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Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes

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ABSTRACT

Viable tumor cells actively release vesicles into the peripheral circulation and other biologic fluids, which exhibit proteins and RNAs characteristic of that cell. Our group demonstrated the presence of these extracellular vesicles of tumor origin within the peripheral circulation of cancer patients and proposed their utility for diagnosing the presence of tumors and monitoring their response to therapy in the 1970s. However, it has only been in the past 10 years that these vesicles have garnered interest based on the recognition that they serve as essential vehicles for intercellular communication, are key determinants of the immunosuppressive microenvironment observed in cancer and provide stability to tumor-derived components that can serve as diagnostic biomarkers. To date, the clinical utility of extracellular vesicles has been hampered by issues with nomenclature and methods of isolation. The term "exosomes" was introduced in 1981 to denote any nanometer-sized vesicles released outside the cell and to differentiate them from intracellular vesicles. Based on this original definition, we use "exosomes" as synonymous with "extracellular vesicles." While our original studies used ultracentrifugation to isolate these vesicles, we immediately became aware of the significant impact of the isolation method on the number, type, content and integrity of the vesicles isolated. In this review, we discuss and compare the most commonly utilized methods for purifying exosomes for post-isolation analyses. The exosomes derived from these approaches have been assessed for quantity and quality of specific RNA populations and specific marker proteins. These results suggest that, while each method purifies exosomal material, there are pros and cons of each and there are critical issues linked with centrifugation-based methods, including co-isolation of non-exosomal materials, damage to the vesicle's membrane structure and non-standardized parameters leading to qualitative and quantitative variability. The down-stream analyses of these resulting varying exosomes can yield misleading results and conclusions.

1. Introduction

Our group previously demonstrated the release of 50–200 nm membranous vesicles by tumor cells into their extracellular environment [1], which have been referred to as exosomes, microvesicles or extracellular vesicles depending on specific characteristics, including size, composition and biogenesis pathway. Since our original demonstration, the release of vesicles has since been demonstrated multiple cell types and systems. In cancer patients, these nanometer-sized vesicles released by tumor cells accumulate in biologic fluids, including blood, urine, ascites, and pleural fluids [2]. These cell-derived vesicles exhibit an array of proteins, lipids and nucleic acids derived from the originating tumor. These tumor-derived exosomes not only represent a central mediator of the tumor microenvironment, but their presence in the peripheral circulation may serve as a surrogate for tumor biopsies, enabling non-invasive diagnosis and real-time disease monitoring [3].

Although the release of exosomes occurs in other types of cells under specific physiological conditions, the increased release of vesicles and their accumulation appear to be important in the malignant transformation process. Recently, circulating vesicles from normal individuals, patients with benign ovarian disease and patients with ovarian cancer have been investigated using the Nanoparticle Tracking Analysis system (Nanosight) [4]. The presence of circulating vesicular materials was demonstrated in all individuals; however, ovarian cancer patients exhibit approximately 3–4-fold more vesicular material. In these cancer patients, the size range of these vesicles was between 50 and 250 nm, with the major peak at 98–99 nm (Fig. 1). The identification of specific

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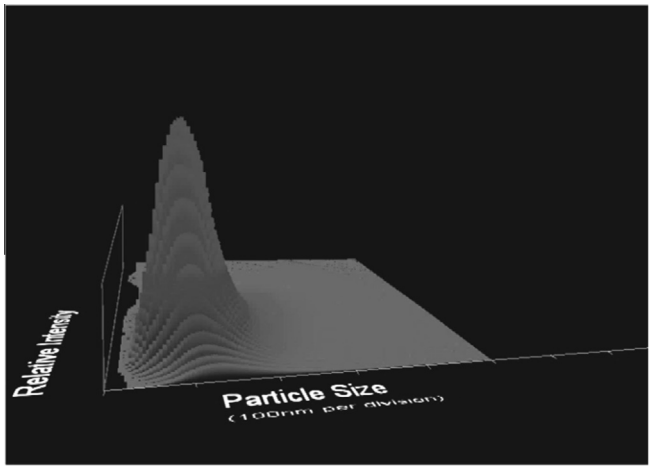

Particle Size / Relative Intensity 3D plot

Fig. 1. Distribution of total vesicles in serum of a patient with stage III adenocarcinoma of the ovary. Serum was centrifuged at 400×*g* for 10 min and this resulting supernatant was centrifuged at 15,000×*g* for 15 min. The supernatant was then diluted 1:4 in PBS and analyzed using a Nanosight NS300 in light scatter mode. The Nanoparticle tracking analysis software defined the number and size range of the vesicles within the sample, plotting the particle size versus relative intensity versus number.

tumor-derived vesicles using fluorescent-label antibody against tumor markers, indicate that, even in advanced stage patients, only approximately 10% of the total exosomes are tumor-derived; the remainder of the increased vesicles are likely the result of the host response to the tumor. Some of the enhanced exosome numbers derived from the host's response to the tumor appears to be derived from immune cells (Fig. 2).

These circulating vesicles have been identified by various terms, including high molecular weight complexes, membrane fragments, exosomes, microvesicles, microparticles, and extracellular vesicles, as well as by functional names. The term "exosome" was coined in 1981 for "exfoliated membrane vesicles with 5′-nucleotidase activity" [5]. This term, "exosome," originated from the discovery of neoplastic cell line-derived exfoliated vesicles, which mirrored the 5′-nucleotidase activity of the parent cells [5]. In ovarian cancer patients, these tumor-derived exosomes were found to express molecular markers that were linked with tumor plasma membranes, including placental type alkaline phosphatase and mdr-1 [6–8]; however, proteins not generally associated with plasma membranes, such as p53, GRP78 and nucleophosmin, have also been identified with these circulating vesicles [9,10]. These findings emphasize the aberrant sorting of components into exosomes in cancer and may differentiate cancer vesicles from their normal counterparts.

Several years after these early characterizations of exosomes from tumor cells, two groups studying maturation in cultured reticulocytes (sheep [11] and rat [12]) examined vesicles released via the canonical pathway upon multi-vesicular endosome fusion with the cell surface. The vesicles were isolated by ultracentrifugation and the pelleted vesicles were found to contain the transferrin receptor that was also found in native reticulocytes [13]. These reports proposed that this represented a mechanism for the elimination of certain cellular components as the reticulocytes matured and differentiated. These investigators "re-defined" these cell-derived vesicles as "exosomes" to differentiate them from "endosomes." The disparate natures of these studies are reflected in the various names that were proposed and which are still used to identify the cell surface-released and endocytic vesicles of different origins. It is of note that these reticulocyte studies (11–13) were exclusively *in vitro* and based on normal cell types, undergoing a specific differentiation pathway. Likewise, the characteristics currently used to define "microvesicles" were derived from studies on normal B cells *in vitro* and may not translate to vesicles derived from other cell types, particularly tumor cells [14]. While many investigators use these restrictive definitions for cell-derived vesicles without understanding their origin, significant overlap exists between structures identified as "exosomes" and "microvesicles," in terms of size, markers, cargoes and function, particularly in the context of transformed cells. Within the circulation, it may not be possible to differentiate 50–100 nm "exosomes" from 50 to 200 nm "microvesicles." Investigators have attempted to define exosomes versus microvesicles, based on size (30–100-nm lipid bilayer vesicles), density (1.12–1.19 g/ml) and expression of specific biomarkers (including tetraspanins) [15].

Using the Nanosight in fluorescent mode to analyze culture-derived tumor vesicles, we have demonstrated the presence of

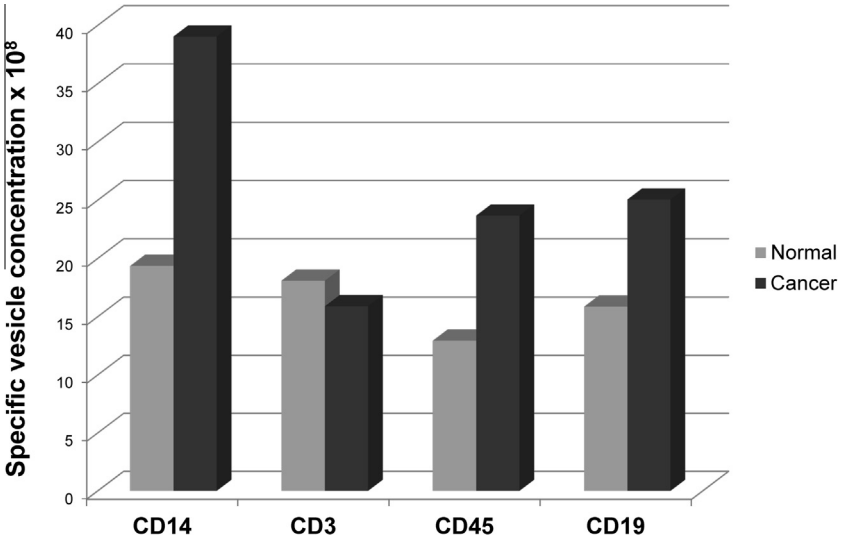

Fig. 2. Total exosomes were isolated from the serum of a normal female control and an ovarian cancer patient by size exclusion chromatography. Specific exosome populations from immune cells were isolated from each serum by immunoaffinity capture using immobilized antibodies against CD14, CD3, CD45, and CD19. The number of exosomes obtained with each antibody was determined using the Nanosight NS300 in light scatter mode.

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"exosome specific" markers on vesicles over the entire 50–250 nm size range, as well as the presence of markers defined as specific to "microvesicles" [4]. In many studies, uncharacterized cell-derived vesicles (in terms of markers or size) are termed "microvesicles," while numerous studies define "exosomes" solely based on density and the presence of the cell surface markers, tetraspanins. These overlaps in vesicle properties and terms suggest these distinctions are not clear-cut. Vesicles isolated from the extracellular environment of tumors (such as from the peripheral circulation), either *in vitro* or *in vivo*, exhibit overlapping similarities in size (defined by dynamic light scattering), morphology (defined by electron microscopy), density (defined by of sucrose gradient centrifugation), and protein markers of both the endosomes and plasma membranes (defined by western immunoblotting and mass spectrometry) [16–18]. While many of the definitions are still used, we now recognize their flaws. Multiple groups have demonstrated that the apparent size and shape of exosomes are artifacts of fixation and drying associated with electron microscopy (EM). Such artifacts have led to the development of new EM techniques, such as cryo-EM that provides a more accurate definition of size and shape [19]. The principal markers of exosomes are tetraspanins, which as plasma membrane associated components are present on most vesicles, regardless of their origin. Thus, it is clear that tetraspanins do not differentiate exosomes and microvesicles. The importance of the endocytic pathway of vesicle formation has also been questioned as knock-out studies with Rab proteins only diminished vesicle release by approximately 30% (based on exosomal protein) [20].

2. Historical isolation

During the 1970s with the development of monoclonal antibody technology, many investigators directed their work to the identification of new biomarkers for antigen-based immunoassays. Our original work focused on the identification of specific isoforms of placental-type alkaline phosphatase (PLAP) associated with ovarian cancer [1]. We defined the presence of heat-stable PLAP in the sera and ascites of patients with ovarian cancer and using size exclusion chromatography, we attempted to purify PLAP from the blood and ascites of these patients [21]. While the expected molecular weight of PLAP was approximately 70,000 Daltons, the enzyme activity appeared in the void volume of Sephadex G200 columns (molecular weight greater than 600,000 Daltons). Subsequent separations using high exclusion limit, agarose-based gels revealed a molecular weight between 100 and 150 million Daltons. Further analyses of a large group of female patients in a blinded study found that this high molecular weight PLAP was observed in all women with ovarian cancer and in women who were pregnant; however, the 100–150 million Dalton PLAP fractions were not observed in any other groups [21].

Released tumor-derived exosomes have been characterized for multiple human cancer types and they are not exact replicates of the plasma membrane or other membranous compartments of the originating tumor cells, but they represent "micromaps" with enhanced expression of tumor antigens, as well as other macromolecules, including major histocompatibility antigens [2]. Our initial electron micrographs of these membranous complexes revealed that they were vesicular [22]. Early analyses of these "high molecular weight" complexes with various extraction approaches, including butanol, indicated that they consisted of lipids and proteins. Using specific markers of intracellular membranous components, these complexes expressed components associated with the plasma membrane (5′-nucleotidase), but failed to exhibit nuclear or mitochondrial marker enzymes (including acid phosphatase and succinic dehydrogenase) [23]. This suggested that the presence of this membranous material was not simply the result of cell death. The membranous complexes isolated from cancer patients were also shown to possess markers associated with tumors. Further, analyses using metabolic inhibitors demonstrated that the release of these membranous structures was energy requiring, further indicating that the release of this material was an active cellular process and not merely the consequence of cell death.

3. Impact of isolation

With current mass spectrometry-based proteomic and amplified, ultra-high sensitivity RNA technologies, it is clear that exosomes are comprised of distinct subpopulations of macromolecules, including proteins and RNAs, associated with cell specific functions. Recent data indicate that these macromolecules can be transferred to target cells and can mediate intercellular interactions, non-classical protein secretion and signaling between neighboring cells resulting in pathologic conditions by becoming functional in their new microenvironment. While tumor-derived exosomes can induce events associated with the pathology of cancer, the renewed interest in exosomes has been based on their potential diagnostic utility. A group led by Mathivanan and Simpson [24] have established a compendium of exosomal proteins and RNA (initially ExoCarta and now updated to Vesiclepedia), since numerous studies have demonstrated the presence of tissue/cell type-specific proteins associated with exosomes. The current data from 358 studies have been cataloged in Vesiclepedia and currently includes 43,731 proteins, 20,796 mRNA, 2400 microRNA and 342 lipids. The presence of tissue/cell-specific marker proteins associated with specific exosome populations can serve as surrogates, identifying the presence of the originating cell. Similarly, we described microRNA (miRNA) associated with circulating exosomes derived from ovarian cancer patients and demonstrated their diagnostic use [25], which would extend its utility to screening asymptomatic individuals.

The various groups investigating exosomal components lack consensus on the methods for isolating exosomes from biologic fluids. These methods include ultracentrifugation, density gradient centrifugation, chromatography, filtration, polymer-based precipitation and immunoaffinity. Based on the high sensitivity of current molecular techniques, even minor components of exosomes can be detected and identified. Thus, the co-isolation of contaminating non-exosomal material can generate a significant artifact. In a similar fashion, the failure to completely isolate exosome fractions or the loss of exosomal materials due to damaged membrane integrity resulting from the isolation method can skew the exosomal protein and RNA profiles. Since exosomes from different sources can exhibit differences in protein/lipid compositions, greater intraluminal content or different degrees of non-specific component aggregation to their surface, these can exhibit distinct sedimentation properties.

4. Ultracentrifugation

Ultracentrifugation is generally regarded as the "gold standard" for isolating exosomes; however, comparisons of the results from the literature demonstrate inconsistencies in reproducibility of isolation data. Classically, in ultracentrifugation, a centrifugal force is applied to a mixture of macromolecules in solution, such that the more dense molecules sediment from the axis of the centrifuge compared to less dense components. This method involves centrifuging a biologic fluid at high *g*-force, 100,000×*g* or greater, to pellet the vesicles. The force that is applied to the sample is a function of the speed of the centrifuge rotor and the radius of the

centrifugation. During our initial isolation of circulating vesicles, differential centrifugation, based on methods developed for membrane protein isolation from homogenized tissues, was used [23]. This approach utilized multiple centrifugation steps (low, medium and high speeds). Initially, intact cells and large debris were removed by low speed centrifugation (400×*g*). The supernatant was then subjected to centrifugal forces, in the range of 10,000–20,000×*g* to remove large debris (and intact organelles). This supernatant is then subjected to centrifugation at high speed (100,000–150,000×*g*). This same differential centrifugation approach is currently applied to the isolation of exosomes. While the stated methodology is relatively straight-forward, the type, quantity and quality of the vesicles isolated by ultracentrifugation is highly sensitive to multiple parameters, including the *g* force, the rotor type (fixed angle or swinging bucket), the angle of rotor sedimentation, radius of the centrifugal force, pelleting efficiency (rotor and tube *k*-factors), and solution viscosity. It may not be possible to account and control all of these parameters.

Based on our initial studies, we identified "high molecular weight" forms of ovarian tumor antigens (in excess of 50 million Daltons). Due to their size, we attempted to sediment these high molecular weight antigens by ultracentrifugation prior to any separation from other blood components. However, an unexpected property of this exosome-associated PLAP present in sera and ascites of ovarian cancer patients was that only a small fraction sedimented with centrifugation at 150,000×*g* for 2 h [26]. Subsequently, we found that after the vesicular void volume was obtained by chromatography using a high exclusion limit Sepharose column, greater than 90% of the PLAP was pelleted at 150,000×*g* for 1 h. This may be a general phenomenon for any membrane vesicle in the presence of plasma/serum components [26]. Despite this early observation regarding the issues associated with solution viscosity on the sedimentation of vesicles, only recently has this issue re-surfaced [27]. Recent studies have confirmed that ultracentrifugation of exosomes is directly influenced by the viscosity of the biofluid, with plasma having the highest viscosity at 20 °C (1.65 centipoise, cP), followed by serum (1.4 cP), then culture media (1.1 cP) (PBS exhibits a viscosity of 1.0 cP) [27]. These viscosities of serum and plasma were in agreement with previously published data [29]. It is note-worthy that ultracentrifugation is never performed at 20 °C and solution viscosity is a function of temperature. This study defined the Pearson correlation for exosome recovery versus viscosity as −0.912 ($p < 0.001$), such that higher viscosity resulted in lower sedimentation efficiency [27]. This greater viscosity in biofluids (plasma and serum) appears to be related to the concentration of circulating proteins, resulting in high internal friction and more energy to sediment vesicles [28].

For the isolation of vesicles from biofluids, both swinging bucket (SW) and the fixed angle (FA) rotors have been used [29,30]. Since the tubes in SW rotors are held horizontal from the rotational axis during rotation, they have a longer sedimentation path length than FA rotors and exhibit in a lower pelleting efficiency. While SW rotors appear to yield better resolution of vesicles with similar sedimentation coefficients, FA rotors are more efficient at the separation of vesicles with distinct sedimentation coefficients [30]. Several recent studies have focused on the importance of the k factors for different rotors. Together with rotor type, the *k*-factor (clearing factor) for various rotors need to be defined and noted, since the *k*-factor is the relative pelleting efficiency of a specific centrifuge rotor at maximum rotation speed [30]. Since different studies use the same *g*-force and time to isolate exosomes, it is critical to note that different rotors with distinct *k*-factor have been used [30]. The difference in pelleting efficiency of rotors commonly used, run for the same time period, can result in drastically different yields of exosomes from the same sample [31]. Further while recent studies have focused on different rotors, the tubes used for these rotors can also affect the *k*-factor, in some cases producing 2–3-fold differences.

Results suggest that an unadjusted protocol for a rotor with a lower *k*-factor does not pellet exosomes with the same efficiency [27]. Therefore, it is essential that centrifugation times are adjusted to compensate for different rotor types. Further, one needs to factor in differences in the tube's *k*-factor [31]. Despite adjusting for these factors, when studies compared protein and RNA yields from duplicate samples, they discovered that the exosomes still failed to pellet with the same efficiency. Thus, these findings demonstrate that there are other differences between the rotors other than in *k*-factors and sedimentation times [31]. For the isolation of exosomes and their cargoes by ultracentrifugation, the exosomal yields do not follow mathematical prediction and thus, it may be impossible to standardize this approach.

Aside from these issues in sample viscosity and rotor and tube *k*-factor standardization, the centrifugation procedures that have been used to isolate circulating exosomes can involve up to five centrifugation steps, with at least two of these centrifugations requiring centrifugal forces in excess of 100,000×*g* for multiple hours. Numerous studies have indicated that ultracentrifugation can either result in the incomplete sedimentation of vesicles or the sedimentation of non-vesicular materials [31]. It is also unclear what effect pelleting a fluid membrane structure against a solid surface at high *g* forces for prolong time periods has on membrane integrity and vesicle content.

5. Density gradient centrifugation

Since exosomes have been traditionally characterized as having densities between 1.1 and 1.19 g/ml, the use of density gradient centrifugation has been extensively employed to "refine" the isolated vesicles. This method is based on ultracentrifugation in combination with sucrose density gradients (or synthetic matrix) or sucrose cushions to float the relatively low-density exosomes away from other vesicles and particles. The theory is that by removing non-vesicular particles that contaminate the vesicle preparations, the use of density gradients can introduce stringency [31]. To achieve this, density gradient centrifugation separates particles of different densities. The original assumption was that ultracentrifugation sediments exosomes, as well as other protein and/or protein–RNA aggregates; these contaminating materials can then be separated based on density.

This approach has been generally described as requiring a 16 h centrifugation time. However, in 2012, several studies using density gradient centrifugation demonstrated that certain vesicles must be centrifuged for 62–90 h to reach equilibrium density [32,33]. Thus, traditional density gradient centrifugation may not be adequate to sediment all populations of exosomes and can introduce an additional artifact. If the centrifugation time is not adequate, contaminating materials may remain in the same density fractions as the exosomes, particularly since this density range is broad. Finally, another common approach is to apply the vesicle pellet from ultracentrifugation to the top of the sucrose gradient [34,35] or a sucrose cushion [36] prior to centrifugation.

It has also been proposed that the use of density gradients can serve to separate the 50–100 nm exosomes from the "larger" microvesicles; however, we now recognize that the sizes of the vesicle types overlap. If vesicles exhibit similar compositions, their density does not change simply based on diameter, since the mass density or density of a material is defined as its mass per unit volume. Mathematically, density is defined as mass divided by volume:

$$\rho = \frac{m}{V}$$

where ρ is the density, m is the mass, and V is the volume. Since different classes of vesicles may have overlapping densities, current density-based procedures lack the stringency to achieve specific vesicle purification and may represent enrichment at most.

One additional aspect of density gradient centrifugation that is not generally addressed is the use of ultracentrifugation, whether the exosomes are initially pelleted and the resuspended pellet is applied to the gradient or after the gradient centrifugation to isolate and concentrate the exosomes. The use of ultracentrifugation to pellet the exosomes then introduces all of the issues raised in the above section.

6. Size exclusion chromatography

Size-exclusion chromatography (SEC) is a method where a solution of molecules is separated based on the component's size, not molecular weight; generally in the context of macromolecules, such as proteins and protein complexes. Grubisic et al. demonstrated a significant correlation between elution volume and the molecule's hydrodynamic radius [37]. This observed correlation based on hydrodynamic volume is the accepted basis of SEC calibration.

SEC is performed using heteroporous beads constructed of a neutral, cross-linked polymeric support, packed into a column. These beads consist of numerous pores or tunnels of varying sizes. Based on the hydrodynamic diameter of each molecule in the solution, they pass through these porous beads, analogous to a "maze." For molecules with smaller hydrodynamic radii, the more pores they can access will result in longer times required to move through the column of porous beads. In contrast, a component with a large hydrodynamic radius can access fewer pores and elutes earlier from the column. Thus, separation of macromolecules occurs by differential exclusion or inclusion of the macromolecules as they pass through the column.

These heteroporous bead resins are generally defined based on their capacity to separate hypothetical, globular proteins with various hydrodynamic radii [38]. The lower value of this range represents the radius below which all molecules are completely internalized within the volume of the beads. These small components will elute in a total volume representing the entire pore volume and the inter-particle volume. The upper value is the range where molecules with high hydrodynamic radii are entirely excluded from entering any pores of the beads, resulting in no separation. These hydrodynamically large molecules elute in a volume equal to only the inter-particle volume (approximately 35% of the column volume). There is a linear range (elution volume) between these two extremes at which separation of molecules with intermediate hydrodynamic radii occurs. The elution volume (Ve) decreases linearly with the log of hydrodynamic volume [38]. Since exosomes exhibit extremely large hydrodynamic radii, relative to individual proteins, lipoproteins, and protein aggregates, including immune complexes, they appear at the exclusion limit or void volume, defined as the upper end of the column "working" range. Since macromolecules, including exosomes, fail to exhibit a fixed size in all dimensions as they rotate in solution, this results in a probability that a molecule might skip a pore, which would be normally retard its passage through the beads, creating elution curves that resemble Gaussian distributions (Fig. 3) [38].

The advantages of SEC are that it results in clear separations of large molecules from the small molecules and various eluting solutions can be applied without interfering with the separation, all while conserving the integrity and biological activity of the molecules being separated [39]. High ionic strength buffers can be used to eliminate non-specific contamination from other blood-derived components. SEC is performed under low pressure, even by gravity flow, insuring that the vesicle structure and integrity is unaffected. SEC is scalable, such as increasing the column length will increase resolution and increasing column diameter enhances the capacity of the column, allowing the analyses of sample volumes from microliters to liters. For analytical separations, the sample volume needs to be 1/15 to 1/20 of the total volume (calculated as $\pi r2h$). However, this can result in dilution of the eluate and may require an additional concentration step. Using SEC, there are well-defined separation times and narrow bands, leading to excellent reproducibility and sensitivity. There is no loss of sample during SEC, since solutes do not interact with the stationary phase. The limited disadvantages are that only a small number of bands can be differentiated, due to the relationship to column volume versus sample volume and the time scale of the chromatogram. Generally, for optimal separation of components within a solution, components must exhibit a 10% difference in molecular mass for significant resolution. While the low pressure of SEC is advantageous, it also leads to long run times. The time to complete each run, including column set-up, elution, column washing, and re-equilibration, limits SEC's application for high-throughput separations and makes processing multiple samples difficult.

While SEC can distinctly separate most contaminating components in plasma or serum from the exosome fraction, it is possible that some chylomicrons may co-purify, since a small percent of these large lipoproteins fall in the 150–300 nm size range. However, previous analyses of SEC-isolated tumor-derived vesicles indicated that any such contamination is minor. Chylomicrons are low is protein, rich in triglycerides, and exhibit significant fluidity. These lipoproteins are only 1–2% protein, 85–88% triglycerides, 8% phospholipids and 1–3% cholesterol, which is inconsistent with the characteristics of SEC isolated exosomes. Lipid and protein analysis of SEC-isolated exosomes (compared with enriched plasma membranes) demonstrate they are high in protein, high in cholesterol (227 ± 40 μg/mg protein), low in phospholipids (669 ± 149nmol/mg protein) and exhibit a 10-fold increase in sphingomyelin/total phospholipid ratio (9.2 ± 2.8%) [40]. These alterations create a "rigid" vesicle as indicated by increases in fluorescence polarization (0.325 ± 0.008) and microviscosity (5.7) [40]. These compositions and rigidity of exosomes are inconsistent with those of any lipoprotein type.

We have compared the extracellular vesicle populations obtained from biologic fluids of ovarian cancer patients by both the ultracentrifugation technique and our original chromatographic method [41]. This comparative study demonstrated that these *in vivo* derived vesicles from both techniques isolated cup-shaped vesicles, with a density between 1.13 and 1.17 g/ml, a diameter between 50 and 150 nm, and expressing CD63, Alix, VPS35, galectin 3, HSP90, fibronectin, and placental alkaline phosphatase [41]. In a subsequent study comparing exosome isolation methods, we further described a more uniform vesicle size and superior recover of vesicle components (proteins and RNA) by chromatography versus centrifugation [42]. This advantage of the chromatographic approach versus centrifugation-based methods may relate to the fact that vesicles are not subjected to shearing force that can damage the vesicle integrity.

7. Filtration

The isolation of exosomes based on their size can be achieved by the use of ultrafiltration membranes. Based on their size, exosomes can be separated from other soluble protein and aggregates using matrices with defined molecular weight or size exclusion limits. These vesicles can, for example, be selectively isolated based on a molecular weight greater 2 million Daltons, followed by isolation with a diameter less than 200 nm. This allows the separation

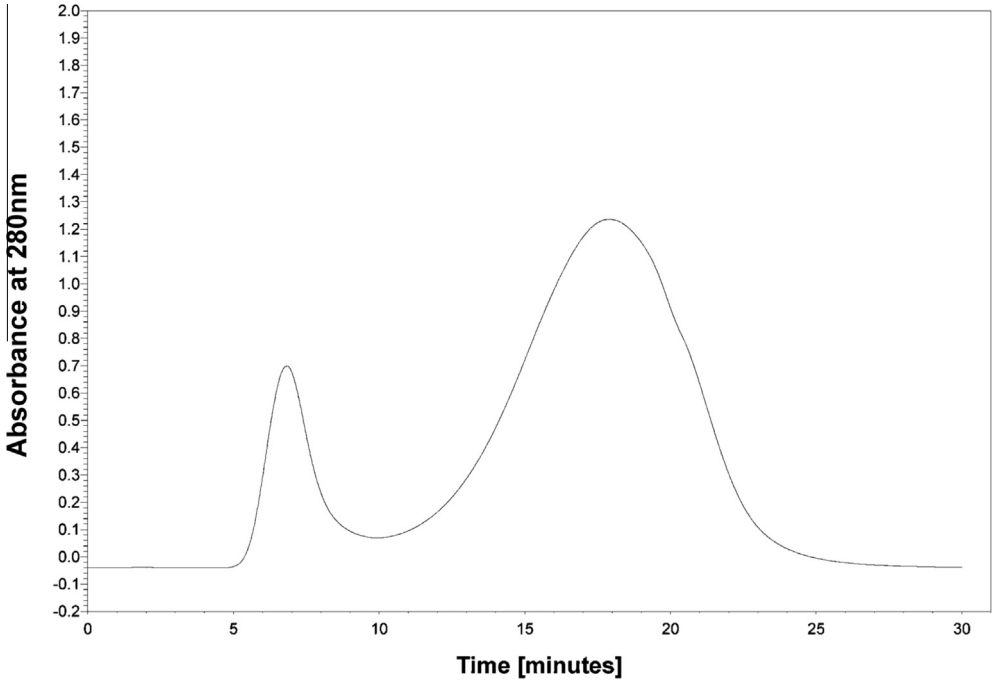

Fig. 3. Chromatogram of an ovarian cancer patient's serum separated using size exclusion chromatography using Sepharose 2B. The elution was performed at a flow rate of 1 ml/min, collecting 1 min fractions and monitoring the elution at 280 nm. The void volume, containing exosomes, appears in the initial peak.

of smaller aggregates and soluble components from exosomes. The use of filtration membranes with specific pore sizes can provide for vesicles larger than the specified size being excluded. Commonly used filters can have pore sizes of 0.8 μm, 0.45 μm or 0.22 μm, where vesicles greater than 800 nm, 450 nm or 200 nm are retained, respectively. After the larger vesicle populations are removed, a second filtration step can be included from the flow-through to remove components less than a specified size, while the specific vesicle population is retained and concentrated by the membrane. There are several issues with filtration methods. First, while some ultrafiltration membranes are constructed of "low protein" binding materials, exosomes can still adhere to the membranes and become lost for down-stream analyses. This filtration does also not completely remove smaller components. Second, in order to pass materials through filtration membrane pores, force is applied above (pressure or centrifugal force) or below (vacuum) to move materials through the membrane. As the materials in the solution become more concentrated, additional force is necessary and membrane pores may become blocked by the filtered materials. To date the consequences of this force on vesicle deformation and potential disruption of vesicles is unknown.

8. Polymer-based precipitation

While the use of polymer solutions to isolate exosomes is relatively new, its use to isolate viruses and other macromolecules has existed for more than 50 years. The use of the polymer, polyethylene glycol (PEG), to precipitate viruses was reported by Hebert to concentrate plant viruses, using a solution of PEG and sodium chloride [43]. This approach has subsequently been used to isolate bacterial viruses and animal viruses. Studies have demonstrated quantitative recoveries of even labile viruses, such as Epstein Barr virus at greater than 65%. The advantages of PEG precipitation is the gentle effect on particles, the ability to precipitate at neutral pH and high ionic concentrations, and the absence of other organic materials. Further careful manipulation of the PEG concentration can be utilized to precipitate particles within narrow size sizes. The use of PEG precipitation has been demonstrated to be compatible with transmission EM and cloning/sequencing for molecular analyses. A study by Colombet et al. compared PEG precipitation for viruses with ultracentrifugation and found that the PEG protocol resulted, on average, the recovery of 2-fold more viruses [44]. These findings further emphasize the diminished recoveries of nanometer sized particles, compared with other methods.

For the application of PEG solutions to isolate exosomes from biofluids, several commercial products have been developed; the most commonly used and known is ExoQuick from System Biosciences. The use of these commercial products is technically easy, does not require specialized equipment and can be rapidly performed. For isolating exosomes, the polymer-based precipitation method generally consist of combining the biofluid with a precipitation solution, containing polyethylene glycol (PEG) with an average molecular weight of 8000 Da, incubating this mixture overnight at 4 °C and centrifuging the mixture to form a pellet at low speed. The precipitating solution is generally a 50% by weight solution of PEG (concentrations ranging from 30% to 50% PEG can also be used). This PEG precipitation solution is generally prepared in a phosphate buffered saline (PBS) solution, although the PBS is not absolutely required. PBS is isotonic and physiologically compatible, and maintains pH and osmolality near physiological levels.

Despite the advantages of polymer-based approaches, there are two issues [42]. First, most studies have demonstrated that polymer-based precipitation methods co-isolate non-vesicular contaminants, including lipoproteins. Second, once isolated, the presence of the polymer material may not be compatible with down-stream analyses. While the use of polymer-based precipitation may be appropriate following an initial enrichment of exosomes, the presence of contaminating non-exosomal materials can be problematic. Multiple studies have demonstrated that polymer-based precipitation produces high yields of circulating proteins and RNAs; however, many of these proteins and RNAs are not exosomal. These issues have led to the development of pre- and post-isolation steps. The pre-isolation step is associated

with the removal of subcellular particles from the plasma or serum prior to precipitation. Lipoproteins, high-density lipoproteins (HDLs) and low-density–density lipoproteins (LDLs), are the major lipid-based particles in the plasma and serum and both contain multiple RNA populations [45]. For post-isolation removal of the polymer, use of Sephadex G-25 spin columns has been incorporated. Based on the 8000 molecular weight range of PEG, the G25 retains the polymer, while the exosomes are excluded in the void volume.

9. Immunoaffinity capture

Immobilized antibodies recognizing specific antigens can be used to isolate vesicles from biofluids. The most commonly used targets are tetraspanins, which allows the isolation of exosomes; however, this approach is limited to the isolation of total exosomes. These antibodies can be covalently attached to plates, beads, filters, or other matrices [46,47]. The theory on which immunoaffinity capture is based is that characteristic surface proteins are present on specific vesicles [48,49]. The approach is analogous is the immunoselection used to isolate ‘‘circulating tumor cells.’’ In this approach, antibodies to surface proteins bind specific targeted exosome populations (positive selection) or bind and remove specific irrelevant exosomes (negative selection).

In a study by Tauro et al. [50], exosomes that were purified from biological fluids and *in vitro* cell cultures were compared between those isolated by ultracentrifugation, density gradient centrifugation and immunoaffinity, since centrifugal approaches invariably exhibit proportions of other membranous vesicles that co-purify with exosomes. These investigators used conditioned media from a colorectal cancer cell line to performed ultracentrifugation, OptiPrep™ density-based separation, and immunoaffinity capture using anti-EpCAM coated magnetic beads (IAC) for exosome isolation. For each technique, the investigators demonstrated that all isolates contained 40–100 nm vesicles based on transmission electron microscopy and were positive for exosome markers (Alix, TSG101, HSP70) based on Western blotting. However, their proteomic analyses revealed that based on the number of MS/MS spectra identified for exosome markers, immunoaffinity capture was determined to be the most effective method to isolate exosomes. Further, analyzing known exosome markers, Alix, TSG101, CD9 and CD81, they demonstrated that using immunoaffinity capture resulted in a significantly higher level (at least 2-fold), compared to ultracentrifugation and density gradient centrifugation.

Since we were investigating cancers of epithelial origin, our initial immunoaffinity approach was based on anti-EpCAM. Based on our previous work, we are aware that antibody binding to exosomal antigens is distinct from binding their counterparts expressed on cells [21]. This may relate to differences in glycosylation of exosomal proteins or other aberrant posttranslational modifications or differences in lipid/protein ratios of the membranes of exosomes versus cells, resulting in differential exposure of antigenic epitopes on integral membrane proteins or changes in protein motility due to the rigidity of exosomal membranes compared to cellular membranes. These may effectively reduce the binding of exosomal target proteins with antibodies linked to beads and reducing the levels of specific exosomes isolated. Other investigators have utilized other surface markers or exosome markers to isolate exosomes from biologic fluids. Selection for A33-positive exosomes has been reported for isolating exosomes from colorectal cancer patients [49,50]. Other investigators have also used anti-CD63 for immunoaffinity selection of circulating exosomes [51]; however, as a general exosome marker, the use of CD63 isolates all circulating exosomes, cancerous and noncancerous.

While currently used to isolate total exosome populations, immunoaffinity has the ability to isolate specific targets with high specificity [52], which is critical for the characterization and specific isolation of unique exosome populations. There are disadvantages to this approach. First, an appropriate surface target must be identified and an antibody recognizing the extracellular domain must be available. Second, due to tumor heterogeneity, it is possible that not all cells within a tumor may express the target antigen, such that exosomes derived from those cells will not be selected. Further, as tumors progress, there can be antigen modulations, such that a tumor may initially express the target antigen; however, over time the expression of that antigen may be lost. Third, the antigenic epitope may be blocked or masked. While exosomal expression of tetraspanins can be detected using Western blotting, in some cases, fluorescent analyses with intact exosomes fail to demonstrate tetraspanins. Thus, for optimal isolation of specific exosomes, it is essential to target multiple antigens, such that loss of one will not prevent immunoaffinity capture.

10. Considerations for with common methods

It is essential to recognize that ultracentrifugation, density gradient centrifugation, SEC and polymer-based precipitation do not preferentially isolate tumor-derived exosomes. These are general approaches that isolate all circulating vesicle populations. While immunoaffinity capture has the capacity to target specific populations of exosomes, most current affinity approaches use general exosome markers for targeting. Several groups, including ours, have used anti-EpCAM to target exosomes derived from epithelial tumor cells. Since most cells normally within or in contact with the vasculature do not express EpCAM, the presence of circulating EpCAM positive exosomes would be expected to be limited to tumors.

During the past three decades, we have evaluated different approaches for exosome isolation from blood and other biofluids that would allow accurate and reliable isolation of intact exosomal proteins for proteomic analyses, while also retaining their biologic activities. For isolation of total exosomal protein for further analyses, the polymer-based precipitation yielded an increased concentration of total protein; however, not of the tumor-specific marker. The use of size exclusion chromatography to isolate circulating exosomes resulted in the second highest concentration of exosomal protein. For the analysis of tumor-specific exosomal proteins, we have examined the level of the specific membrane protein marker, placenta-type alkaline phosphatase (PLAP) [42]. Our analysis indicated that, when standardized to volume of the initial sample, immunoaffinity capture using anti-EpCAM beads isolated exosomes exhibiting the highest level of the tumor marker [42]. The next highest level of PLAP was associated with exosomes isolated by chromatography, with ultracentrifugation exhibiting the least recovery of PLAP. In a similar manner, the use of anti-EpCAM beads to isolate exosomal RNA resulted in yields similar to chromatography [42]. Ultracentrifugation produced the lowest ratios for RNA integrity, potentially due to co-sedimentation of protein impurities [42].

11. Summary

Released cellular vesicles have major roles in the pathogenesis of diseases. A critical role of exosomes is the signaling mediated by specific interactions between tumors and their target cells. The release of exosomes is essential for events, including the horizontal transfer of genes and gene products, expanding the boundaries of the cell into the extracellular space and to other cells specifically targeted. Knowledge of the molecular specificity

of the vesicles of different cellular origins will be instrumental in the identification of their pathogenic roles and to their potential use as diagnostic tools. View Within ArticleEarly detection of cancers is usually important to improve the survival rate of cancer patients. This assumes that cancer biomarkers are known, identified and reliable. Even though the number of potential biomarkers identified for cancers is increasing, the reality is that the actual number of markers used in clinic is limited due to the problems encounters during the validation step of those biomarkers. Usually diagnosis and monitoring of a variety of solid tumors requires invasive tissue biopsies; exosomes isolated from simple blood draws or ascites fluids collection offers an alternative route. However there is still a need in identifying and validating reliable exosome-derived biomarkers to use in diagnosis, prognosis, and treatment of cancer.

The key to reliable exosomal biomarkers lies in their optimal isolation. Based on current data, while ultracentrifugation is the "gold standard" isolation method, it is problematic. The type, quantity and quality of the vesicles isolated by ultracentrifugation is extremely sensitive to parameters, such as *g* force, rotor type, angle of rotor sedimentation, radius of centrifugal force, pelleting efficiency (rotor and tube *k*-factors), solution viscosity and vesicle density. It is not possible to account for, control and standardize all of these parameters. Aside from these variables, the consequence of pelleting exosomes at high g factors against a solid support for long time periods on vesicle integrity is unclear. However, while EM studies of vesicles isolated by ultracentrifugation demonstrate the continued presence of vesicles, they generally exhibit the appearance of extensive debris and diminished recovery of vesicles (in terms of quantity and quality). Most evidence clearly indicates a significant failure to recover exosomal protein and RNA following ultracentrifugation and density gradient centrifugation [27,29,30,42,49,50]. SEC appears to be a better alternative for exosome isolation, since there is no loss of exosomes or no damage to the vesicle structure. However, like the centrifugation approaches, total exosomes are isolated by this approach. Most studies support the finding that immunoaffinity capture can isolate both total exosomes and pathology-specific exosomes, while maintaining vesicle integrity and cargo content [42,49,50]. Since all of these isolation approaches have limitations, new technologies are being developed. While still being developed and not commonly available, a promising technology is field flow fractionation (FFF) [53]. FFF is based on laminar flow of particles in a solution, where a mixture of particles is propelled through a channel, perpendicular to the direction of flow, resulting in separation of the particles present in the suspension. Like SEC, FFF separation is dependent on their hydrodynamic diameters; however, this method is unique from other separation methods, since it can separate components over a wide colloidal size range.

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