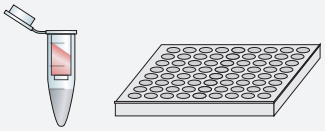
2. EVs PURIFICATION



- dUC
- SEC
- precipitation
- immunoaffinity
- others

- EVs hetereogeneity
- highly pure EVs samples

3. SAMPLE PREPARATION



- FASP
- in-solution
- iST/SP3
- multi-plate
- robotic handling

- increased high throughput
- samples of limited amounts

4. MASS SPECTROMETRY

- EVOSEP
- micro-flow
- DDA/DIA
- BoxCar
- ion mobility/FAIMS

- improved sensitivity levels
- improved dynamic range

5. COMPUTATIONAL ANALYSIS

- deep learing
- peptide fragmentation
- RT prediction
- open search engines
- IceR

- comprehensive data sets
- accurate quantification

6. DATA REPOSITORIES

- spectral libraries
- neoantigens
- PTMs
- expression
- db cross-ref

- high quality EV proteomes
- integrative multiomic analyes

	Advantages	Disadvantages	Sample Volume	Characteristics
Differential ultracentrifugation	▪ Low cost (if an ultracentrifuge is available) ▪ Robust method ▪ Widely use (allowing comparison) ▪ No technical expertise required	▪ Low throughput ▪ Potential damage to EVs ▪ Efficiency affected by several parameters ▪ Non vesicular impurities	100s of mL	Intermediate recovery and specificity
Density gradient ultracentrifugation	▪ High purity	▪ Low throughput ▪ Low yield	Up to 1 mL	Low recovery/high specificity
Size-exclusion chromatography	▪ Low contamination with free proteins ▪ EV integrity maintained ▪ Fast and simple procedure ▪ Scalability	▪ Co-isolation of non-EV particles (e.g. protein aggregates, lipoproteins) ▪ Low resolution	Up to few mL	Intermediate recovery and specificity
Ultrafiltration	▪ Fast and simple procedure ▪ Easily combined with other methods	▪ Low resolution ▪ Protein contamination ▪ Potential damage to EVs	100s of mL	High recovery/low specificity
Precipitation	▪ Fast and simple procedure ▪ Cheap ▪ Commercially available kits. ▪ High yield	▪ High contamination (e.g. proteins, proteins aggregates, PEG) ▪ Low resolution	> 100 µL	High recovery/low specificity
Immunoaffinity	▪ High purity ▪ High specificity (capable of isolating subpopulations) ▪ Commercially available beads.	▪ Expensive ▪ Contamination with antibodies	Up to 1 mL	Low recovery/high specificity
AF4	▪ High resolution (capable of isolating subpopulations)	▪ Low yield ▪ Technical expertise required	100 µL	Low recovery and high specificity
Microfluidics	▪ Highly automated ▪ Can be combined with EV characterization ▪ High purity	▪ Expensive ▪ Low yield ▪ Technical expertise required	> 10 µL	Low recovery/high specificity