Review Article

Canonical and selective approaches in exosome purification and their implications for diagnostic accuracy

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Abstract: Extracellular vesicles (EVs) play a pivotal role in cell to cell signalling in both physiological and pathological conditions. Based on their biogenesis, three main classes of EVs are recognized: exosomes, microvesicles and apoptotic bodies. Exosomes are cell-derived vesicles (EVs) present in many body fluids (blood, urine, milk, cerebrospinal fluid) ranging in size from 30 to 150 nm. Due to their involvement in numerous physiological and pathological events, cell derived exosomes in bodily fluids represent a unique source of clinically relevant and non-invasive biomarkers. Since biomolecule content present in exosomes reflects the state of the parent cell, exosome analysis and characterization may provide valuable information about the presence of aberrant processes in the cells from which they originated. Because of the large and heterogeneous scientific community working with exosomes, several purification strategies have been applied so far, which yield EV fractions largely differing for quantity and quality. Most of the present exosome isolation approaches based on ultracentrifugation (UC), ultrafiltration (UF) or precipitation, are inefficient and hard to standardize, thereby creating low reproducibility in sample quality and potentially misleading results because highly sensitivity downstream analytical techniques, such as mass spectrometry, can detect even minute traces of co-isolated contaminants. Furthermore, loss of certain exosomal fractions during purification process or damage of exosomal membrane integrity can also alter final protein and RNA profiles. As a consequence, there is a strong interest in consensus principles for the exosome purification and the search for reliable methods for selective isolation of EV sub-populations. In the present manuscript, we critically overview the most commonly used techniques used for exosome preparation such as ultra-centrifugation, size-based isolation methods, precipitation and immunoaffinity (IA) and their respective applicability for purification of exosomes from clinically relevant samples.

Keywords: Affinity purification; antibodies; extracellular vesicles (EVs); gradient centrifugation; immune-based chromatography; liquid biopsy

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Introduction

Cell to cell signalling is crucial for maintaining homeostasis in multicellular organisms. Information exchange can be accomplished through direct cell-to-cell contact or through transfer of different secreted molecules. In addition, most eukaryotic cells release membrane derived vesicles that can impact both neighbouring and distant cells (1). Extracellular vesicles (EVs) are classified based on their cellular origin and/or biological function or based on their biogenesis. As

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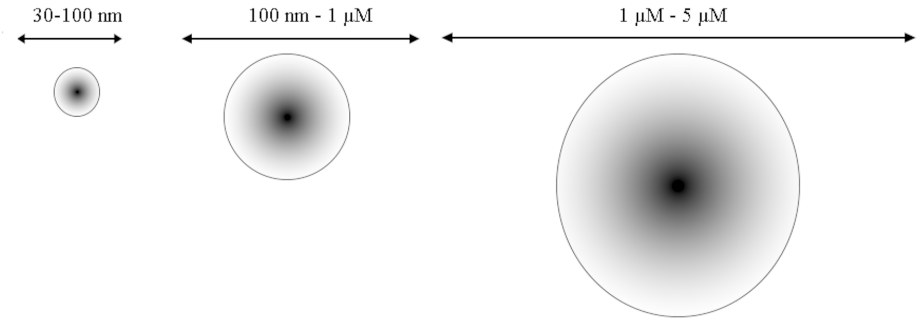

	Exosomes	Microvesicle	Apoptotic body
Origin	Endolysosomal pathway; intra-luminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	Cell surface; outward budding of cell membrane	Cell surface; outward blobbing of apoptotic cell membrane
Markers	Tetraspanins (such as TSPAN29 and TSPAN30), ESCRT components, PDCD6IP, TSG101, flotillin MFGE8	Integrins, selectins, CD40 ligand	Extensive amounts of phosphatidylserine
Content	mRNA, microRNA (miRNA) and other non-coding RNAs; cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules	mRNA, miRNA, non-coding RNAs, cytoplasmic proteins and membrane proteins, including receptors	Nuclear fractions, cell organelles

Figure 1 Complexity of the circulating extracellular vesicles. In the biological fluids circulate EVs of different origin and characteristics. The exosome specific purification is further complicated by the presence of particles as different as cholesterol vesicles (LDL/HDL) and nucleic acid-protein (argonaut-RNA) complexes. They are difficult to be separated because of their partially overlapping chemical-physical features. EVs, extracellular vesicles.

determined by their biogenesis, the three main classes of EVs are exosomes, microvesicles and apoptotic bodies (2) (*Figure 1*). Exosomes are cell-derived vesicles present in many body fluids (blood, urine, milk, ascites and cerebrospinal fluid) ranging in size from 30 to 150 nm (3). First reports dealing with the formation of EVs emerged in the 1980s from two independent research groups. They described the formation of multivesicular bodies in reticulocytes that released such vesicles into the extracellular space (4,5). One decade later, the release of these vesicles has been described for lymphocytes and dendritic cells through a similar route (6,7). Since then, exosomes have been purified from nearly all mammalian cells including primary cells of the immune (8) and nervous system (9,10) and stem cells (11,12). Interestingly, this mechanism is not exclusively restricted to mammals, but secretion of EVs has been identified in plants (13), lower eukaryotes (14,15) and prokaryotes (16).

Numerous studies deal with physiological and pathological roles of EVs and exosomes in living organisms. So far

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they have been linked with transfer of proteins, lipids, nucleic acids and possibly active metabolites (1,17-20), cell self-renewal (11), tissue repair (21), immune surveillance (6,22) and blood coagulation (23). Besides these physiological processes, the role of exosomes has been shown in genesis of tumors (24,25) and their metastasis (26), spread of pathogens (27,28), transfer of toxic peptides linked with neurodegenerative diseases such as amyloid-β-derived peptides (29) and α-synuclein (30), as well as the spread of the abnormal pathogenic cell surface prion protein PrPC (31). EVs can also mediate inter-species horizontal transfer of coding mRNAs and the consequent translation of human genetic material into murine proteins (32), resulting in *in vivo* protective effect against cisplatin-induced kidney injuries (33) and liver morphological and functional recovery (34). Exosome exert their effect in a pleiotropic manner by directly activating cell surface receptors for proteins and merging with bioactive lipids of the receiving cell, a process which results in direct delivery of their internal cargo in the host. This can include transcription factors, oncogenes, small and large non-coding regulatory RNAs [such as microRNAs (miRNAs)], mRNAs, and infectious particles (1,11,25,32,35). Due to their involvement in a myriad of cellular processes, exosomes could be considered as multifunctional signalling complexes for controlling fundamental cellular and biological processes.

Several proteomics studies performed on different types of material (exosomes from primary cells, cell cultures, tissue cultures and biological fluids) yielded extensive data on protein abundance on different types of vesicles and these data are available in several public on-line repositories such as Vesiclepedia (www.microvesicles.org) (36), EVpedia (www.evpedia.info) (37) and ExoCarta (www.exocarta.org) (38). Protein content of EVs is made up of pan-exosome markers common for most of exosomes, but also proteins and protein post-translational modifications that specifically reflect the cellular origin and the mechanism of secretion (39-41). In general, exosomes contain cytoskeleton, cytosolic, heat-shock and membrane proteins, as well as proteins involved in vesicle trafficking and protein-lipid complexes (42). However, the proteomic profiles obtained in different studies depended much on the manner of exosome isolation and, since different methods yielded different EV populations and exosome sub-populations, the proteomic data are hard to compare.

Due to their involvement in numerous physiological and pathological events, cell derived exosomes in bodily fluids represent a unique source of clinically relevant and non-invasive biomarkers. The biomolecule content present in exosomes mirrors that of the parent cells and, therefore, exosome analysis may provide valuable information about presence of aberrant processes in the cells from which they are originated. Particularly peripheral blood and urine are rich and easily accessible sources of circulating exosomes. Analysis of exosomal content in peripheral blood provided access to exceptional amounts of biomarkers of diagnostic and prognostic value and, consequently, exosomes seem a particularly convenient target for liquid biopsy procedures (43). However, present exosome isolation approaches based on ultracentrifugation (UC), UF, and precipitation, are often inefficient, hard to standardize, thereby creating variability in sample quality and often leading to loss of important biomolecules (44).

State of the art

Much of our understanding of exosome biology has been obtained from heterogeneous or impure exosome preparations. Many of the published studies fail to assess the purity of the isolated exosome populations before performing functional assays or omics analyses despite the precise indications listed by the scientific community (36,45,46). This condition jeopardizes the interpretation of findings and the possibility to use EVs for therapeutic applications (47). The priority is therefore the identification of more reliable methodologies for the preparation of homogeneous exosome populations not contaminated with further membranous particles—such as apoptotic bodies (400–2,500 nm), shed microvesicles (50–1,500 nm), oncosomes (>1,000 nm)—released by cells into body fluids and sometimes sharing some of the "standard exosome" biomarkers (48-51).

Commonly purification methods include UC, density gradient (DG) centrifugation, chromatography, filtration, polymer-based precipitation and immunoaffinity (IA) (50). Both the quantity and the quality of the purified EVs critically depend on the applied purification strategies (50) which can yield exosomal preparations contaminated with vesicles and protein complexes, exosome with damaged membrane integrity, or the simple loss of exosomal fractions during the purification process. All these conditions can result in misleading data interpretation of the genomics and proteomics profiles.

Despite the several commercial polymer-based kits available on the market and that promise the fast precipitation of the exosome fraction, the gold standard for

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exosome isolation remains based on differential UC (52), mostly integrated with DGs, UF, size exclusion chromatography (SEC) or IA steps (50). UC exploits multiple centrifugation steps performed at increasing centrifugal strength to sequentially pellet cells (250–400 ×g), cell debris (2,000 ×g), microvesicles (10,000–20,000 ×g), and exosomes (100,000–150,000 ×g). Of course, the characteristics of the original samples strongly influence the purification final output. Cell culture supernatants represent a relatively simple and homogeneous starting material in comparison to body fluids characterized by high-abundance of contaminating proteins, lipoprotein particles and EVs originated by different cell types as well as by physical properties that might interfere with standard isolation protocols. Because this complexity represents a further element that can perturb EV data comparison, also the standardization of the protocols for the preparation of biological samples to undergo EV fractionation is under discussion (51,53). In parallel, the community search for specific biomarkers that would enable the selective and straightforward IA purification of exosome populations directly from biological samples (54) (*Table 1*).

UC and DG UC

UC has been for long time considered the method of choice when it comes to exosome isolation. In UC, centrifugal force is applied to a mixture of macromolecules in solution sedimenting them according to density. This involves applying to the sample a high g-force (100,000 ×g or greater) to pellet the vesicles. The most widely accepted method for exosome isolation is differential centrifugation (55,56). Successive centrifugation steps with increasing speeds of centrifugal force are intended to pellet consecutively the apoptotic bodies and cell debris, the shedding vesicles and the exosomes. Since UC was considered the "gold standard" in exosomal purification, it still accounts for the most commonly exosome isolation technique employed by users in exosome research (54). Although this methodology should be relatively straightforward, quantity and quality of the obtained material are highly sensitive to several parameters such as g-force, rotor type (fixed angle or swinging bucket), the angle of rotor sedimentation, the radius of centrifugal force, the pelleting efficiency (rotor and tube k-factors), and the solution viscosity (44,57,58). Viscosity difference among various biological fluids used for exosome purification influences the internal friction within the solution resulting in variable optimal centrifugation velocities needed for pelleting (50,59). The design of the centrifuge rotor also influences the efficacy of pelleting, as swing bucket rotors show lower pelleting efficacy but appear to produce better resolution of vesicles with similar sedimentation coefficients (57). Beside rotor design, ultracentrifuge clearing factor (k-factor) plays an important role. K-factor could be used to predict the time required for pelleting as it results from the correlation between k-factor and the sedimentation coefficient (in Svedberg units): $t=\frac{k}{s}$. Many applied protocols use the same pelleting time and speed, but the difference in k-factor of the used devices leads to drastically different yields of exosomes from the same samples (48). It is therefore essential to adjust the centrifugation time to compensate for different rotor types. All these factors are hard to standardize and control and often account for the observed dissimilarity in data even when same protocols are applied. Numerous studies have indicated that UC can either result in the incomplete sedimentation of vesicles or the sedimentation of non-vesicular materials (48). On the top of that, it is still unclear how prolonged pelleting against solid surface affects the fluid membrane in terms of integrity and vesicle content, although the functionality of gradient-purified exosomes has been thoroughly described in some cases (60).

Improvements of classical UC, such as DG UC are now available. In DG UC, exosome separation depends on their size, mass, and density. A reduced volume of the sample is first loaded on a DG medium formed in a centrifuge tube in which density decreases progressively from the bottom to the top, then the DG is subjected to an extended round of UC. The most common DG-based methods exploit either sucrose or iodixanol [OptiPrep™ (Sigma-Aldrich, St. Louis, MO, USA)] (61). In particular, iodixanol-based gradients have been described improving the separation of exosomes from viruses and small apoptotic bodies (62). Upon applying the centrifugal force, solutes move through the DG medium towards the bottom according to their respective specific sedimentation rates, generating discrete solute zones. The separated exosomes can then be conveniently recovered by differential fraction collection. Both continuous and discontinuous gradients are used (63). The limiting factor of DG is the restricted volume that can be loaded, a factor which in turns limits the preparative capacity of the method (64). Furthermore, this protocol requires long running time to reach equilibrium, with reports spanning from 16 to 62–90 h (65,66). DG can lead to incomplete sedimentation of all

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Table 1 Principles and shortcomings of the methods used for EV purification

Purification technique	Method principle	Method shortcomings
Ultracentrifugation	Sedimentation of biomolecules according to density using high g-force	Quantity and quality of the obtained material is highly sensitive to: g-force, rotor type (fixed angle or swinging bucket), the angle of rotor sedimentation, radius of centrifugal force, efficiency of pelleting (rotor and tube k-factors) and viscosity of solution
		Hard to standardize and control all the parameters gives different data even when same protocols are applied
		The effect of prolonged pelleting against solid surface on the fluid membrane integrity and vesicle content is still unclear
Density gradient ultracentrifugation	Separation according to density in a pre-constructed density gradient medium	Narrow loading zone which in turns limits the capacity of the method
		Long running time to reach equilibrium
		Incomplete sedimentation of all exosomal fractions
		Artefacts from to contaminating material in the same density fractions due to inadequate sedimentation time
Ultrafiltration	Distribution of particles in a solution across a polymer-based membrane primarily dependent on size and molecular weight of the particle	Use of force may cause deformation and breakage of large-size vesicles influencing the results of down-stream analysis
		Adherence of material to the membranes
Size-exclusion chromatography	Macromolecules are sorted through porous stationary phase according to their size	Possible co-purification of some chylomicrons, since a small percent of these large lipoproteins fall in the 150–300 nm size range
Field-flow fractionation	Separation of the particles present in the fluid, depending on their differing mobility under the force exerted by the field	Very little work has been carried out to optimize the field-flow fractionation in characterizing and separating exosomes
		So far proved to be suitable only for analytical characterization of small samples pre-purified using a conventional protocol and not as an actual preparative method
		Relies on the availability of very expensive pieces of equipment and highly trained personnel necessary to analyse the data
Precipitation	Addition of water-excluding polymers retain that water and force less-soluble components, such as exosomes, out of the solution	Possibility to co-precipitate other non-exosome contaminants, such as proteins and polymeric materials
Immuno-affinity techniques	Immuno-affinity interaction between ligands on the vesicle surface and specific antibodies	Identification of appropriate surface target by an antibody recognizing the extracellular domain
		Heterogeneity of exosome populations hinder the universal applicability of the approach
		Antigen can be blocked or masked
		Tetraspanins from exosome membrane are detected by Western blot but in some cases not by florescent detection of intact exosomes
		Multi-targeting of antigens is necessary to avoid the loss of some of the fractions

EV, extracellular vesicle.

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exosomal fractions or artefacts from contaminating material co-migrated in the same density fractions due to inadequate sedimentation time. Furthermore, since the separation is achieved based on density, exosomal fraction can contain other vesicles of different origin but similar characteristics, such as cholesterol vesicles (LDL/HDL) (50,67-69).

Size-based isolation techniques

Different approaches of vesicle separation according to their size have been employed including UF, SEC, ion-chromatography, and filed-flow fractionation (FFF) (64).

UF in exosome preparation is no different form standard applications of this technique in other analytical fields. The principle of UF relies on the possibility to separate the particles present in a complex solution by exploiting their dimension. Based on their diameter, exosomes can be separated using membrane filters of defined size-exclusion limits (70) usually in a multi-step process designed to specifically remove contaminants with different characteristics. UF requires less time than UC and no special equipment is necessary for performing the separation (71). However, the exerted pressure may cause deformation and breakage of large-size vesicles influencing the results of down-stream analysis (72). Recently, it has been demonstrated that the filter material can significantly influence the final yields, with 10 kDa regenerated cellulose filters being the most efficient solution for exosome purification from plasma and urine (63).

Nano-membrane concentrators have been successfully employed for purification of exosomes from urine, more rapidly and effectively as UC (73). Short centrifugation time enabled the isolation of intact vesicles from as little as 0.5 mL of urine, as confirmed by electron microscopy and Western blot. This approach simplified and accelerated the isolation of urinary exosomes from clinical samples demonstrating its potential diagnostic applicability.

Sequential filtration for exosome isolation has been used mainly for cell culture supernatant as source of exosomes. This approach implies passing the source of exosomes through a series of membranes with different molecular weight cut-off to eliminate cells and cell debris (100 nM) and then soluble proteins (500 kDa) while in the final step the exosome containing solution is concentrated using a 100 kDa membrane (64). Sequential filtration allows the isolation of exosomes with high purity and apparent functional integrity as the result of low manipulation forces and in combination with sucrose DG has been successfully employed for purification of therapeutic exosomes for clinical trials (74,75).

SEC has always been a popular means for EV enrichment because it separates efficiently them from the bulk of soluble macromolecules present in biological samples using mild physical conditions which let unaffected vesicle integrity and structure (50). In SEC, porous stationary phase is utilized to sort macromolecules and particulate matters out, according to their size. Components in a sample with small hydrodynamic radii are able to pass through the pores, thus resulting in late elution. Components with large hydrodynamic radii including exosomes, are excluded from entering the pores. SEC was used for isolation of intact exosome from mesenchymal stem cells conditioned medium. Structural integrity of exosomes isolated in such a way was confirmed by transmission electron microscopy while other features such as size distribution and molecular markers were confirmed by dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and Western blot respectively (76). SEC is also useful to remove OptiPrep remnants from gradient-purified exosomes (63).

The net EV ζ-potential was exploited to induce protamine-dependent EV precipitation from serum, saliva, and cell culture media without the need for UC (77). This bulk separation based on total charge should not undermine the fact that the proteasome displayed on exosomes would correspond probably to unique fingerprints for each vesicle sub-class and, consequently, would have very peculiar surface chemical characteristics, such as the net charge. However, there are only few reports of conventional ion-exchange chromatographic approaches applied to exosomes. Recently, Kim *et al.* (78) demonstrated that anion exchange chromatography—in contrast to cation-based resins—is suitable for binding EVs and convenient for material concentration and consequently for scaling-up the sample volumes used in preparative purifications. However, the authors did not evaluate if non-vesicle contaminants co-eluted in the unique EV-containing peak recovered at 0.5 M NaCl. The results of the limited characterization of the EV surface biomarkers suggested that vesicles with different characteristics co-existed even though the complexity of the original sample (cell culture supernatant) was reduced. In a preliminary work (79), anion-exchange monolith resin seemed to provide better resolution and distinct EV elution peaks. Unluckily, also in this case no sufficient biochemical characterization of the peak contents was performed to assess the quality of the purified fraction. In a further work in which monolith anion exchange chromatography was used, it appeared that EVs can

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co-elute with a fraction corresponding to virus particles (80). Altogether, these reports indicate that ion exchange can be useful for a preliminary enrichment of EVs from large volumes of samples but are insufficient to infer conclusions about fraction purity and possibility to separate among EV subclasses.

FFF allows the field-dependent separation of the particles present in a fluid according to their variable mobility under the exerted force conditions. FFF separation is made according to particle hydrodynamic diameters and can discriminate very precisely among complex sample components over a wide colloidal size range. Asymmetrical flow field-flow fractionation (AF4) technique was proposed originally 10 years ago to isolate macrovesicles (81,82). Low resolution AF4 was effective in mild purification of miRNA-containing fractions (exosomes and high-density lipoproteins) directly from patient sera (83). However, only recently its potential for purification and quantification of different exosome sub-populations was confirmed by means of studies which coupled it to multi-detection systems based on UV, dynamic and multi angle light scattering, and transmission electron micrographs (84-87). The apparent limit of this technology, although very efficient in terms of sub-group separation, is that so far proved to be suitable only for analytical characterization of small samples pre-purified using a conventional protocol and not as an actual preparative method. Furthermore, it relies on the availability of very expensive pieces of equipment and highly trained personnel necessary to analyse the data sets.

Precipitation of exosomes

Settling out of exosomes from biological fluids can be achieved by altering their solubility or dispersity. This is usually achieved by water-excluding polymers such as polyethylene glycol (PEG) (71). Water-excluding polymers retain the water and force less-soluble components out of the solution (71). Samples containing EVs are incubated, usually at low temperature (4 ℃) and for relatively long time (overnight), with a precipitation solution containing polymers such as PEG 8000, after which the exosome-enriched precipitate is isolated by means of either low-speed centrifugation or filtration (71). Precipitation of exosomes is easy to do and it does not require any kind of specialized equipment, only that cells and cell debris are removed from samples. This simplicity enables straightforward integration of the method in both research labs and clinical applications (72). Currently, several commercial exosome precipitation kits compatible with different biological fluids, such as plasma, serum, ascites, urine, cerebrospinal fluid, and culture medium are available on the market (88). These systems have been largely employed for the purification of exosomes, for instance from urine samples yielding high quantities of miRNAs and mRNAs (89). The major downside of polymer based exosome precipitation is the abundant co-precipitation of non-exosome contaminants, such as proteins and polymeric materials (54). This shortcoming has been repeatedly evidenced by comparative assessments of exosome purification protocols (see below).

Affinity and IA purification

As it has been pointed out, UC, DG, SEC and polymer-based precipitation do not preferentially isolate specific EV sub-classes (50). It means that by using conventional purification methods potentially informative exosomes, such as the tumor-derived ones that never exceed 10% of the total circulating exosomes (50), will represent a marginal fraction even in a highly pure fraction. The consequence is that significant variations of cargo biomarkers could remain unappreciated because of the dilution effect of the "informative vesicles" in the bulk of physiological EVs and this condition is clearly exasperated when the sample is highly contaminated by other biological components. Affinity chromatography can increase recovery specificity if the bound molecules are biomarkers expressed solely on the outer surface of (sub-groups of) the target vesicles. For instance, lectins and heparin can bind EVs (90-93) and several lectins have been identified as having high affinity towards saccharide residues on the surface of urinary exosomes and have been used successfully as means for exosome precipitation (85). Downstream analyses such as atomic force microscopy, DLS, Western blot and RNA analysis confirmed physical and molecular properties of isolated vesicles (85) but lectins have not been exploited to selectively enrich some exosome sub-classes. Nevertheless, the recent demonstration that fibronectin exposed on myeloma cell exosomes is the molecule that acts as the ligand for heparan sulfate (94) opens the opportunity to more selective purification methods.

Binders specific for membrane lipids have been used for separating exosome sub-populations according to their membrane composition. The phosphatidylserine-binding Tim4, a protein expressed on macrophages, was immobilized on magnetic beads for enabling IA capture. The approach was successfully used but only to capture total EV fractions, from both conditioned media

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and biofluids (95). A more interesting application has been reported by Tan *et al.* [2014]. The authors showed that cholera toxin B chain (CTB) and annexin V (AV), which bind to GM1 ganglioside and phosphatidylserine, respectively, succeeded in isolating two distinct EV groups from plasma. The approach has been further developed by the addition of Shiga toxin B chain (STB)—specific for globotriaosylceramide—as a new EV-binding substrate and sequential immunopurification resulted in the isolation of EV populations with unique contents (96,97). The approach has been recently used to isolate three different exosome populations starting from patient ovarian ascites (97).

IA purification exploits the highly selective and strong interaction between antibodies and ligands displayed on the vesicle surface. Due to the extremely specialized structure that antibodies have developed for recognizing specifically their antigens, IA capture possesses the potential capacity to differentiate between minimal conformational diversities. However, the implementation of immunopurification as the standard method for the selective isolation of exosome sub-populations (for instance derived from cells belonging to different tissues or differing for maturation stage) will be possible only when reliable biomarkers for exosome subclasses will be identified and the corresponding antibodies will be produced and validated. In a seminal work, Clayton *et al.* (98) succeeded in demonstrating that exosomes derived by different antigen-presenting cells displayed specific combinations of biomarkers on their surface and that immunocapture enabled to recover analytical amounts of vesicles on the surface of magnetic beads. These biomarkers were often shared but varied in terms of expression level among vesicles originated by different cell types. Apart from well characterized antigens [the members of the human epidermal growth factor receptor family (99), EpCAM, Mart-1, TYRP2] displayed on the outer side of exosomes generated by different tumors, even at the present IA exploits mostly either antibodies against exosome universal biomarkers such as CD63 for the terminal purification step (94,100-102) or is limited to capture EVs originated from some well-characterized cell types which possess specific rather than exclusive molecular markers. For instance, anti-EpCAM antibodies have been successfully used to discriminate epithelial tumor-specific from other circulating exosomes assuming that healthy cells do not express such a biomarker (44). In another application, MHC class II was targeted to immunoprecipitate exclusively the exosomes derived from B cells from a pre-purified EV pool and the exosomes originated from antigen-presenting cells present in bronchoalveolar lavage fluid of asthmatic patients, respectively (103,104). Combinations of antibodies directed against different biomarkers have been also used (105) and the present effort is towards more systematic analyses, such as that allowed by using the multiplexed platform nPLEX (106). Although the survey was limited by the number and quality of available antibodies (by far lower than the device potential), it demonstrated that for each cell type and its corresponding exosomes there are combinations of right antibody/antigen pairs suitable for selective separation and diagnostics. Other EV stratification approaches based on multiple IA have been proposed (107) and they share the procedure depicted in *Figure 2*. Different EV classes were separated from the culture medium of endothelial and endothelial progenitor cells by using a pool of antibody-conjugated microbeads in combination with quantum-dots conjugated with a second set of specific antibodies (108). Quantum dot was also used to amplify the signal of another multi-antibody capture system which enabled the identification of as few as 100 exosomes/μL of tumor patient serum samples (109). In another experimental setting, gold-coated glass sensor chips were functionalized with antibody microarrays specific for the extracellular domains of exosome membrane proteins for monitoring the EV distribution by surface plasmon resonance imaging (110). IA of exosomes obtained by employing variable combinations of antibodies in a microfluidic system is reported also in He *et al.* (111) but the lack of detailed information relative to the used experimental methodology impairs to evaluate the quality of this contribution. Recently some new accessible membrane antigens have been proposed as biomarker candidates for prostate (gamma-glutamyltransferase 1, LAMTOR1, transmembrane protein 256) and pancreas (glypican-1) cancers (112-114). The task of successful exploitation remains not trivial since EV biomarkers can be slightly modified with respect to the structure of the homologue present in the original cell (115) or in related exosomes (116). Since this condition can impair the epitope recognition of conventional antibodies, new reagents should be probably developed.

In the perspective of obtaining the further stratification of the exosome fraction, the introduction of IA protocols that use new antigen/antibody pairs for the isolation of exclusive exosome groups is highly encouraging. A33 was initially identified as a biomarker suitable for targeting and immunocapture of exosomes released by human colon tumor cells (117). Successively, by applying sequential

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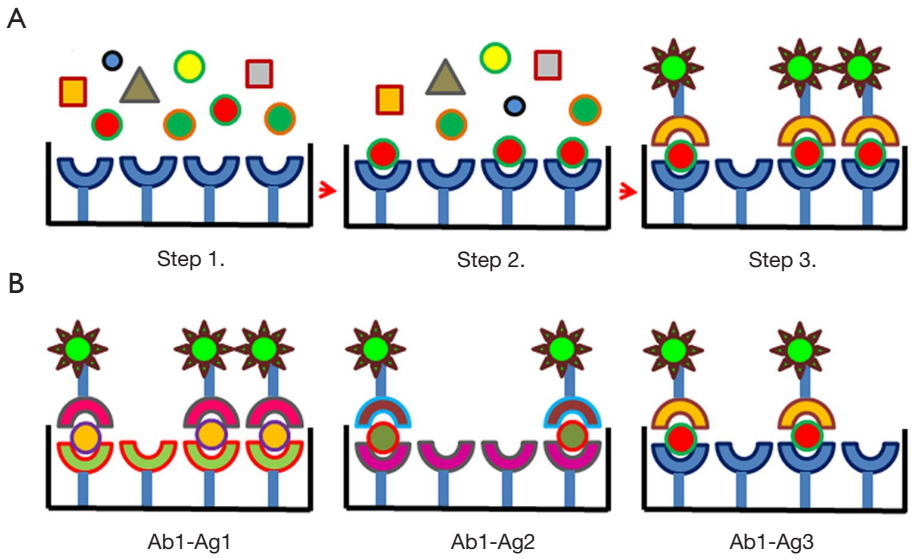

Figure 2 Diagnostic sandwich platforms for EV sub-population quantification. Antibodies specific for vesicle surface antigens can be used to capture specifically those sub-populations which exclusively express the biomarkers. These sandwich approaches (ELISA-type or on magnetic beads) need couples of antibodies to be performed (A). One antibody will be bound to the well surface and used for selective capture of the target vesicles (step 1). After washing of the contaminants (step 2), the second antibody specific for a further antigen and functionalized with a reporter carrier (for instance, quantum dot, a fluorescent protein, or an enzyme able to catalyze a color reaction) will be added (step 3). Each single well/chip spot can be functionalized with a different antibody specific for a sub-class biomarker (B) to selectively capture different vesicle sub-classes. An antibody linked to a reporter and recognizing either a selective or a shared antigen can be finally used for detection and quantification of the different fractions

immunocapture with anti-A33 and anti-EpCAM to the same human colon carcinoma cell supernatant sample, it was possible to separate two exosome populations (putatively released from basolateral and apical cell surface, respectively) which shared only a minor fraction of their miRNA cargo between them and with the microvesicle fraction separated by differential centrifugation (49,118). The phosphorylation state of IRS-1 in neural derived exosomes was assessed after selective streptavidin-dependent immunoprecipitation of exosomes labelled with biotinylated antibodies against anti-neural cell adhesion molecule-1 (NCAM-1) and anti-neural cell adhesion molecule L1 (L1CAM/CD171) antibodies (119). L1CAM-dependent immunocapture was further successfully performed for recovering α-synuclein-containing exosomes released from central neuron cells (120). EVs from urine of kidney transplanted patients were sorted by using magnetic beads functionalized with anti-CD133 commercial antibodies and their presence allowed to monitor renal functionality (121). Similar magnetic beads functionalized with anti-CD34 antibodies enabled the recovery of specific AML blast-derived exosomes directly from patient plasma (122). Magnetic beads functionalized with monoclonal antibodies against complement receptor type 1 (CR1) were used to immunoprecipitate specifically podocyte derived-exosomes (123). Finally, IA performed using anti-CD45 has been exploited to remove hematopoietic exosome contaminants from virus particle samples (124). Other results cannot be easily assessed because of poor methodological description but indicate the growing interest in this research field (125,126).

Immunocapture is also the most used enrichment method for the recovery of small amounts of EVs for diagnostics goals in conventional set-ups (ELISA plates, magnetic beads and fluorescent beads for flow-cytometry), in microfluidic devices such as Exochip and ExoSearch (51,127), lateral flow immunoassay (LFIA) (128), and in monolithic silica micro-tips (129). These tools have been usually conceived to capture possibly any meaningful exosome present in a sample and therefore they exploit antibodies against antigens "universally" expressed in such vesicles, such as CD63 (130-133), CD81 (128,134), CD9 (54,112,128,129), or against generic epithelial tumor markers such as EpCAM (133,135,136) and its combination with CD24 and CA-125 (127).

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Comparative analyses

The different EV purification methods result in variable exosome yield and contamination levels. Recently it has also reported that they can induce the detachment of EV components form the vesicle surface (100). Several research groups evaluated the available protocols and commercial products to establish their reliability and to point out their critical aspects (50,54). When UC, OptiPrep™ DG separation, and IA capture using anti-EpCAM-coated magnetic beads (IA) were compared (44,137), it resulted that IA was the most efficient method to isolate exosome and exosome-associated proteins. It yielded at least twofold more material than the two other alternative methods as monitored by mass spectrometry assessing the enrichment of marker proteins associated with exosome biogenesis, function, sorting, intracellular trafficking, and internalization in a recipient cell. IA separation performed better than chemical-physical methods also in previous works (138). The drawback of the IA approach is its dependence on the availability of convenient antibodies. If this factor is limiting, the authors advise using DG centrifugation (137) or SEC (138). With respect to UC, DG yields are significantly lower in terms of total protein, but the exosome samples are not contaminated as those recovered after standard UC. OptiPrep™ DG centrifugation outperformed UC and both ExoQuick™ (EQ, System Biosciences, Palo Alto, CA, USA) (139) and Total Exosome Isolation™ (TEI, ThermoFisher, Bridgewater, NJ, USA) (140) precipitation methods also in another systematic assessment (52). Also in this case, the methods which provided higher yields in term of total protein (EQ and TEI) performed very badly in terms of exosome purity, as demonstrated by immune electron microscopy, western blot, RT-qPCR, and NTA. EQ and the peptide-based precipitation method exploiting the ME™ kit (141) as well as other commercial kits resulted producing the most contaminated material also in further comparative surveys which demonstrated the highest quality of the samples obtained by immunopurification (54,142). Apart from publications apparently directly sponsored by commercial companies, we found only one comparative survey in which precipitation kits were evaluated positively (143), but the authors used conventional UC as a reference instead of the more suitable protocols presently used by EV specialists (46,51).

Conclusions

The awareness about the necessity to have reproducible methods to purify exosome fractions not contaminated with other sample components as well as the interest to discriminate among distinct subpopulations to analyse separately their content supported innovative technical solutions for EV fractionation. Pre-clearing steps have been proposed to diminish sample complexity and disrupting undesired interactions with other biological components (144-146) and combined techniques, in which a final immunocapture enables high selectivity after cycles of (gradient) centrifugation, filtration and SEC, represent the state-of-the-art more than conventional multi-step centrifugation. In particular, since the medical and scientific interests moved to selectivity rather than larger yields, methods based on affinity and IA became more and more attractive and demonstrated to be sufficient for recovering highly purified exosomes even directly from complex biological media, without preliminary steps (97,120,123,129,147). In this perspective, the trend seems to be using combinations of biomarkers rather than single ones to identify more specifically distinct EV sub-populations (98,113) and separate them using different device configurations (106,128). The progressive identification of EV/exosome sub-group biomarkers could lead to the development of multi-parameter flow cytometry-based sorting of exosome/EV subpopulations (*Figure 3*) (148,149), in a way resembling the characterization and separation of hematopoietic cells at different degree of their differentiation. In this optic of multi-parametric labelling, non-exclusive, quantitative biomarkers could contribute to differentiate between exosome subgroups. The limiting factor could become the production of suitable antibodies able to recognize (enough) exosome-specific epitopes.

The conventional procedure would consider: (I) inferring potential exosome biomarkers by biology and proteomics results; (II) producing the corresponding recombinant proteins or their soluble domains; (III) evaluating their actual binding to EV epitopes. An alternative method is the blind direct antibody selection on exosomes to recover conformational-specific reagents. This approach is feasible *in vitro* panning pre-immune recombinant antibody libraries. We applied it successfully to recover nanobodies able to discriminate between very similar cell types (150) and obtained promising preliminary results panning directly on

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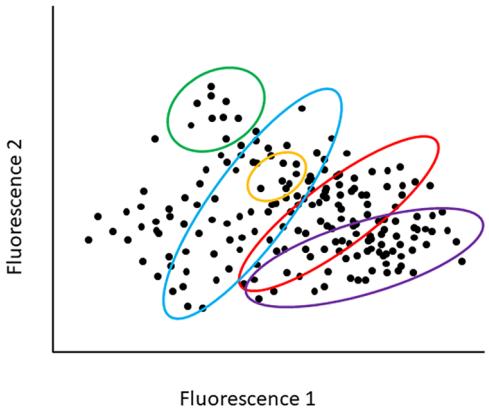

Figure 3 Multi-dimensional flow-cytometry and selective sorting of EV sub-populations. Vesicle sub-populations could be identified and discriminated not due to the expression of a single exclusive biomarker but because they possess unique combinations of several biomarkers. Consequently, only multi-dimensional analysis—such as multi-dimensional flow-cytometry—would allow for the identification and sorting of vesicle groups characterized by different biomarker profiles.

exosomes (Popovic *et al.*, in preparation) (151). The resulting recombinant antibodies are inexpensive to produce and simple to engineer and their straightforward labelling (152) is compatible with multi-parameter flow cytometry.

Another interesting development could consist in a combined chromatographic protocol in which SEC and IA are integrated with ion-exchange (*Figure 4*). In particular, monolith columns could be the material of choice because of their pore diameter can be selected according to the needs, the structure is preserved even at high pressure, and volumes are scalable. The reliability of this material for exosome purification was already demonstrated by the applications in which the capture antibodies were immobilized into monolithic tips (129) and EVs were recovered by mini-SEC (122). Columns formed by successive monolith discs with different chemical, physical, and affinity features (SEC + ion exchange + multiple IA chromatography) could be customized for the recovery of exosome subclasses.

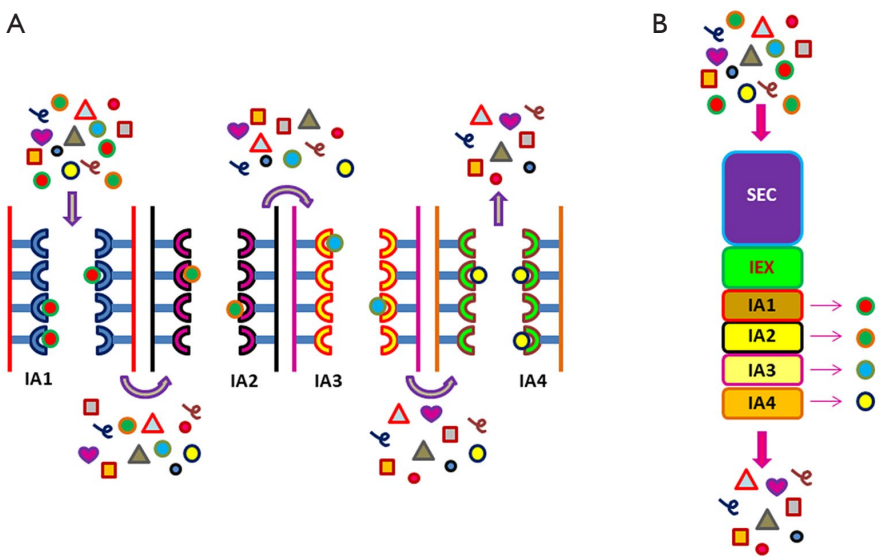

Figure 4 Multi-step chromatographic separation of sample components. (A) Antibody-based affinity capture of EV sub-populations. Antibody selective recognition of surface antigens enables the specific capture of vesicle sub-populations in separated chromatographic elements. A heterogeneous initial sample composed by both vesicles and molecular contaminants belonging to different biological classes will be uploaded on a column functionalized with antibodies specific for one single biomarker. This first step will enable to separate the vesicle class expressing that biomarker—that will bind to the column—from the vesicles negative for that biomarker that will pass through the column together with the contaminants. The process can be repeated by exploiting further antibodies, each time specific for a further single discriminatory antigen. This multi-step IA chromatography will purify sets of vesicle populations which differ for some surface biomarkers, a condition that indicate their different origin and probably will correspond to different molecular cargo content; (B) multi-step separation based on different chromatographic properties. An array of mini chromatographic units has the capacity of exploiting distinct chemical-physical properties to separate the sample components. For instance, an initial SEC can reduce the overall complexity and favor the binding of the EVs to an IEX unit. Once eluted, the EV sub-populations can be immobilized on and separately recovered from independent IA units functionalized with antibodies specific for exclusive biomarkers. IA, immunoaffinity.

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