

## **Extracellular vesicle isolation and characterization: toward clinical application**

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### **Review Series**

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# Extracellular vesicle isolation and characterization: toward clinical application

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## Introduction

Scientific and clinical interest in extracellular vesicles (EVs) has increased rapidly as evidence mounts that they may constitute a new signaling paradigm (1). EVs are secreted by most cells and carry diverse cargoes including proteins, RNA species (including mRNA, miRNA, lncRNA, and other RNA species), DNAs (mtDNA, ssDNA, dsDNA), and lipids that can be transported and exchanged between cells as a means of intercellular communication at both paracrine and systemic levels (2–7). It is clear that EVs carry preassembled complex biological information that elicits pleiotropic responses in recipient cells. Such responses underpin EV participation in the maintenance of normal and pathophysiological conditions (4, 8), including cancer (9, 10), neurodegenerative diseases (11), rheumatic diseases (12), and infectious diseases (13–19). For a comprehensive review of our current understanding of the role of EVs in normal physiology, including reproduction, embryonic development, tissue repair, bone calcification, and the nervous system, see Yáñez-Mó and colleagues (20).

Due to their bioactive cargoes, EVs have innate therapeutic potential in diverse areas, such as cell-free cancer immunotherapy (21, 22) and regenerative medicine (23, 24). In light of their intercellular communication capability, it is not surprising that naturally derived EVs are also being exploited for the delivery of exogenous therapeutic reagents, such as small molecule antiinflammatory drugs (e.g., curcumin to activated monocytes; ref. 25), macromolecular drugs such as siRNA (26, 27), and vaccine-like tumor-associated antigens for presentation in the immune system (28, 29). For a review of EV application to cancer vaccines see Tan et al. (30). EVs also provide an as yet largely untapped source of diagnostic, prognostic, and predictive biomarkers (10, 12, 31). For a summary of recent clinical and preclinical investigations of EV-based therapies, see refs. 32 and 33.

Here, we will discuss several fundamental issues in the field of EV research, including how many EV subtypes occur naturally, whether they differ depending on cellular origin and physiological state, and how they differ with respect to biochemical properties and functional activity. Strategies for purifying EVs will be presented, with an emphasis on proteomic profiling as a means of categorizing EV subtypes.

## How many EV subtypes are there?

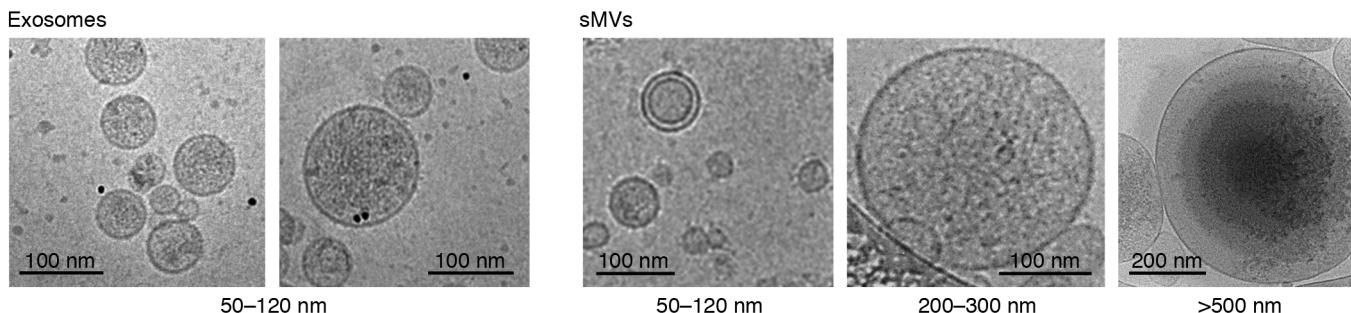
It is well recognized that cells release essentially two EV subtypes that can be readily separated by differential centrifugation (DC): the larger size class (referred to as “microvesicles” or “shed microvesicles” [sMVs]) are heterogeneous (50 to ~1,500 nm) and sediment at approximately 10 to 14,000 g, while the smaller size class (referred to as “exosomes”) are relatively homogeneous in size (50–120 nm) and sediment at approximately 100,000 g (refs. 34–37 and Figure 1). Other vesicle types, such as apoptotic bodies (50–2000 nm) that are released by cells undergoing apoptosis (38), blood-derived vesicles (130–500 nm) that are released upon platelet activation (“platelet dust”) (39–41), and autophagosomes, (42) will not be covered in this review.

Vesicle annotation is an ongoing problem that has plagued the field over the past decade with varying names describing the two EV subtypes — this polemic has led to international efforts to standardize nomenclature and the quest for specific protein markers to distinguish one EV subtype from another (ref. 43 and *Journal of Extracellular Vesicles* [<http://www.journalofextracellularvesicles.net/>]). For position papers on standardization procedures for EV purification and minimal requirements for EV definition and function, see Witwer et al. (44) and Lotvall et al. (45), respectively.

It has been proposed that sMVs and exosomes arise from different biogenesis mechanisms, with sMVs directly budding from plasma membranes, while exosomes have endocytic origins and are formed as intraluminal vesicles (ILVs) by inward budding of the limiting membrane of late endosomes or multivesicular bodies (MVBs). MVBs subsequently fuse with the plasma membrane and release their sequestered ILV contents

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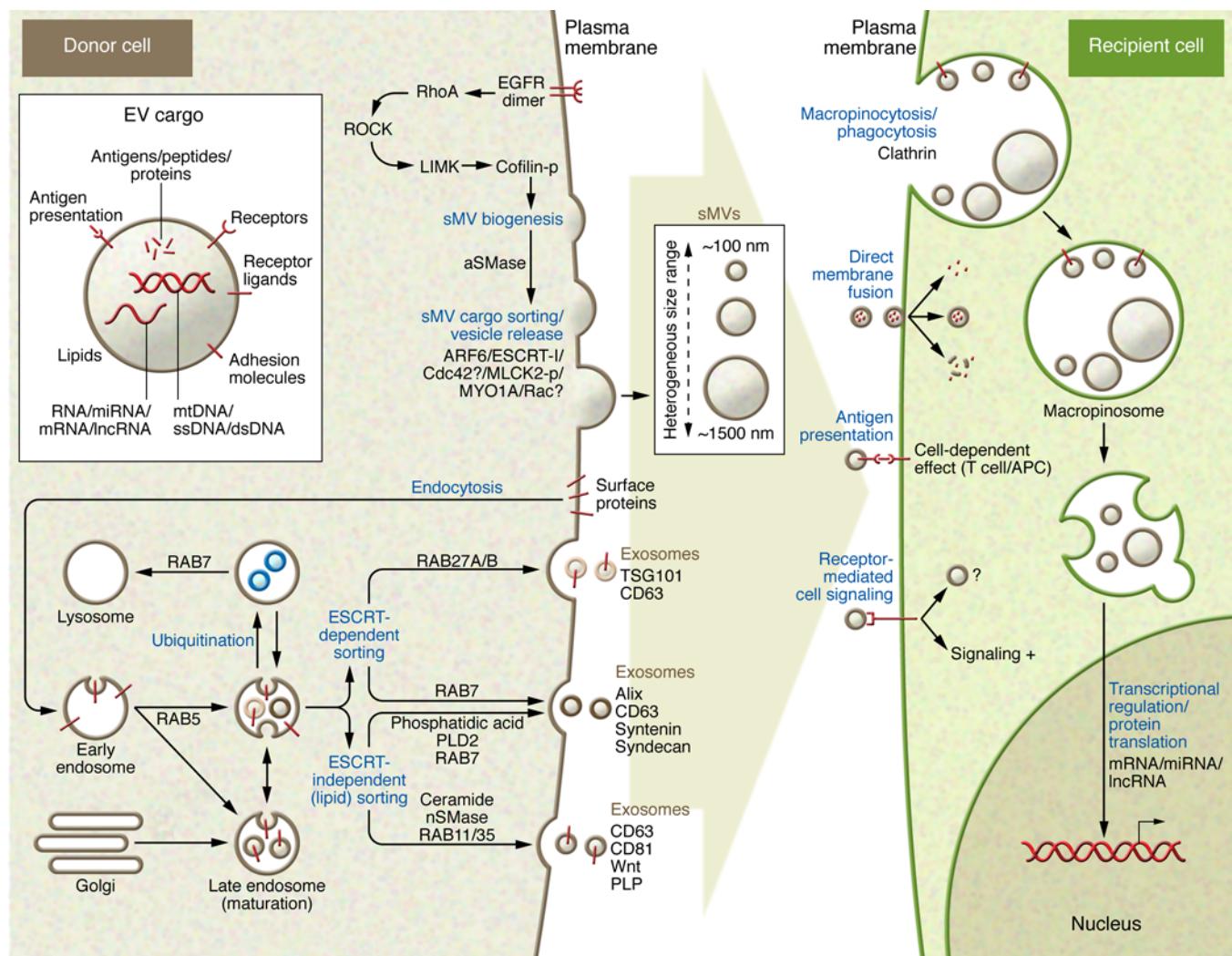


**Figure 1. Characterization of distinct EV subtypes by cryoelectron microscopy.** Electron micrographs of EV subtypes released into human colon cancer cell line LIM1863 cell-culture media. EVs were harvested from cell-culture media by sequential centrifugal ultracentrifugation, as described (37). EVs range in diameter from small (50–120 nm) (exosomes and sMVs) to intermediate (~200–300 nm) (sMVs) and large (>500 nm) vesicles (sMVs).

as exosomes into the extracellular environment (Figure 2). Accruing literature reveals that the ubiquitous release of EVs by tumor cells and plasma tumor-associated EV levels in disease patients increase with cell invasiveness or disease progression (e.g., ovarian carcinoma, ref. 46; uveal melanoma, ref. 47; breast cancer, refs. 48, 49). For some time it has been known that the endosomal sorting complexes required for transport (ESCRTs), first discovered and named for their role in sorting membrane proteins from endosomes to lysosomes (50), make up the major machinery for MVB/ILV (and exosome) biogenesis (51). In addition to ESCRT-driven MVB/ILV formation (52, 53), it now appears that ESCRT-independent MVB/ILV formation may also occur. These ESCRT-independent mechanisms involve lipid-metabolizing enzymes such as neutral sphingomyelinase (nSMase), which hydrolyzes sphingomyelin to ceramide (54), and phospholipase D2 (PLD2), which hydrolyzes phosphatidylcholine into phosphatidic acid (55). ADP-ribosylation factor 6 (ARF6) and its effector, PLD2, are reported to regulate syntenin/Alix-driven exosome biogenesis, while ARF6-dependent activation of PLD2 also enables plasma membrane blebbing and release of sMVs (34, 55). While formation of MVBs and their intrinsic ILVs appears to involve multiple ESCRT-dependent and -independent molecular machineries, it is not clear whether these mechanisms are cell type dependent and whether they act simultaneously on the same or different MVBs within a given cell type (for excellent reviews of EV biogenesis mechanisms, see refs. 5, 51, 52). Evidence accumulated over the past 5 years suggests that ESCRTs not only play a central role in exosome biogenesis, but also regulate biogenesis of sMVs (51). However, mechanistic differences between ESCRT-dependent biogenesis of exosomes and sMVs are yet to be clearly defined. In the case of sMV biogenesis, the RhoA/Rho-associated kinase-dependent (ROCK-dependent) signaling pathway appears to be specifically involved in sMV formation in cancer cells. Interestingly, Cerione and colleagues show that attenuation of exosome release in highly aggressive MDAMB231 breast cancer cells by ectopic expression of dominant-negative mutant of the CHMP3 protein (the mammalian homolog of yeast VPS24 protein, which is essential for exosome secretion) does not impair the amount of sMV release (56). Likewise, processes that control sorting of specific molecules (protein and RNA species) into different EV subtypes, target cell recognition, and EV uptake are not well

understood. While many molecules have been implicated in selective packaging into EVs of different proteins (e.g., ARF6, refs. 57, 58; arrestin domain-containing protein 1 [ARRDC1]) (59), posttranslational modifications (60, 61), and RNA species (62–64), the sorting mechanisms await further definition (for reviews, see ref. 65). A key issue hindering progress in understanding these key areas of EV biology has been the technical challenge of isolating homogeneous EV subpopulations suitable for molecular analysis.

While it is generally acknowledged that there are two broad categories of EVs, emerging evidence suggests that the exosome class contains subpopulations. For example, we found two distinct populations of exosomes released from the human colon carcinoma cell line LIM1863 grown in culture (66), one positive for the exosome surface marker A33 glycoprotein, the other positive for epithelial cell adhesion molecule (EpCAM). These exosome subpopulations were isolated from cell culture using sequential immunocapture with anti-A33 and anti-EpCAM mAbs coupled to magnetic beads. The two populations are identical in size and morphological properties based on electron microscopic analysis, and both contain stereotypical exosome marker proteins TSG101, Alix/PDCD6IP, and HSP70. However, gel electrophoresis followed by liquid chromatography-tandem mass spectroscopy (GeLC-MS/MS) analysis revealed that each exosome population has a distinct protein profile consistent with release from either apical (EpCAM-Exos) or basolateral (A33-Exos) surfaces of these highly polarized cells (66). Further, both exosome protein profiles were clearly distinguishable from those of sMVs isolated from the same cell culture. In a follow-up study using the same exosome and sMV preparations, we showed that these EV subtypes also have distinct miRNA-enrichment signatures, suggesting that these miRNA cargoes are biologically significant (67). These findings raise questions as to the nature of the underlying mechanisms responsible for selective packaging of exosome cargo. In another study, Ogawa and colleagues report two types of exosomes in human whole saliva that vary in size (20–80 nm and 30–250 nm) and morphology and contain stereotypical markers (TSG101, Alix, HSP70), but have different protein compositions (68). It is interesting that in both examples, two distinct exosomal populations were isolated in highly polarized cells (colon tumor line or oral mucosa). While tantalizing, the biological significance of these findings awaits further investigation.



**Figure 2. Proposed molecular machineries involved in EV (exosome/sMVs) secretion and recipient cell uptake.** Components of donor cells are incorporated into EVs (exosomes, sMVs) that contain diverse cargoes, such as signaling proteins, transcriptional regulators, various RNA species, DNA, and lipids. Multiple machineries are thought to be involved in exosome biogenesis of ILVs and MVBs. ESCRT components are involved in an ESCRT-dependent intracellular pathway that traffics MVBs and their ILV contents (exosomes) out of the cell, while other ESCRT-independent pathways (lipid dependent) have also been described. Members of the Rab GTPase family have been shown to modulate exosome secretion and are thought to act on different MVBs along ESCRT-dependent and -independent endocytic pathways. It is not known whether each of these pathways acts on different MVBs or on the same MVB concomitantly or how exosome/ILV-loaded MVBs differ from those destined for lysosomes. Even less is known about the molecular machinery regulating outward budding of the plasma membrane and sMV release, apart from a requirement of ARF6, acid sphingomyelinase activity, and some ESCRT components (ESCRT-I). EVs may interact with recipient cells by direct signaling through ligand/receptor molecules on their respective surfaces or by their uptake via direct fusion of EV and recipient cell plasma membrane (110, 111) through lipid raft-, clathrin-, and calveolae-dependent endocytosis, macropinocytosis, and phagocytosis (112–117). EVs can be involved in antigen presentation and in the transfer of both MHC molecules and antigens, thereby participating in immune regulation (118–120). APC, antigen-presenting cell.

## Importance of working with highly purified EV populations

As discussed above, EVs are very likely to be carriers of information between cells as well as having great promise as specific pharmaceutical targeting vehicles and as sources of diagnostic and prognostic markers. However, studies to elucidate these roles can be confounded by the presence of EV subtypes with different mechanisms of biogenesis, organelle origin, and constituent makeup. It is therefore clear that functionality and diagnostic/therapeutic uses can only be truly defined once the range of EV subpopulations from a given source are fully described and isolated for complete analy-

sis of constituent molecules. If functional differences between EV subtypes do indeed exist, it is unclear how this might affect possible side effects if impure samples are used in a clinical setting. For bi-distribution and biokinetic studies, highly purified vesicle populations are an absolute necessity. Thus, for the field to progress and live up to expectations, there is an imperative to overcome the technical challenges associated with purifying EVs to homogeneity as well as develop procedures for their accurate quantification.

There is a large body of literature describing protocols for purifying EVs (2, 37, 69–71), assessing their purity (72), and identifying shortcomings (71); for a comparison of yields of protein

**Table 1. Commonly used methods of isolating EVs**

Method	Detail/references	EV yield	Purity	Scalability
DC	Used to isolate crude EV/exosome mixtures from conditioned media (CM) by DC at ~100,000 g after cell debris/intact cells are removed by low- <i>g</i> force centrifugation steps (~500 g/2,000 g). After an initial low- <i>g</i> force spin (10–14,000 g) after cell debris removal, crude sMVs can be isolated from the CM (34, 36, 66); purified exosomes can be subsequently harvested from the resultant sMV-depleted CM by centrifugation at 100,000 g. This approach does not separate EVs from possible high M <sub>r</sub> protein oligomers/protein-RNA complexes and viruses (35, 44).	Medium (high molecular mass protein complexes, issues with nonselectivity and sample heterogeneity)	Low	Medium (μl-l)
DGC	Fractionates EVs on the basis of buoyant density using a discontinuous gradient of a sucrose solution or less-viscous iodixol, OptiPrep. Generally results in reduced levels of nonvesicular protein contaminants (35, 72). DGC does not affect complete separation of sMVs and exosomes whose buoyant densities overlap (35, 70).	Medium (sample loss during fractionation)	High	Low (μl-ml)
Sucrose cushion centrifugation	This method is a variation on sucrose DGC (69), typically used for morphological analysis of EVs (e.g., electron microscopy) because it minimizes mechanical stress encountered in DC methods.	Medium (sample loss during fractionation, exosomes, and viral particles of similar densities copurify)	High	Medium (μl-ml)
HPLC gel permeation chromatography	A well-established, high-yield method for purifying functional EVs for tissue regeneration studies (121). Requires specialized equipment and is time consuming, but the general principles can be applied to simple, readily made chromatography columns operated under gravity or by inexpensive pumps. Widely used for isolating EVs from plasma samples (90) and has been adapted for high-throughput clinical samples (95). This method overcomes many of the problems associated with EV isolation from plasma/serum using DC/DGC – e.g., coisolation of EVs with large M <sub>r</sub> protein aggregates and lipoproteins (90, 94, 95).	Medium (loss of small size exosomes)	Medium	High (ml-l)
AC	AC methods for isolating EVs rely on an affinity tag (mAb that targets an EV surface antigen, biospecific peptide [ref. 122], or proteoglycan affinity reagents [refs. 76, 77, 123]) covalently fused to either magnetic or agarose beads. mAbs that have been successfully employed as bait are described in refs. 35, 70, 102, and 124–132. AC was reported to be more effective than DG and DGC for isolating EVs from LIM1863 cells (35).	Low (Ab selection/availability dependent)	High	Low (μl-ml)
Microfluidic devices	Three categories of microfluidic devices: (a) trapping exosomes with an immune-affinity approach (Exochip, iIMER, μNMR, nPLEX); (b) sieving (nanoporous membranes); (c) trapping exosomes on porous structures (nanowire on micropillars) (133, 134).	Medium	Low	Low (μl)
Synthetic polymer-based precipitation	Several synthetic water-soluble polymers, commonly used as protein/virus/particle precipitants, are now used to rapidly isolate crude EV mixtures from CM and biofluids (135–137). These methods afford a rapid EV isolation/concentration step for the purpose of diagnostic assay of known EV-associated biomarkers.	High (coisolation of nonvesicular contaminants, ribonucleoprotein, and lipoprotein complexes)	Low	Low (μl-ml)
Membrane filtration	Typical commercial devices for this method include stirred ultrafiltration cells (operated under N <sub>2</sub> pressure and containing a magnetic stir bar for mixing) and ultrafiltration spin columns/tubes operated using low centrifugal force. Nanomembrane ultrafiltration spin devices, equipped with low protein-binding membranes, have recently been applied in clinical laboratories for the isolation of EVs from multiple, low-volume, urinary (138, 139), and blood plasma samples (140). This approach has been modified to enable the fractionation of highly purified sMVs and exosomes from the same CM of colon carcinoma LIM1863 cells (37).	Low (sample loss during fractionation, choice of filter units [e.g., vertical versus tangential] for fractionation important)	High	Medium (μl-ml)

and RNA species using different isolation procedures see Alvarez et al. (44, 73). Isolation strategies typically used, along with approximate yields and scalability, listed in Table 1, include DC, density-gradient centrifugation (DGC), sucrose cushion centrifugation, gel-permeation chromatography (GPC), affinity capture (AC), microfluidic devices, synthetic polymer-based precipitation, and membrane filtration. It is obvious from this long list that there are varying methodologies for purifying (enriching) EVs,

but, as yet, no gold standard method that would allow researchers to conduct interlaboratory comparisons. In an effort to evaluate commonly used methods for isolating exosomes, Tauro and colleagues compared DC, DGC, and AC procedures using colorectal cancer cell line LIM1863 culture supernatant as source material (35, 70). Using a mass spectrometry approach to profile protein compositions and label-free spectral counting (4, 66, 74) to evaluate the effectiveness of each method, AC using magnetic

**Table 2. Methods for quantifying EVs**

Method	Equipment	Analysis and range	EVs analyzed	Major advantages	Major limitations	References
Nanoparticle tracking analysis (NTA)	LM20, LM10, LM14, NS500	Size distribution (10 nm–2 $\mu$ m) concentration ( $10^7$ – $10^9$ particles ml $^{-1}$ )	Exos, sMVs	Accurate for both monodisperse and polydisperse; calibration particle standards	Size > 70 nm (fluorescent-NTA, >50 nm)	41, 141–146
Dynamic light scattering (DLS)	Nano ZS	Size distribution (0.3 nm – 1 $\mu$ m)	Exos, sMVs	Accurate for monodisperse samples; lower size (<30 nm)	Large particles can compromise the results, inaccurate for polydisperse samples	141, 146
Resistive pulse sensing (RPS)	qNano	Size distribution (30 nm–1 $\mu$ m), concentration ( $10^7$ – $10^{10}$ particles ml $^{-1}$ )	Exos	Surface charge	For unknown size distribution, insufficient for detection of all particles, size > 70 nm	142, 143
Flow cytometry (FACS)	BD flow cytometer	Distribution Size (200 nm – 1 $\mu$ m) concentration ( $10^6$ – $10^9$ particles ml $^{-1}$ )	Exos, sMVs	Low particle concentration (10 $^6$ particles ml $^{-1}$ )	Size > 200 nm. For exos, not absolute size measurement	80, 85, 143, 144, 147
Electron microscopy (EM)	TEM: JEOL JEM-2010 Cryo-EM: Tecnai G2 F30, Tecnai F20, Titan Krios	0.1 nm– $\mu$ m range	Exos, sMVs	TEM/cryo-EM: direct visualization and observation of EVs, EV structure/morphology; cryo-EM: preserves membranes in native state	TEM: fixation induces shrinking of EV structure, equipment, cost	4, 5, 43, 107

cryo-EM, cryoelectron microscopy; TEM, transmission electron microscopy; exos, exosomes.

beads coated with a mAb directed to the exosomal surface was clearly superior to DC and DGC for isolating exosomes. Additionally, as demonstrated for mAb purification, AC methodology is more scalable than precipitation/centrifugation-based methods (75). Clearly, a major drawback of AC is the availability of a suitable mAb directed toward a specific vesicle surface protein as “bait.” Interestingly, Balaj and colleagues describe an elegant AC method for purifying EVs from cell-culture media and human plasma using heparin-AC based on their earlier observation that heparin blocks EV uptake in cells (76, 77). Given that heparin/heparin sulfate can interact with intact cell surfaces (78), it is unclear whether heparin-AC methodology can discriminate between exosomes and sMVs. In any event, this method promises to be a one-method-fits-all approach for isolating EVs from cell-culture media and blood, especially if used as a final polishing method when combined with other EV-fractionation approaches (e.g., ref. 37).

Using sequential centrifugal ultrafiltration (SCUF), we recently developed an unbiased EV-fractionation method to address the question of how many EV subtypes might be released from cells into culture media (37). This SCUF method employed hydrophilic PVDF membranes of various pore sizes over the range 0.1 to 0.65  $\mu$ m and low *g* centrifugal force. Fractionated EVs were examined morphologically using cryoelectron microscopy, and vesicle particle size was measured by dynamic light scattering; comparative protein profiling was undertaken by GeLC-MS/MS. Our findings revealed that only two EV subtypes are released from LIM1863 colon cancer cells (these make up more than 98% of total vesicles in sample): one characteristic of exosomes (Alix $^+$ /TSG101 $^+$ /CD63 $^+$ /CD81 $^+$  and relatively homogeneous in size [range 30–100 nm]), and the other characteristic of sMVs (heterogeneous in size ranging over 30 to 1300 nm diameter). Strikingly, sMVs were negative for Alix/TSG101/CD63 and CD81. The buoyant densities of exosomes and sMVs, determined by OptiPrep DGC, were 1.10 to 1.11 g/ml and 1.18 to 1.19 g/ml, respectively. A major finding from

this was that 350 proteins were uniquely identified in sMVs, when compared with exosomes, many of which have the potential to provide markers for this EV subtype (e.g., members of the septin family, kinesin-like protein [KIF23], exportin-2/chromosome segregation like-1 protein [CSE1L/CAS], and Rac GTPase-activating protein 1 [RACGAP1]). While these marker proteins have been observed in many different colon cancer cell line-derived sMV preparations (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI1129DS1), their universality for this EV subtype must await further studies using an extensive range of cell types and body fluids. Interestingly, while both exosomes and sMVs induced invasion of recipient fibroblast cells in the Transwell-Matrigel invasion assay, sMVs exhibited approximately 3-fold greater invasive activity than exosomes (37). In another study, Menck and colleagues report that breast tumor-derived microvesicles (sMVs) induce human breast cancer invasion (MCF-7 and SK-BR-3 cells) to a significantly greater extent than exosomes (79). While further studies are necessary, there is an emerging appreciation that sMVs and exosomes might be functionally distinct.

## How to measure EV purity?

One of the most vexing problems in EV biology is how to accurately measure and assess EV purity (72). This becomes a critical issue when evaluating EV dosage for functional studies, recipient cell activation, and achieving therapeutic efficacy. How does one measure EV purity? In biochemical terms, this is akin to measuring EV “specific activity,” i.e., by expressing the concentration of a specific EV surface-marker antigen (e.g., by ELISA assay or Western blot/FACS/EV array) or EV protein concentration as a ratio of vesicle concentration (i.e., “protein-to-particle” ratio; ref. 72). There are numerous methods for measuring vesicle/particle numbers (80), including optical methods, such as nanoparticle tracking analyses, dynamic light scattering, and flow cytometry, and

nonoptical methods, such as resistive pulse sensing, transmission electron microscopy, and Raman spectroscopy (81). For a summary of these methods, along with advantages and limitations, see Table 2. While at present there is no single method allowing accurate phenotyping, sizing, and enumeration for the whole range of EV types, a microarray platform has been recently described for multiparametric MS-based quantitation and fluorescence-based phenotyping (82). For accurate protein concentration measurements, commonly used methods include 1D-SDS-PAGE coupled to densitometry employing SYPRO Ruby staining (35, 70) and micro-BCA (72); while both methods are linear over a protein concentration range of  $\mu\text{g}/\text{ml}$  to  $\text{mg}/\text{ml}$ , the SYPRO Ruby method is linear and sensitive at sub- $\text{ng}$  levels per gel band. However, several caveats make the task of measuring EV number and protein content difficult. For example, current particle tracking approaches are typically biased toward a designated EV size range (i.e., do not evaluate the entire global field) and cannot discriminate EVs from nonvesicular material (e.g., membrane fragments). On the other hand, obtaining accurate protein concentrations of EVs is not without its problems either; for example, the potential problem of noncovalently bound proteins copurifying with EVs has yet to be adequately addressed. There is also the issue of discriminating between copurifying artifactual proteins (contaminants) and the possibility of physiologically important noncovalent EV surface proteins, or oligomeric protein complexes. It is anticipated that robust “membrane shaving” experiments, which involve gentle treatment EVs with proteases to digest (shave) surface proteins while maintaining integrity of EV luminal proteins, will reveal much about the luminal contents of EVs and also resolve issues such as (a) whether RNA species are selectively packaged as integral components of EVs (83) and not just bound to the outer surface of the vesicle complexed with RNA chaperone proteins (i.e., in the extracellular space), and (b) how to discriminate between integral EV proteins and copurifying artifact proteins (e.g., high-molecular weight oligomers and nonselective sticky/adhesive proteins).

Another question that affects EV dosage calculations is whether EV particle number/protein concentration remains static during cancer (disease) progression. While there is accumulating evidence that in late-stage cancers more EVs are released (46–49, 84, 85) and EV cargo contents (e.g., proteins) increase (74, 84, 86, 87), the question of whether EV volumes and total cargo content increase during cancer progression remains unresolved.

### Isolation of EVs from body fluids

While most early studies evaluating methods for isolation and purification of EVs were performed on material harvested from cell-culture media (see Table 1 for a summary of commonly used isolation methods), there is tremendous scientific and clinical interest in body fluid-derived EVs (e.g., blood, urine, malignant ascites) due to their clinical information (refs. 8, 88, and Supplemental Table 2). However, the isolation of EVs from blood, for example, presents formidable challenges, such as limited availability of valuable biospecimens, the presence of high-abundance proteins and lipoprotein particles, the physical properties of this matrix (e.g., viscosity and density), and the presence of a multitude of EVs originating from many different cell types in blood (although the vast majority originating from erythrocytes and platelets

[platelet dust; ref. 39]). Hence, the desired EV subtype originating from a specific disease may represent only a small percentage of the total EV population, with this percentage depending upon the nature of the standard operative procedure (SOP) employed for blood collection (i.e., extent of platelet vesiculation). Additionally, EVs may be coated with glycoproteins or glycolipids, which may cause aggregation and low yields if DC isolation methods are employed. Needless to say, if the EV field, as it applies to clinical diagnosis/disease prognosis, is to mature and reach its full potential, then standardization of critical parameters, such as blood collection, processing and storage, methods for isolating EVs from plasma, and EV sizing and enumeration, need to be addressed. For example, there are contrary reports in the literature as to the preferred plasma collection method (i.e., usage of anticoagulants citrate, EDTA, or heparin) (89–91). In this regard, in 2013, two international bodies — the International Society for Extracellular Vesicles (ISEV) and the International Society for Thrombosis and Haemostasis (ISTH) — in their guidelines and recommendations regarding standardization of sample collection and handling, discourage the use of heparin-based anticoagulants (44, 92). More recently a Microvesicle Analysis Interest Development Group was established under the auspices of the International Society for Advancement of Cytometry (ISAC) to discuss efforts for standardizing EV sample preparation and flow cytometry measurement approaches (93). There is a considerable body of research supporting the notion that size-exclusion chromatography (SEC) coupled with membrane filtration is the preferred procedure for isolating EVs from plasma/serum (71, 90, 94, 95); the preferred method for harvesting EVs after SEC, i.e., precipitation versus centrifugation, requires further evaluation (95). For urinary EV isolation procedures, see Alvarez and colleagues (73) for a comparison of different methods based on DC and commercial exosome precipitation protocols. Sáenz-Cuesta et al. further compared different EV isolation protocols with blood and urine samples and several types of analysis (flow cytometry, nanoparticle tracking analysis, and electron microscopy), taking into account facilities of a nonspecialized core laboratory (88). A detailed list of methods for isolating EVs from various body fluids is given in Supplemental Table 2.

With the emerging interest in the use of EVs as clinical diagnostic reagents, several high-throughput diagnostic platforms have been developed, ranging from classical sandwich ELISA-based microarray chip technologies (87, 96) to the use of sophisticated platforms involving immunomagnetic exosome RNA (iMER) analysis (97), miniaturized micro-nuclear magnetic resonance ( $\mu\text{NMR}$ ) microfluidic chip system (98), Exo-chip (99), and label-free high-throughput nano-plasmonic exosome assay (nPLEX) using surface plasmon resonance (SPR) (100) (see Table 3 for a list of EV-based diagnostic approaches). When coupled to high-throughput procedures for harvesting EVs from peripheral blood (95), these platforms promise to extend EV research into routine diagnostic and therapeutic settings. While several clinical studies have employed multiplexed protein markers (e.g., 37 proteins; ref. 96) and a combination panel of proteins and miRNAs (4 and 4, respectively; ref. 101) for disease diagnosis, Melo and colleagues (86) report that a single protein molecule (glycican-1 [GPC1]) anchored to circulating EVs can be employed to diagnose late-stage pancreatic

**Table 3. Application of EVs in clinical diagnosis**

Method	Analysis and detection	Biofluid	EV type	Current application(s)	Reference
Exos sandwich ELISA assay	Sandwich ELISA, 1-3 Abs	Plasma	Exos	Pancreatic ductal adenocarcinoma ( <i>n</i> = 40)	87
FACS	Exos-bound beads	250 $\mu$ l serum	Exos	Pancreatic ductal adenocarcinoma ( <i>n</i> = 190)	86
	Exos-bound beads	1–1.5 ml serum	Exos	Pancreatic cancer ( <i>n</i> = 131)	101
EV array	Sandwich ELISA-based method, Multiplex capability – different Abs (37) probed to capture Exos	10 $\mu$ l Plasma	Exos	Advanced non-small cell lung carcinoma ( <i>n</i> = 109)	96
ExoScreen	Amplified luminescent proximity homogeneous assay using photosensitizer beads	5 $\mu$ l serum	Exos	Colorectal cancer patients ( <i>n</i> = 194)	148
Ready-made chromatography columns	Sephadex CL-2B column (CellG)	Plasma (up to 1.5 ml)	Exos	Healthy donor plasma	95
Exochip	Immunoaffinity-based microfluidic device for on-chip	Serum (400 $\mu$ l)	Exos	Pancreatic cancer patients ( <i>n</i> = 5)	99
iMER	Immunomagnetic Exos RNA analysis	Serum	Exos	Glioblastoma multiforme patients ( <i>n</i> = 17)	97
$\mu$ NMR	Miniaturized micronuclear magnetic resonance	Plasma	Exos	Glioblastoma multiforme patients ( <i>n</i> = 30)	149
nPLEX	Transmission SPR through periodic nanohole	Ascites	Exos	Ovarian cancer patients ( <i>n</i> = 20)	100

cancer using only a single drop of blood for analysis. At this stage, there is no international consensus as to which EV isolation procedure or diagnostic platform is likely to be translatable into the clinic (32, 33).

### Distinguishing EVs based on protein expression: current status

As discussed above, two broad categories of EVs are released from the human colon cancer cell line LIM1863 (37), exosomes and sMVs. Over the past decade, our laboratory has accumulated exhaustive proteomic profiling data sets for highly purified EV subtypes (exosomes and sMVs) from the human colon cancer cell lines LIM1863 (35, 37, 66), HCT116, SW1222, LIM1215 (102), SW480, SW620 (74), human NIH3T3 fibroblast, and Ras-transformed NIH3T3 fibroblast cells (103), and the Madin-Darby canine kidney (MDCK) cell line and an MDCK cell line transformed with oncogenic H-Ras (21D1 cells) (4). These extensive proteomic data sets (RAW files) are publically accessible through PeptideAtlas (104). Interrogation of these data sets (Supplemental Table 1) reveals that the stereotypical exosome protein markers TSG101, Alix/PDCD6IP, and CD63 are exclusively enriched in exosomes; they are not present in sMVs. Other proteins exclusively enriched in exosomes that warrant further examination as potential selective markers include Disco-interacting protein 2 homolog B (DIP2B), members of the 4-transmembrane protein family TSPAN6 and TSPAN3 (105), arrestin domain-containing protein 1 (ARRDC1), immunoglobulin superfamily member 8 (IGSF8), and CD82. Numerous proteins found exclusively in sMVs (e.g., KIF23, RACGAP1, chromosome segregation 1-like protein, exportin-2 [CSE1L/CAS]) warrant further study as to

their potential use as discriminatory markers for sMVs. The high abundance of RNA binding proteins such as the family of heterogeneous nuclear ribonucleoproteins (hnRNPs) in both EV subtypes is consistent with their proposed role in selective loading of RNA cargoes (106). This study is a first attempt at defining protein markers that would allow discrimination between exosomes and sMVs; however, this study was restricted to human colon cancer cell lines and the dog kidney MDCK cell line. Needless to say, for general applicability, these studies need to be extended to other tissue-specific cell lines and biopsy samples.

### The future

Currently, there is general consensus in the field that there are at least two types of EVs: exosomes (~50–120 nm diameters) and sMVs (~50–1500 nm in diameter). Due to the extensive size heterogeneity of sMVs, it is not yet clear whether this fraction makes up a single vesicle subtype with respect to composition and functional properties in a continuum of oligomeric states, perhaps in dynamic equilibrium. For example, are greater than 1- $\mu$ m diameter oncosomes and greater than 0.5- $\mu$ m sMVs functionally identical? Further research is required to fully define the complete functional range of EV fractions from a single source. To date, most EV research has been conducted using vesicles isolated from cell culture. Encouragingly, recent cryo-electron microscopy with receptor-specific gold staining of an EV fraction from healthy patient plasma reveals an overall particle size distribution similar to that observed in cell culture (107). High-resolution mass spectrometry (37) and deep sequencing (67) of highly purified exosome and sMV populations from colon tumor cells show distinct protein and miRNA profiles for the two EV

subtypes. While these results have provided the opportunity to develop specific markers for discriminating EV subtypes in a colon cancer context, further studies using highly purified EVs from a broad spectrum of tissue types are required to confirm general applicability for these markers. In this regard, the exciting development of a heparin-AC method (77) promises to overcome limitations of many EV isolation strategies, especially those that lack a specific immuno-AC step, in our opinion the preferred method for isolating EVs (35). Whether the heparin-binding affinity of exosomes and sMVs differ sufficiently to allow discrimination of subtypes by this method remains to be determined. However, when coupled with other methods such as SCUF (37) or DC, heparin affinity-based isolation techniques should afford an excellent final polishing step.

In the clinical context, a fundamental issue is the technical challenge of EV quantification. While great strides have been made (see Table 2) in ability to accurately measure both EV particle numbers and protein content (72), there is still a pressing need to standardize EV enumeration procedures across laboratories. This is particularly important for allowing comparison of functional data and defining EV dosage for clinical trial purposes. At present, there is much debate as to whether EV dosage should be defined by number of vesicle particles, amount of vesicle protein, or expressing dosage as a vesicle number to protein ratio. This question must be addressed for clinical applications to be advanced. As outlined above, improvements in EV-subtype iso-

lation strategies (Table 1) promise to yield highly purified EVs for biomarker discovery. As novel EV subtype markers continue to emerge, the opportunity to employ high-resolution fluorescence microscopy (108, 109) should improve our understanding of EV subtype biogenesis. Needless to say, a better understanding of EV biology together with standardized methods for EV quantification, isolation and storage, molecular characterization, and establishing potency assays will greatly enhance the future promise for EV-based diagnostic and therapeutic applications.

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