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Proteomic analysis of exosomal cargo: the challenge of high purity vesicle isolation

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Abstract

The re-discovery of exosomes as intercellular messengers with high potential for diagnostic and therapeutic utility has led to their becoming a popular topic of research in recent years. One of the essential research areas in this field is the characterization of exosomal cargo, which includes numerous non-randomly packed proteins and nucleic acids. Unexpectedly, a very challenging aspect of extracellular vesicle exploration has turned out to be their effective and selective isolation. The plurality of developed protocols leads to qualitative and quantitative variability in terms of the obtained exosomes, which significantly affects the results of down-stream analyzes and makes them difficult to compare, reproduce and interpret between research groups. Currently, there is a general consensus among the exosome-oriented community concerning the urgent need for the optimization and standardization of methods employed in the purification of these vesicles. Hence, we review here several strategies for exosome preparation including ultracentrifugation, chemical precipitation, affinity capturing and filtration techniques. The advantages and disadvantages of different approaches are discussed with special emphasis being placed on their adequacy for proteomic applications which are particularly sensitive to sample quality. We conclude that certain methods, exemplified by ultracentrifugation combined with iodixanol density gradient centrifugation or gel filtration, although labor-intensive, provide superior quality exosome preparations suitable for reliable analysis by mass spectrometry.

Key words

exosome, extracellular vesicles, isolation methods, mass spectrometry, proteomics, ultracentrifugation, precipitation, filtration, affinity capturing, sample purification, sample contamination

Introduction

Exosomes, very small vesicles with great potential, are one of the components of the extracellular milieu involved in intercellular communication. The increasing interest in this field, evinced by the almost nine hundred new exosome-related articles indexed in PubMed in 2015, is fueled by reports indicating their considerable role in the development of and response to treatment of several common diseases, including cancer. Preliminary studies into different biological aspects of exosomal biogenesis, composition and function, although still insufficient, have led to their unquestionably becoming one of the most promising sources of new diagnostic biomarkers and therapeutic strategies in the near future.

The term "exosome" has been in common usage since the early 1980s, when *Trams et al.* [1] reported cellular secretion of small membrane vesicles with varying diameters up to 1000 nm that carried active 5'-nucleotidase. Since then, the meaning of the term has evolved and nowadays it mainly relates to nanovesicles with a diameter in the 30–130 nm range and a phospholipid bilayer membrane that are formed by inward budding of late endosomes/multivesicular bodies and that are secreted during their fusion with the plasma membrane (reviewed in detail in [2,3]). However, after over thirty years of intense exosome-related research, precise categorization of extracellular vesicles (EV) is still a topic of discussion [4–6]. From a practical point of view, appropriate evaluation and classification of vesicles is of prime importance, as the generally available methods/protocols lead to the isolation of a mixture of different subtypes of EVs and even some non-EV carriers. Faced with the urgent need for standardization of the nomenclature and methods of verification which are fundamental for proper interpretation of the data, the International Society for Extracellular Vesicles (ISEV) recommended a collection of criteria that should be taken into consideration during study of EVs [7]. These recommendations are a vital issue, since vesicles such as exosomes are isolated from different body fluids, e.g. blood (plasma/serum) [8,9], urine [10–12], cerebrospinal fluid [13], saliva and breast milk [14], as well as from *in vitro* cell culture media [15,16], and these fluids vary in physical and chemical properties which may contribute to accumulation of unreliable results and artifacts. Currently,

exosomal studies mostly concentrate on cargo characterization which concerns RNA [17–19], DNA [20,21] and protein [22,23] profiling, with reference to the potential function of exosomes as mediators of intercellular communication [24,25] and their role in immune response and disease development [26–28]. The type of vesicle source and the requirements of downstream applications usually determine the choice of isolation method, which in most cases is based on ultracentrifugation or precipitation with polyethylene glycol-like substances. The plurality of the recently reported protocols for exosomal isolation is reflected in the increased number of reviews focused on this issue. Some of these provide an interesting introduction to extracellular vesicle isolation, deftly presenting the most popular strategies[29,30], while others consider suitability for specific downstream applications such as exosomal microRNA profiling [31] or clinical diagnostics [32]. However, at the same time we have noticed a lack of articles dedicated mainly to proteomic applications. In this review, we have focused on the currently available exosomal isolation and purification methods which are particularly useful in mass spectrometry-based analysis. The mass spectrometry (MS) technique was implemented with great enthusiasm in biological research more than three decades ago as a powerful tool for the identification and quantification of proteins, also allowing the detection and characterization of post-translational modifications. MS has established itself as a solid method for proteomic research. However, in spite of extensive development of instrumentation, methods of fragmentation and bioinformatics backup, specific properties of biological samples–their complexity and instability, still present a challenge for mass spectrometrists. Unfortunately, in most cases the limiting factor is not equipment capabilities but sample quality (the content of salts, detergents and highly abundant or non-protein components, pH) that affects the measurement [33,34]. New instrumental techniques make restrictive demands on sample preparation strategies owing to their higher sensitivity to both low-abundance peptides and contaminants.

Exosome isolation methods

Ultracentrifugation

Ultracentrifugation is a traditional and widely practiced method for the isolation of exosomes that was the basis of early exosomal studies [35,36]. Currently, a total protocol includes several preparation steps that precede exact exosomal pellet collection. Initial centrifugation of a culture supernatant (or a body fluid) allows the removal of larger contaminants such as dead cells, while the second centrifugation purifies the solution from cell debris [37–40]. In some cases, this part of the protocol is modified by supernatant filtering through a 0.22 μm filter to remove all particles larger than 200 nm, including apoptotic bodies and aggregates of smaller vesicles [37,41,42]. Finally, the pre-cleaned supernatant is centrifuged at high speed (100,000 × g, so-called ultracentrifugation) for 1–2h, then the exosomal pellet is washed with PBS and the last centrifugation is repeated. Although this basic protocol is implemented in exosomal studies as universal, there are some properties of this method (often disregarded) that can affect the results and that should be taken into consideration before starting an analysis. The efficiency of isolation depends not only on g force [43], but also on the type of rotor, its *k*-factor and solution viscosity. Fixed angle rotors usually have shorter sedimentation path length than swinging bucket rotors and as a result have higher pelleting efficiency, but on the other hand the pellet is less condensed and less spread on the tube wall. *K*-factor, which is a measure of rotor pelleting efficiency, can be useful for the comparison and standardization of centrifugation conditions applied in different labs via an estimation of the time required for exosome sedimentation using a specific rotor ($t = \frac{k}{s}$, t-time in hours, k-*k*-factor, s–sedimentation coefficient in Svedberg units) [44]. The final parameter is viscosity that is especially important in extracellular vesicle studies due to the diversity of potential exosomal sources. *Momen-Heravi et al*. [45] revealed a significant difference between the sedimentation efficiency of plasma, serum and culture media, and concluded that higher viscosity resulted in lower sedimentation efficiency; therefore, ultracentrifugation time and speed should be modified to compensate for differences in viscosity. Alternatively, to maintain

constant centrifugation parameters and improve/standardize sedimentation efficiency, the simple dilution of a viscid sample may also be a possible option. In this context, it is clear that "standard" protocols should be treated with appropriate caution. Although the preliminary preparation of a sample reduces contamination of the final pellet with larger extracellular vesicles, ultracentrifugation remains non-specific for exosomal isolation and some undesirable components could also be obtained. From the point of view of cargo characteristics, especially in the proteomic field, co-sedimentation of protein aggregates and co-isolation of proteins derived from body fluids or culture media is a serious disadvantage of this method. For mass spectrometry applications as well as protein measurements for exosomal quantification, an additional step of purification is necessary; otherwise, non-exosomal proteins will definitely affect the analysis.

Precipitation

Another well-known method of exosome isolation successfully adapted from viral studies [46] is polymer-based precipitation. Its popularity results mainly from convenient commercial solutions (ExoQuick™ from System Bioscience or Total Exosome Isolation Kit from Life Technologies) which make isolation less laborious when compared to ultracentrifugation. This method is based on the formation of a polymer network under specific conditions (salt concentration, low temperature) that entwines all components present in the sample and causes a decrease in solubility. The whole procedure is abbreviated to mixing equal volumes of a polymer solution and the sample of interest and leaving the mixture for overnight incubation. Afterwards, the precipitated exosomes are recovered by low-speed centrifugation and resuspended in PBS for downstream applications [40,47,48]. In some cases, isolation is performed using a home-made PEG buffer containing 33.4% PEG 4000, 50 mM HEPES (pH 7.4), 1 M NaCl [49,50]. Nevertheless, the general principle of operation remains the same. It should be noted that precipitation, just as ultracentrifugation, belongs to the group of non-specific methods of exosomal isolation and robust pre-and post-purification is equally important in both cases. However, besides the potential non-exosomal contaminants that have been mentioned above, the sample contains polymer molecules which are incompatible with the application of mass

spectrometry. Polyethylene glycol (PEG) is reported as one of the most common interfering factors in proteomic analysis. It is well-known as an ion suppressor in the ion source and is observed on a mass spectrum as +44 ion series [34,51]. As a non-volatile solute, PEG prevents matrix crystallization that is essential for MALDI-based analysis and can easily contaminate LC-columns [52,53]. Under such circumstances, sample preparation/purification based on in-gel digestion [54,55] or its alternatives is advisable rather than direct MS-or LC/MS-analysis. A recently reported variation of PEG-related precipitation taking advantage of a polyethylene glycol and dextran mixture allows researchers to save time and increase recovery efficiency in comparison with the standard procedure. However, due to the very high background from co-isolated proteins, phase separation with fresh PEG requires several repeats, which can decrease the reproducibility of isolation [56,57].

Membrane-based filtration

Protocols based on filtration belong to the most diverse group owing to the plurality of the methodological variants. The main principle of this approach is separation dependent on the size of vesicles. In most cases, isolation is realized using ultrafiltration membranes and/or SEC columns and results in one or a couple of fractions enriched with exosomes. However, the essence of both methods is in fact different. Commercially available ultrafiltration units such as Vivaspin® or Amicon® (usually with 100 kDa MWCO) work like a sieve that keeps exosomes on its surface and allows the removal of the majority of a sample buffer, significantly reducing the volume. Depending on the unit type and low-speed centrifugation equipment, one-or multistep concentration can be efficient for large volume samples such as urine [58,59] or cell culture media [42], which often constitutes a restriction for precipitation or immuno-affinity methods. It is worth noting that membranes with low protein-binding properties are more suitable for this approach, since they reduce the attachment of exosomal proteins and facilitate their recovery [58,60]. Similarly, devices based on centrifugation seem to give better results than those which are pressure-driven [42]. Unfortunately, the relative infrequency of isolation based exclusively on filtration and the plurality of the materials used hinders the drawing of a conclusion. Results reported by *Lobb et al.* [42] *and Cheruvanky et al.* [58] indicate that the

effectiveness of filtration verified by standard methods such as electron microscopy, Western blot and tunable resistive pulse sensing is comparable with ultracentrifugation and can be a faster alternative. The cited authors also claim to have achieved a satisfactory reduction of high-abundance proteins derived from the bio-fluid, which makes filtration attractive for mass spectrometry application; however, more comprehensive study is undeniably advisable in this field.

Gel filtration chromatography

Another method of exosomal isolation profiting from size differences between isolated components is gel filtration chromatography (size exclusion chromatography; SEC). Separation of particles of interest is performed using columns packed with heterogeneous polymeric beads. In this case, the most commonly used gel filtration medium is Sepharose containing a crosslinked, beaded form of agarose with diverse diameter range. For isolation, a sample is loaded onto a packed column and allowed to pass through heteroporous resin. There is no interaction between the matrix and sample components; nevertheless, the accessibility of the pores is differential. Larger molecules can enter fewer pores than the smaller ones and are eluted earlier; hence, the retention time is longer, the smaller the molecule is. The main advantages of this method are its good reproducibility and high efficiency in removing interfering contaminants such as non-specifically interacting proteins or high-abundance protein aggregates. Additionally, low pressure or the even more often used gravity-dependent flow allow non-cracked vesicles with regular shape to be obtained. Self-made columns can be adapted for specific experimental conditions; however, the sample volume should not exceed 10 % of the resin volume, which makes this method less useful for larger volumes owing to the costs. It is probable that this limitation is the main cause of the lower popularity of stand-alone SEC isolation, which has also been successfully presented for serum-derived vesicles in relation to mass spectrometry analysis [61,62]. So far, gel filtration has been successfully used for a wide range of samples in combination with ultracentrifugation and/or ultramembrane-based concentration as an effective extra purification step [63–65]. Summing up, from the standpoint of application in mass spectrometry, the main advantage of filtration-based methods is the high purity of the sample, due

to both its high efficiency in high-abundance protein removal and the lack of a requirement for additional chemical components.

Affinity-based capture

Affinity-related methods are used in popular commercially available solutions. Their main principle is the highly selective separation of exosomes using specific antibodies immobilized to various carriers that can be then easily removed from the analyzed biofluid. Currently, available kits make use of immuno-beads, ELISA plates, modified chromatography columns or microfluidic devices capturing exosomes by targeting surface markers such as those from the tetraspanin family (CD63, CD9, CD81), annexin or EpCAM [66]. Magnetic beads, definitely the most widespread tool for immunological separation, have been successfully used for different research applications [68–70] including LC-MS/MS analysis [67] and for now their high utility in exosomal studies is unquestionable. At the same time, promising reports about the diagnostic potential of extracellular vesicles have contributed to intensive development in the field of microfluidic technologies. Recent innovations, e.g. immuno-chips allowing for efficient and quick isolation and characterization of exosomes from cancer patient samples, have paved the way for their practical use in clinical applications [71–74]. On the other hand, highly specialized diagnostic tools usually focus on particular components of the cargo and are not appropriate for extensive research.

The immuno-based isolation methods belong to the most specific group of methods, especially in comparison with the other methods already presented here. They provide a unique opportunity for highly selective studies of concrete subpopulations[67,75], which is an essential feature for diagnostic-oriented studies of tumor-specific vesicles from biofluids, as well as for basic research into exosomal formation, packing and secretion mechanisms. However, high specificity can be problematic for general exosome studies, since the lack of the targeted protein on the surface excludes some vesicles enriched with alternative markers which, in turn, results in a unreliable analysis. The availability of proper antibodies, especially from non-human species, may also be a limiting factor. Furthermore, the immuno-based methods are poorly suited to large-scale capture and the elution buffers required

to release the vesicles can also permanently disrupt the functional activity of the released vesicles. Hence, for preservation of the biological properties of isolated exosomes elution by certain conventional methods reliant on significant alterations of pH and/or the presence of detergents and reducing agents should be avoided. These harsh conditions may affect the three-dimensional structure of surface proteins determining their functionality and influence on lipid membrane properties; for example, permeability. In the case of the commercial kits, information about their suitability for functional biological assays is usually available in the manufacturer's documentation. Otherwise, some milder elution conditions may be tried, such as higher salt concentration or a gentle lowering of pH. Another factor that should be taken into account, especially in the case of proteomic analysis, is the potential co-elution of an antibody with target vesicles. High-abundance Ig can significantly decrease the efficiency of the identification of exosomal cargo components that are found in a solution in much lower concentrations. In some cases, even the SDS-PAGE purification step may be insufficient due to masking of a large area of the gel by heavy and light Ig chain bands, and additional attempts would be necessary. Cautious selection of an available isolation kit and/or time-consuming optimization of the experimental conditions (using beads with permanently bound antibodies or soft elution buffers) can improve mass spectrometry analysis [76,77].

Besides the most popular antibody-related capturing methods, some alternatives, definitely less selective, have also been proposed. An article by *Gosh et al.* [78], showing the potential of synthetic peptides called venceremin (Vn), is worth mentioning here as an example. Vn peptides efficiently capture HSP-containing vesicles via their strong affinity for heat shock proteins and facilitate their collection by low speed centrifugation. Another method developed by *Balaj et al.* takes advantage of heparin affinity for extracellular vesicles and is based on using heparin-conjugated agarose beads [79]. Lectine affinity for exosomal surface glycoproteins, so far occasionally used for urinary extracellular vesicle isolation, is actually readily investigated because of its high potential in clinically usable diagnostic test development [80-82]. All these methods are supported by comprehensive studies and seem to be an interesting alternative to traditional methods; however, it is difficult to predict their

role in future exosomal studies. Finally, it should also be noted that affinity-based isolation methods definitely require initial purification of the sample and in most cases also vesicle pre-concentration; so, in practice they complement ultracentrifugation or precipitation methods rather than offer a self-contained method.

Major steps and key features of the above-mentioned methods of exosome isolation are compared schematically in Figure 1. In addition, the advantages and disadvantages of these methods are summarized in Table 1.

Biological purity of exosome samples

Proteomic analysis of exosomal cargo using mass spectrometry should take into account two aspects of sample purity: biological-important for the specificity; and chemical-determining the yield of results. The former relates to the effective separation of exosome populations from other membrane components and, as far as possible, reduction of background caused by co-isolated protein/lipoprotein aggregates. The latter aspect concentrates on the quantity of a sample, its chemical homogeneity and reduction of MS-interfering substances that directly influence the measurement.

In point of fact, irrespective of the chosen isolation method and type of sample, pre-treatment comprising sequential centrifugation and/or filtration is obligatory. As already mentioned regarding the ultracentrifugation protocol, the first low-speed centrifugation, according to the most popular protocol of *Théry et al.* [38], is performed at 2,000 × g for removal of death cells and larger cellular debris. The following centrifugation at about 10,000 × g in theory separates membrane fragments and most of the non-exosomal vesicles, although in practice also some small amounts of exosomes can be detected in the pellet [83]. A similar function is fulfilled by filtration through a 0.22 µm filter device that should eliminate all components with a diameter exceeding ca. 200 nm and this can be implemented instead of the centrifugation or as its completion. The resulting supernatant/filtrate is a promising starting point for isolation of a pure population of exosomes; however, for some down-

stream applications, e.g. mass spectrometry or functional studies, additional purification steps are strongly recommended. At this stage, it is desirable to remove non-specifically interacting proteins, especially those high-abundance ones such as albumin or Tamm-Horsfall protein that co-sediment with the vesicles. Two methods are especially used for this purpose: density gradient centrifugation and gel filtration. Density gradient centrifugation is a well-known technique that allows the separation of particles according to their size and density using a specific gradient medium. This method, commonly used for isolation of subcellular components such as mitochondria, peroxisomes or endosomes [84–86], has been successfully applied for extracellular vesicles purification. Generally, there are two main strategies in this approach: (i) continuous density gradient-where the density of the medium gradually increases from the top to the bottom of the tube (zonal variant–gradient is created before centrifugation, isopycnic variant–gradient is established during centrifugation); and (ii) discontinuous density gradient-where the density of the medium increases in discrete steps from the top to the bottom of the tube (cushion variant–simplified method in which the particle are centrifuged through the medium layer) [87,88]. A sucrose-based medium is traditionally the most popular medium for exosomal studies and is mostly used in the cushion centrifugation variant. Samples pre-concentrated by ultracentrifugation or ultramembrane filter units are added to 30 % sucrose D_2O solution and ultracentrifuged at 100,000 × g for at least 60 min. Subsequently, the cushion layer is collected, diluted with PBS and pelleted for isolation of pure vesicles with characteristic density in the range of ca. 1.13–1.19 g/mL [38,89–91]. Currently, iodixanol is also approved as an alternative medium for sucrose [92,93], owing to its high efficiency in the separation of components with a very similar density range, like exosomes and HIV-1 particles [94], as well as in removing protein aggregates and preventing vesicle clumping, especially in the case of the gradient variant [95]. Another frequently used method for improving the purity of exosomes is the above-discussed gel filtration. In the most typical protocol, pre-cleared and (in the case of large initial volumes) also pre-concentrated samples are loaded on Sepharose® 2B columns and washed with PBS. Early fractions containing exosomes are pooled and ultracentrifuged at about 100,000 × g [64,96]. It

is worth noting that vesicles prepared in this way are devoid of protein contaminants, morphologically intact and usable in functional studies, as has been well documented by *Muller et al.* [97] via experimental confirmation of the immuno-suppressive ability of cancer patient exosomes that inhibit expression of CD69 in activated CD4+ responder T cells. Sepharose®-based purification has also been successfully applied by *Hong et al* [98] during functional studies on the influence on NK cells of TGF-β1-rich exosomes isolated from plasma patients with acute myeloid leukemia.

A separate issue regarding studies of exosomes isolated from cell culture supernatants is the risk of sample contamination by vesicles from other species, mainly bovine, derived from culture media supplements that can significantly affect the results. That was thoroughly investigated by *Shelke et al*. [99] Generally, there are two strategies to avoid such undesirable co-isolation. The most popular method is the application of exosome-depleted serum that can be either purchased as a ready-to-use solution (the expensive option) or home-made from a standard FBS (the time-consuming option). The depletion procedure is based on prolonged ultracentrifugation of culture medium, containing up to 30% (v/v) FBS, that after sterilizing filtration can be diluted to the working concentration and/or stored for several weeks at 4°C [38]. Successful depletion from undiluted FBS was also reported (18h for 95% effectiveness) [99], but this option is not used widely yet. Another possibility is performing experiments with serum-free media [90]. At first glance, this option seems to be very tempting, since at the same time problems with serum derived vesicles and high-abundance proteins are avoided. Unfortunately, serum starvation is a serious stress factor that causes complex cellular responses and influences essential biological processes such as cell cycle regulation, apoptosis or autophagy, which may affect a research object in unpredictable ways [100]. Application of 1% bovine serum albumin instead of FBS could be a compromise solution for some cell lines [38]. However, the effect of deprivation is strictly dependent on its duration and cell type; therefore, these two factors should definitely be taken into account during the planning of an experiment.

It is highly recommended to verify the effectiveness of the selected strategy via the robust characterization of the obtained vesicles. In fact, electron microscopy visualization of sample

components is a standard that allows the assessment of their size, morphology and integrity as well as the estimation of sample heterogeneity. These data may be supplemented by size distribution measurement of a larger population by nanoparticle-tracking analysis, dynamic light scattering, or resistive pulse sensing. The presence of specific exosomal markers, e.g. tetraspanins (CD9, CD63, CD81), TSG101, annexins or syntenins, should be demonstrated by Western blot or flow cytometry, while the absence or underrepresentation of non-exosomal proteins associated mainly with intracellular compartments such as GRP94, cytochrome C or GM130 should also be documented [7]. From the viewpoint of mass spectrometry analysis, preservation of exosomal functionality is not necessary, so in this case selection of the method depends on the effectiveness of the isolation of specific, membrane-intact vesicles with the lowest possible contamination with non-exosomal proteins and other interfering particles. In that context, special attention should be paid to the evaluation of sample purity, which can be performed using a simple method proposed by Webber and Clayton [101] based on the ratio of exosome counts to protein concentration. There is an inverse relationship between the degree of exosome purity and the protein yield; hence, the higher the ratio the better. As a reference value for the good quality of a preparation, 3×10^{10} particles per μg of protein has been proposed. Paradoxically, due to the very high sensitivity of MS tools, the scale of an experiment is primarily dependent on validation methods requirements which exceed those for actual proteomic analysis. However, irrespective of the downstream application, all purification steps should be considered in terms of their possible impact on the final composition of the sample and the form of vesicles.

Processing of exosome samples for MS analysis

Sample preparation for mass spectrometry analysis is a culmination point in the biological material processing that may be crucial for the peptide identification yield and, as a consequence, for the success of the whole experiment. Over the years, this issue has been the subject of many comprehensive studies [102–104] that can be an interesting introduction into the methods presented

below in the context of extracellular vesicle examination. It is worth noting that, irrespective of the selected procedure, obtaining a high quality sample is a real challenge. As the MS analysis of exosomal cargo is traditionally performed according to the bottom up strategy, the main point of the sample preparation is enzymatic digestion that can be implemented in three variants directly connected with contaminant removal (Figure 2). Without doubt, the most popular variant in exosomal studies is the in-gel digestion procedure [93,105–111]. In brief, a predetermined amount of protein is separated by gel electrophoresis and stained gel pieces are excised from the gel. After a series of dehydration and rehydration steps interlaced with reduction and alkylation reactions, trypsin solution is added for overnight digestion. Finally, peptides are extracted with acidified acetonitrile solution fully compatible with mass spectrometry tools [112]. The prevailing option is simple but efficient one-dimensional gel electrophoresis that allows for effective removal of most common contaminants and initial fractionation of a sample according to the molecular weight of proteins. Less popular, but also in use, is two-dimensional electrophoresis, where molecules are additionally separated by their isoelectric point [93,107]. The high price to pay for more precise separation are the greater restrictions for a sample (ionic detergents are not allowed which can be challenging for the lysis), extending the time of the whole procedure and the requirement for expensive equipment [113]. For protein visualization, typical Coomassie blue-like solutions as well as more sensitive silver staining tools have been successfully implemented [93,107]. The starting amount of protein varies in the cited reports and ranges from 10 μg [110] to about 100 μg [108] for 1D, and to over 300 μg [93,107] for 2D PAGE. In summary, in-gel digestion is a well-established method that, despite some disadvantages such as its labor-intensiveness and the high risk of contamination with keratins, is still widely and successfully used owing to its robustness and high efficiency in impurities removal [114].

Two other methods of sample preparation, generally popular, but rarely used in exosomal studies, are: in-solution [115,116] and filter-aided digestion [117,118]. The former is probably the most user-friendly [119]. Proteins can be purified from detergents, salts or even lipid components by precipitation using chloroform/methanol solutions as well as trichloroacetic acid or acetone [104]; however, it should

be remembered that some of these can introduce selective modifications, e.g. acetone may introduce modification of peptides in which glycine is the second residue, resulting in +40 Da adducts [120]. After pellet resuspension in MS-neutral denaturing buffer, the whole further procedure, including reduction, alkylation and digestion, can be performed using a single tube, which significantly reduces sample loss. This feature is particularly important in the case of small amounts of samples, quite typical for vesicle studies. However, after precipitation some proteins can be difficult to re-dissolve, thereby hindering the yield enzymatic digestion. Another unfavorable factor is the high complexity of the final sample, because in-solution digestion does not provide a pre-fractionation step which is advisable to perform as a support for LC-MS/MS analysis. The better is a peptide separation before analysis, the higher yield of protein identification will be achieved, which is caused by the reduction of masking of low-abundance molecules by those more numerous and more susceptible to ionization. Also, the efficiency of contaminant removal may be lower, compared with the other two methods [121]. The last strategy, developed by *Manza et al.* [122], but improved and disseminated by *Wisniewski et al*. [123], combines the advantages of both those previously discussed. In the Filter-Aided Sample Preparation (FASP) procedure, a protein sample in denaturing urea-based buffer is passed through a filter device with high-molecular weight cut-off. An effluent containing detergents and other impurities is discarded and the trapped proteins are washed with urea-containing buffer and treated with an alkylating agent. Subsequently, trypsin solution is added on the filter top and digestion runs overnight. The peptides thus obtained are easily recovered by short centrifugation. Optionally, this procedure can be extended with additional Lys-C digestion and fractionation related to pH by stepwise elution from SAX Tip-columns [124]. FASP allows pure, high quality and pre-fractionated samples to be obtained; however, depending on the option, it can be time-consuming and labor-intensive. Working simultaneously with more than a few samples may also be problematic.

To sum up, all three presented strategies are suitable for preparing samples with satisfactory quality for mass spectrometry analysis. The choice should be made taking into account the complexity and the size of the sample as well as the type of contaminants to be dealt with. Omission

of the purification step can severely influence the identification yield. Ions from salts and detergents can significantly suppress analyte ions from the compounds of interest. Formation of adduct ions reducing accuracy of the molecular mass and complicating the spectrum is also very common. Although ionization methods vary in their sensitivity to contamination, generally it is highly recommended to avoid contaminants in any MS application [125].

Comparative analyzes of exosome isolation strategies

The intense interest in exosomal research in recent years and the notable diversity of the applied isolation protocols may suggest that, in spite of appeals for work standardization [126], researchers have never been so far from unanimity in this field. The enduring popularity of the traditional ultracentrifugation method and the equally persistent search for alternatives may cause real confusion, especially for novices. In this situation, more pressing seems to be the need for comparative studies, such as those presented by *Lobb et al.* [42], *Tauro et al.* [127], *van Deun et al.* [128], *Kalra et al.* [129], *Lane et al.* [130] or *Alvarez et al*. [131] It has been revealed that the different strategies applied in these reports, i.e. ultracentrifugation, ultracentrifugation with iodixanol density gradient (OptiPrep™), immuno-affinity capture, filtration and precipitation, are useful for isolation of exosomes, however with different efficiency and quality. Generally, none of the methods significantly altered the size distribution or morphology, albeit larger particles were observed by *Lobb et al.* [42] and van *Deun et al.* [128] after precipitation. The results presented by *Lane et al*.[130] indicate that this phenomenon is more probably a result of the co-isolation of some non-exosomal objects, than an effect of exosome aggregation. In terms of the absolute number of particles, precipitation methods produce higher yields of exosomal-size vesicles than the others [42,128], although their efficiency differs depending on the reagent manufacturer [130]. Relative protein amount obtained for equal vesicles number is also several times higher after precipitation compared to use of the traditional ultracentrifugation and OptiPrep™/sucrose density gradient [128]. Nevertheless, the presented studies leave no doubt that this is a result of co-isolation of contaminating proteins such

as albumin, apoliprotein E or THP [42,128]. The purity of a sample, defined as the ratio of particle number per µg of protein, was by far the best for ultracentrifugation in OptiPrep™ gradient [42,128], where the absence of highly abundant serum/plasma components was proven by high-resolution MS analysis [129]. Similarly gratifying results have been obtained for both cell culture supernatant and human plasma in the case of coupling of ultrafiltration-based concentration and size exclusion chromatography [42]. On the other hand, an additional comparative criterion, which could be enrichment of exosomal markers, gave precedence to immuno-affinity capture over ultracentrifugation and OptiPrep™ [127]. Interestingly, in this ranking ultracentrifugation was considered average, since its lower recovery rate was compensated by relatively low protein contamination in comparison with precipitation and acceptable exosomal marker enrichment [42,131], similar to that observed after ultramembrane-based concentration for cell culture supernatants [42]. The total time of sample processing is also favorable in comparison with some other protocols, which still makes ultracentrifugation an attractive option.

In the context of high risk of abundance non-exosomal protein contamination, the commonly used protocols for easy vesicle quantification based on protein concentration measurement [38] seem to be rather unreliable and should be applied very carefully. Hence, less contamination-sensitive methods, albeit also not without flaws [132], e.g. tunable resistive pulse sensing technology (tRPS) [133], nanoparticle tracking analysis (NTA) [134] or flow cytometry [135], may be worth considering in most cases.

Studies by *Tauro et al.* [127], *van Deun et al.* [128] and *Kalra et al.* [129] clearly show that the choice of isolation method has a significant influence on the mass spectrometry analysis and data interpretation. Low-quality samples result in a cluttering of databases with artifacts and false positive identifications of exosomal cargo components. For this reason, it is highly recommended to precede proteomic analysis with meticulous verification of the biological and chemical purity of samples and their documentation at least according to the directives of the International Society for Extracellular Vesicles [7].

Conclusions

Mass spectrometry is a powerful tool for the identification of proteomic components of exosomal cargo that can be crucial for understanding the role and function of extracellular vesicles, as well as for their diagnostic application as a potential source of disease-related biomarkers. However, the high sensitivity of MS is both its strength and its weakness. Even small amounts of impurities can significantly affect the analysis. From the several strategies commonly used for exosomes isolation, Optiprep™ density gradient ultracentrifugation outperforms the others in terms of the biological purity of the final material. Unfortunately, this method-definitely most suitable for restrictive mass spectrometry requirements, is not universally applicable owing to its being a time-consuming and labor-intensive procedure. A promising alternative is ultrafiltration coupled with size exclusion chromatography, which provides vesicle purity comparable to the OptiPrep™-based technique. Irrespective of the selected methods, isolation and purification processes should be carefully planned, taking under consideration the biological and chemical properties of the sample and downstream applications. A low–quality, contaminated sample has a significant influence on protein identification yield. Due to the plurality of isolation and purification strategies, precise description of all steps would definitely help to improve the comparability of results.

Conflict of interest

The authors declare that they have no conflicts of interest.

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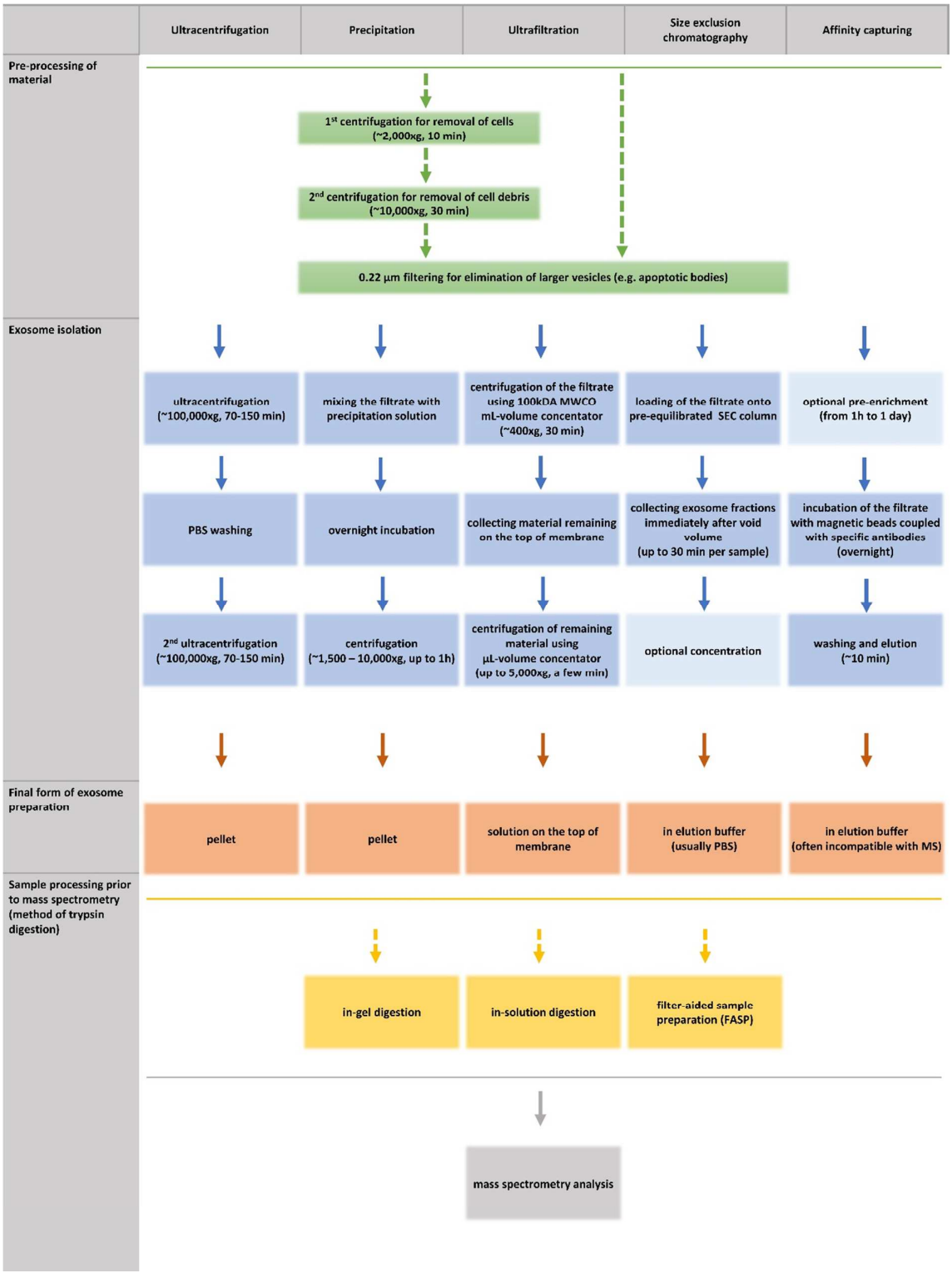

Figure 1. Comparison of representative workflows of the most popular methods of exosome isolation.

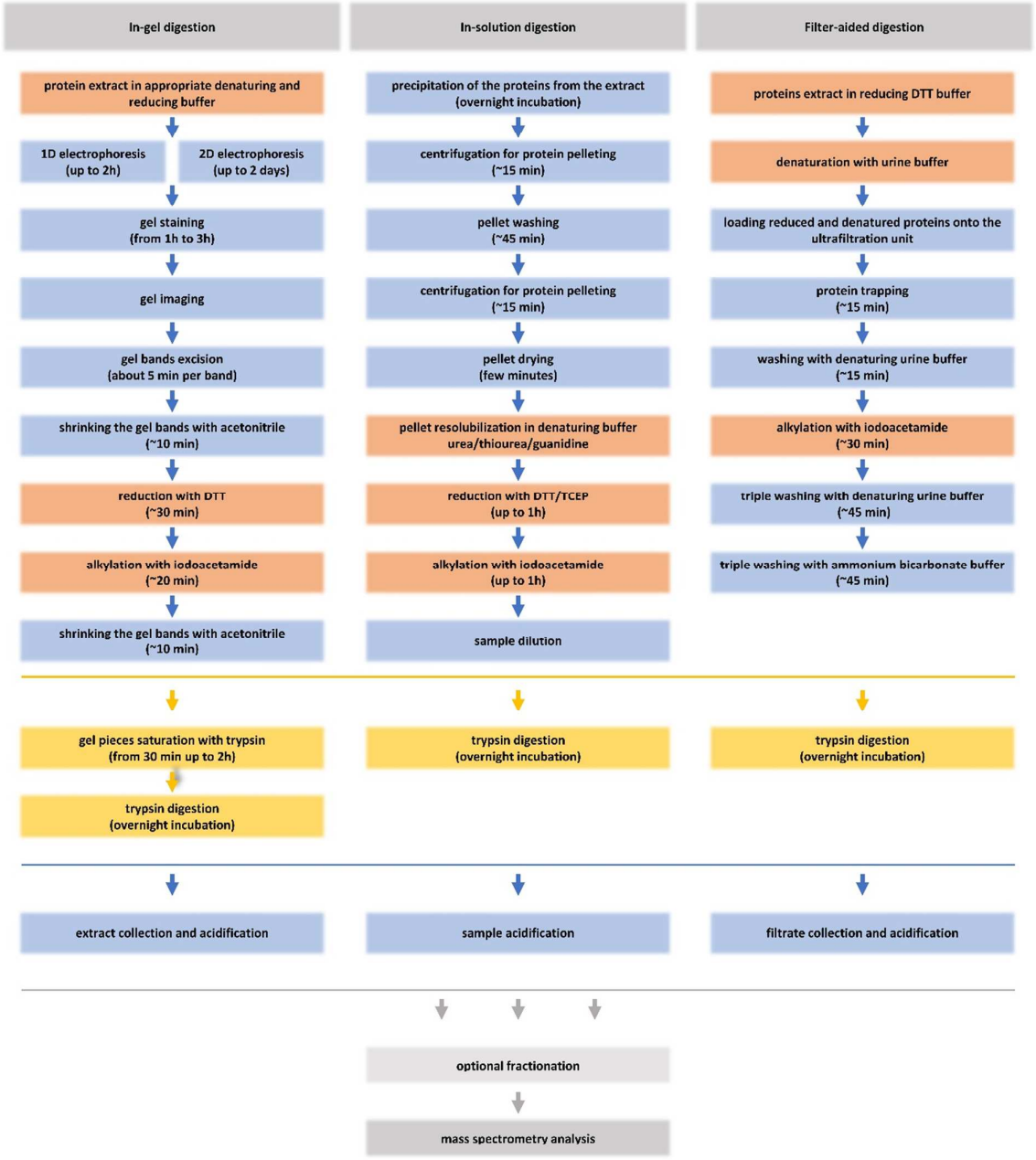

Figure 2. Comparison of representative workflows of the most popular methods of sample preparation for MS analysis.

Table 1. Important features of the most popular methods of exosome isolation.

Method	Advantages	Disadvantages
Ultracentrifugation	-suitable for isolation from large volumes -low cost (assuming availability of an ultracentrifuge) -no chemical additives	-efficacy depends on physicochemical properties of a sample (e.g. viscosity) -time-consuming procedure -low yield
Precipitation	-not labor-intensive -no special equipment required	-sample contamination by polymer particles -possible co-isolation of non-specific proteins -efficiency differs depending on the reagent's manufacturer
Ultrafiltration	-the least time-consuming: total time of isolation about 1h -no volume limitation -relatively low contamination with non-specific proteins -no chemical additives	-efficiency dependent on type of ultramembrane -risk of exosomes lost by becoming stuck in membrane pores -many confounding factors affecting filtration rate (e.g. temperature, viscosity and concentration of sample)
Size exclusion chromatography	-high purity of the final sample: low contamination with high-abundance proteins -low time-outlay (per sample) -low cost -no chemical additives	-significant dilution of the final sample -small scale of isolation–sample concentration is required -low sample throughput–only one sample at a time -labor-intensive
Affinity-based capturing	-enables examination of selected sub-populations of exosomes -very selective -not labor-intensive (as long as pre-enrichment is not required)	-the isolated fraction contains the antigen -contamination of a sample with antibodies -limited possibility of isolation from large volumes, usually sample pre-enrichment required -expensive -elution buffers can contain components incompatible with mass spectrometry