Biologia Serbica, 2019, 41(2): 36-43

DOI 10.5281/zenodo.3532082

Review

Routine and novel methods for isolation of extracellular vesicles

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Received: 15 August 2019 Accepted: 27 August 2019 / Published Online: 8 November 2019

Summary. Extracellular vesicles (EV) play an important role in many physiological and pathological processes. Three main classes of EV are recognized, based on their biogenesis: exosomes, microvesicles and apoptotic bodies. Exosomes are extracellular-vesicles of 30 to 150 nm found in many bodily fluids (blood, urine, milk, cerebrospinal fluid, etc.). Due to their cellular origin and role in physiological and pathological processes, exosomes present in body fluids are considered a unique source of non-invasive and clinically relevant biomarkers. Analysis of exosomes can provide insight into the state of the parent-cell from which they originated. However, there is great heterogeneity in the methodologies used for exosome purification affecting the results of downstream analysis. The most commonly used methods for purification are based on ultracentrifugation (UC), ultrafiltration (UF) and precipitation. However, these are hard to standardize, leading to confounding and misleading results during downstream analyses, especially when highly-sensitive techniques such as mass spectrometry are used. Furthermore, loss of certain fractions or damage of EVs can lead to loss in obtained protein and RNA profile. Consequently, there is an emerging need to obtain consensus protocols for exosome isolation and identification of specific sub-populations. This manuscript will critically review the most commonly used techniques for EV purification such as UC, UF, size-exclusion, precipitation and immunoaffinity (IA) methods. We will also review the use of nano-antibodies for the development of novel IA protocols and identification of new EV biomarkers.

Keywords: extracellular vesicles, exosomes, purification, nanobodies, liquid biopsy.

INTRODUCTION

Maintenance of homeostasis in a multicellular organism requires cell-to-cell communication. This is accomplished either by direct contact or by exchange of different secretory components. In that regard, the majority of eukaryotic cells excrete membranous vesicles that can serve as both paracrine and endocrine signals (Lee et al. 2012). Extracellular vesicles (EV) can be classified based on either their cellular origin and biological role or their biogenesis pathway (Fig. 1). Using biogenesis as a classification EVs can be divided into exosomes, microvesicles and apoptotic bodies (van der Pol et al. 2012). Exosomes are membranous vesicles of cellular origin found in blood, urine, cerebrospinal fluid, milk and ascites. Their size ranges between 30 and 150 nm (van der Pol et al. 2012). Since their discovery in the 1980s (Pan and Johnstone 1983), exosome release has been described for nearly all mammalian cells, including stem cells (Lai et al. 2011), primary immune (Wahlgren et al. 2012) and neuronal cells (Guescini et al. 2010).

The role of exosomes in living organisms has been described as both physiological and pathological. There are reports indicating their function as carriers of proteins, lipids and nucleic acids as well as active metabolites (Lee et al. 2012) in tissue repair (Gatti et al. 2011), cell renewal, blood coagulation (Del Conde et al. 2005) and immune surveillance (Thery et al. 2009). However, exosomes have been linked to pathological processes as well, such as tumorigenesis and tumour metastasis (Rak and Guha 2012), pathogen spread (Hosseini et al. 2013), transfer of amylogenic peptides

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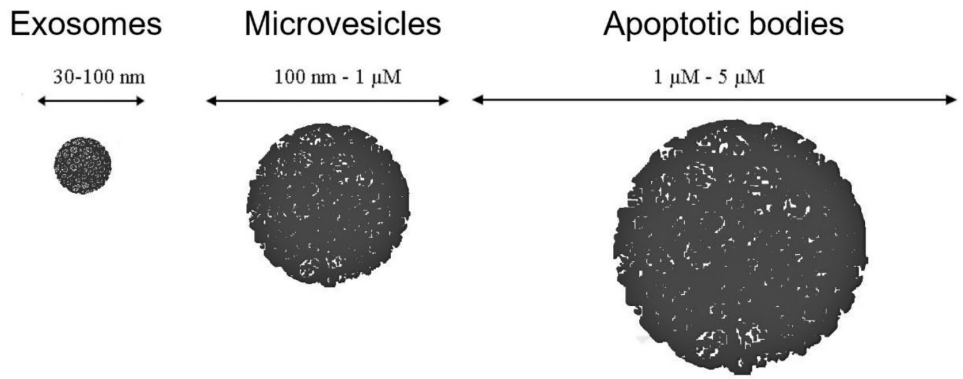

Fig. 1. Different circulating EVs.

in neurodegenerative diseases (Bellingham et al. 2012) etc. Exosomes exert their effect by merging their content with a receiving cell after activation of cell surface receptors. This way they can transfer their internal cargo consisting of RNA (microRNA, mRNA), proteins (transcription factors, oncogenic regulators), metabolites and infectious particles, directly to the host cells (Valadi et al. 2007; Camussi et al. 2011). Because of their role in many different cellular processes, exosomes could be considered to be a unique source of clinically relevant and non-invasive biomarkers. The biomolecular content of exosomes mirrors that of the parental cell enabling insight into the functioning of the cell from which they originated. Exosomes have been proposed as the preferential material for liquid biopsy, in order to evaluate disease prognosis and therapeutic choices (Revenfeld et al. 2014). Nevertheless, the present exosome isolation approaches are very inefficient in terms of purity and profiling capacity. Therefore, standardized purification and characterization methods would critically improve the quality of the diagnostic information. In this perspective, immunoaffinity based exosome sub-class discrimination and purification would therefore represent a major advancement in the field, because relevant biomarkers transported by EVs (ncRNA, mRNA, proteins) could be selectively enriched (Popović and de Marco 2017). To date, this approach has been prevented by our limited knowledge with respect to the membrane-displayed antigens that are specifically expressed in different EV subpopulations. Immuno-affinity-based methods for exosome purification have been shown to be effective for purification of cancer-related exosomes from blood of prostate cancer patients, promoting the diagnostic values of this type of approach.

EXOSOME PURIFICATION

Commonly used purification techniques include ultracentrifugation (UC), density gradient centrifugation (DG), chromatographic methods, ultra-filtration (UF), precipitation using polymer-based reagents and immunoaffinity methods (IA) (Table 1). The yield and purity of such obtained material greatly depends on the applied purification strategy (Taylor and Shah 2015). Many published reports dealing with various aspects of exosomal biology and applications have failed to first assess the purity of the resulting material before further downstream analysis. Contamination of exosomal material with protein complexes and/or damage to the membrane, as well as loss of certain exosomal fractions can cause misleading data interpretation, especially in genomic or proteomic studies. Therefore, a list of minimal criteria for definition of EVs before their further use has been established (Lotvall et al. 2014; Van Deun et al. 2017). This has led to the creation of several proteomic repositories to aid researchers in selecting markers to assess the characterisation of isolated material that are available online (Vesiclepedia (www.microvesicles.org/) (Kalra et al. 2012), EVpedia (www.evpedia.info) (Kim et al. 2013) and ExoCarta (www.exocarta.org) (Simpson et al. 2012)).

CENTRIFUGATION BASED APPROACHES

UC has long been considered to be the method of choice for EV purification. Centrifugal force is applied to the sample resulting in sedimentation of macromolecules from the solution according to their density. EVs are usually pelleted at very high g-forces (100000 × g or more). Differential centrifugation is the most commonly used method of exosome purification (Momen-Heravi et al. 2013). This implies the use of successive centrifugation steps with increasing centrifugal force intended to pellet apoptotic bodies and cell debris, shedding vesicles and exosomes. Despite the fact that this methodology is relatively straightforward, the yield and purity of the resulting material is highly dependent on several factors including g-force, rotor type (swing bucket or fixed angle), the angle of sedimentation of the rotor, sedimentation efficiency and viscosity of the sample solution (Momen-Heravi et al. 2012; Cvjetkovic et

Table 1. Comparison of techniques most commonly used in exosome purification.

Purification method	Principle	Shortcomings
Ultracentrifugation	Sedimentation of EVs according to density using high g force	- Quantity and quality of the obtained material is highly dependent on: g force, rotor type, efficiency of pelleting (rotor and tube k-factors) and viscosity of solution - Hard to standardize and control all of the parameters - The unknown effects of prolonged pelleting against a solid surface on the membrane integrity and vesicle content
Density gradient ultracentrifugation	Separation according to density in a pre-constructed density gradient medium	- Limited capacity of the method - Long running time - Incomplete sedimentation of all exosomal fractions - Artefacts from contaminating material in the same density fractions
Ultrafiltration	Distribution of particles in a solution across a polymer-based membrane 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 contaminants in the same-size range.
Precipitation	Addition of water-excluding polymers retain water and force less-soluble components, such as exosomes, out of solution	- Possibility to co-precipitate other non-exosome contaminants
Immuno-affinity techniques	Immuno-affinity interactions between ligands on the vesicle surface and specific antibodies	- Identification of appropriate surface targets - Heterogeneity of exosome populations hinder the universal applicability of this approach - Multi-targeting of antigens is necessary to avoid loss of some fractions

al. 2014). Ultracentrifuge clearing factor (k-factor) is an important predictor for the time required for pelleting, since it is correlated to the k-factor and sedimentation coefficient (s) according to the equation . Although many published studies relied on the same pelleting time and speed, differences in k-factor for the used devices have led to drastically different reported yields from the same samples (Witwer et al. 2013). In order to circumvent differences in rotor type, adjustments in centrifugation time are required. However, it is difficult to standardize and control all of these factors even if the same protocol is applied. Reports have indicated that US can lead to incomplete sedimentation of EVs as well as co-sedimentation of EVs with non-EV material (Witwer et al. 2013). Furthermore, prolonged pelleting of membranous EVs against a solid surface could affect membrane integrity and vesicular content and the effects of this phenomenon have yet to be studied in detail.

DG UC offers some improvements when compared to classical UC purification since purifications depends on size, mass and density. A DG gradient is formed in the centrifuge tube in such a way that it increases from top to bottom. The gradient is commonly formed using sucrose or iodixanol [OptiPrep™](Kalra et al. 2013). In DG UC, a small amount of sample is loaded onto the preformed gradient. After centrifugal force is applied, solutes move through the DG medium until they reach their respective density. Separated components can then be recovered by differential fraction collection (Vergauwen et al. 2017). One of the main limiting factors of DG UC lies in the small amount of sample that can be loaded to the preformed gradient limiting preparative capacity. Furthermore, protocols for DG UC require long running times to reach equilibrium, so reported methods span a range of 16 to 90 h to complete the preparation (Li et al., 2017). Incomplete sedimentation can also be an issue with this method, as well as co-sedimentation of artefacts of the same density. It is a particular problem when plasma is used a source of exosomes, because HDL/LDL particles can co-migrate with them and contaminate the final preparation (Yuana et al. 2014; Sodar et al. 2016).

SIZE AND CHROMATOGRAPHY BASED APPROACHES

Purification of vesicles has been achieved using different size base approaches such as, ultrafiltration (UF), size-exclusion (SEC) and chromatography based approaches such as ion-exchange (IEX) chromatography (Li et al. 2017).

The use of UF in exosome preparation does not dif-

fer from its standard use in protein chemistry. Particles in a complex solution can be separated according to their dimensions using membrane filters with a defined size-exclusion cut-off (Quintana et al. 2015). Preparative UF is usually a multistep process allowing for the removal of contaminants with different characteristics. UF can be performed in a fraction of the time required for UC and/or DG UC and does not call for special equipment (Zeringer et al. 2015). Unfortunately, the pressure that is exerted during UF, especially on larger-sized vesicles, can lead to damage and influence downstream analysis (Batrakova and Kim 2015). Filter material itself can impact the final quality of the obtained material and a recent report suggests that regenerated cellulose is the most efficient material for exosome purification from serum and plasma (Vergauwen et al. 2017).

Sequential filtration has been frequently employed for exosome purification, mainly when conditioned cell culture medium is used as a source. Sample is passed through a series of membranes with different cut-offs to eliminate cell debris (100 nM) and soluble proteins (500 kDa) with a final concentration step using 100 kDa membrane filter (Li et al. 2017). Sequential filtration allows for isolation of highly purified exosome that maintain functional integrity. It has been reported that sequential filtration followed by DG UC in sucrose gradient can be used for preparation of exosomes for therapeutic applications (Escudier et al. 2005).

SEC has been widely used as means for EV separation because it uses mild physical conditions, allowing for the separation of intact vesicles from other soluble biomolecules present in the sample (Taylor and Shah 2015). A porous stationary phase is used to sort molecules according to their hydrodynamic radii. Components that are smaller in size can enter the pores of the separation matrix, resulting in later elution, while components with larger hydrodynamic radii are excluded from entering the pores, leading to faster elution. Furthermore, SEC is also useful in removing Optiprep remnants after DG UC purification (Vergauwen et al. 2017).

There are actually only a few reports that have demonstrated the use of conventional IEX for exosome preparations. Anion exchange has recently been described as a useful step in exosome purification that enabled concentrating the material for further preparative purification (Kim et al. 2016). Results from the limited characterization included in this report suggested that vesicles with different characteristics co-existed in the partially purified sample although the complexity of the original sample has been reduced (Kim et al. 2016). Monolithic ion-exchange matrices can be used to co-elute vesicles with fractions corresponding to virus-like particles (Steppert et al. 2016). All these reports, albeit with limited down-stream characterization of the samples, suggest that IEX can be a good starting step for enrichment of EVs from larger starting volumes.

PRECIPITATION BASED APPROACHES

Water-excluding polymers, such as PEG can be use to extract exosomes from solutions (Zeringer et al. 2015). Water-excluding polymers retain water forcing less-soluble components to precipitate (Zeringer et al. 2015). Exosome containing solutions are incubated at low temperature overnight with the precipitating solution, commonly containing PEG 8000, after which the formed precipitate is separated by means of filtration or low-speed centrifugation (Zeringer et al. 2015). EV precipitation is easy to perform and does not require any kind of special equipment. It is however, necessary to remove cells and cell debris but this is easily achieved with centrifugation or filtration of the sample prior to adding the precipitant. Currently, there are several commercially available exosome precipitation kits that are compatible with different kinds of biological samples: plasma, serum, urine, cerebrospinal fluid and cell culture medium. The major drawback of this method, which has been repeatedly confirmed in comparative assessments of EV purification protocols, is in fact co-precipitation on non-EV contaminants, such as proteins and other polymeric materials (Zarovni et al. 2015).

IMMUNO-AFFINITY BASED APPROACHES

IA purification is based on the selectivity and strong interaction affinity of antibody-antigen (Ab-Ag) pairs. If the antigen (Ag) is a protein located on the membrane surface of EV, this method can easily be used for EV purification. Since Abs offer extreme specificity, due to their specialized structure, IA capture has the capacity to distinguish between minimally different conformations. However, application of IA to EV purification is often hampered by our lack of knowledge concerning reliable biomarkers that are specific for different EV sub-populations. Some biomarkers that are widely used for exosome capture include universal exosomal biomarkers such as CD8 and CD 63, as well as EpCAM, Mart-1 and TYRP2, and members of the human epidermal growth factor receptor family (Koga et al. 2005). However, because these markers vary in terms of their expression levels, using different Ab combinations can capture exosomes derived from different cells (Clayton et al. 2001). Using universal biomarkers for purification limits the method to isolation of EVs from well characterized cell types and is often used as a final purification step (Caby et al. 2005). Individual markers, such as EpCAM or MHC class II can be used to isolate exosomes derived from specific cell lines such as epithelial tumor cells and B-cells, respectively (Wubbolts et al. 2003; Tauro et al. 2012).

Stratification of different exosome populations is highly encouraged, but relies heavily on the use of new IA protocols

with new Abs-Ags pairs. There are several emerging reports that employed these new targets. For instance, A33 was identified as a marker for exosomes from human colon cancer cells and, in combination with EpCAM, was used to capture two populations of exosomes that shared a minute fraction of miRNA cargo between them (Tauro et al. 2013). There are various other examples as well, such as using anti-CD133 Abs to assess renal function in kidney transplant patients (Dimuccio et al. 2014), or using anti-CD34 Abs to recover specific AML blast-derived EVs from patient plasma (Hong et al. 2014).

In several studies comparing different isolation methods such as UC, DG UC and IA capture, IA was assessed as the most efficient (Greening et al. 2015). One drawback of IA separation is its dependence on the availability of conventional Abs. It is often very time consuming and financially demanding to develop new binders for EV purification.

ISOLATION OF RECOMBINANT SINGLE-DOMAIN ANTIBODIES SPECIFIC FOR EXTRACELLULAR-VESICLES

Heavy-chain-only Abs, which are naturally occurring in camelids (VHH), are stable and functional binders despite the lack of a light chain. VHHs share structure and sequence similarities with VH domains of conventional Abs with the exception of key mutations in their framework2 region that is responsible for interactions between variable domains in conventional immunoglobulins. The increased structural stability and reduced aggregation propensity of VHHs are advantageous in conditions requiring more stable Abs (e.g. increased detergent concentrations, low pH) and makes them effective reagents for imaging and therapeutic applications in oncology, infectious, inflammatory, and neurodegenerative diseases. (de Marco 2011). Large, pre-immune libraries of VHHs are widely available and protocols for panning soluble antigens and whole cells have already been established (Moutel et al. 2016; Crepin et al. 2017).

EVs have been used as material for panning, and this has enabled isolation of binders for the native conformation of an antigen exposed on the EV surface (Popovic et al. 2018). Pre-purified, enriched EV fractions from two different cell lines were used as panning material. The aim was to establish a panning protocol using EVs rather than soluble antigens. This step was critical because it has been reported that antigens present on EVs can be slightly modified with respect to those expressed on the original cell (Taylor et al. 1980). A panning protocol was established that uses very mild conditions (Fig. 2), so that the EVs remain intact; but still enabled isolation of several binders that display distinctive binding patterns. One group of binders clearly identified CD9, while the other group recognized other, thus far unidentified surface markers. Antibodies identified as anti-CD9 clearly competed with commercial anti-CD9 antibodies in flow-cytometry based assays. Furthermore these antibodies enabled capture of vesicles from different sources, cell culture supernatant and human plasma, enabling isolation of EVs and further downstream analysis. This methodology is

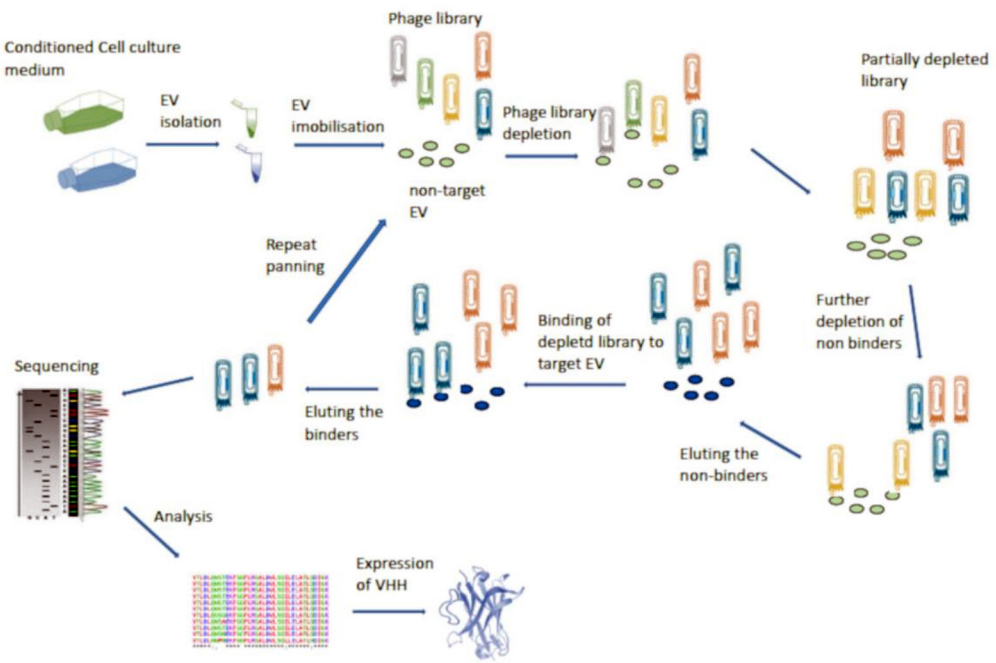

Fig. 2. Strategy for panning a pre-immune VHH library against isolated EVs.

faster and less costly when compared to standard hybridoma protocols. Furthermore, it offers additional advantages such as isolation of binders for EV epitopes in their native conformation and potentially isolation of binders against novel antigens. Isolated nanobodies could be produced on a larger scale and with different tags as cheap, application-friendly immuno-reagents. Moreover, adding different tags, such as GFP would allow for their immediate use in different applications (flow-cytometry, fluorescence imaging, fluorescence microscopy etc.).

This report (Popovic et al. 2018) was the first case of successful isolation of anti-EV nanobodies by direct panning of a phage library on partially purified EVs. This achievement enabled stable immunoaffinity-based EV capture and consequently simplifies the future discovery of novel antibody-vesicle surface biomarker pairs that will be instrumental for the systematic stratification of EV sub-populations and their individual characterization.

CONCLUSIONS

There is an emerging need for reproducible methods able to purify exosome fractions free from contamination by other sample components. Furthermore, such methods should also enable discrimination among distinct subpopulations to allow separate analyses of their contents; and will require novel, innovative technical solutions for EV fractionation. Comparative surveys of EV purification methodologies indicate that EVs can be obtained in a highly pure state through use of a combination of density gradient ultracentrifugation and size-exclusion chromatography or ultrafiltration, but fractionation of EV subclasses is mostly dependent on affinity techniques. Stable immunoaffinity-based approaches for EV capture can simplify the future discovery of novel antibodies for surface biomarkers that will in turn enable systematic differentiation and characterization of EV subpopulations.

Acknowledgments

This work was supported by Grant 172049 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

REFERENCES

Batrakova EV, Kim MS. 2015. Using exosomes, naturally-equipped nanocarriers, for drug delivery. Journal of Controlled Release 219:396–405.

Bellingham SA, Guo BB, Coleman BM, Hill AF. 2012. Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? Frontiers in Physiology. 3:124.

Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. 2005. Exosomal-like vesicles are present in human blood plasma. International Immunology. 17:879–887.

Camussi G, Deregibus MC, Bruno S, Grange C, Fonsato V, Tetta C. 2011. Exosome/microvesicle-mediated epigenetic reprogramming of cells. American Journal of Cancer Research. 1:98–110.

Clayton A, Court J, Navabi H, Adams M, Mason MD, Hobot JA, Newman GR, Jasani B. 2001. Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. Journal of Immunological Methods. 247:163–174.

Crepin R, Gentien D, Duche A, Rapinat A, Reyes C, Nemati F, Massonnet G, Decaudin D, Djender S, Moutel S, et al. 2017. Nanobodies against surface biomarkers enable the analysis of tumor genetic heterogeneity in uveal melanoma patient-derived xenografts. Pigment Cell & Melanoma Research. 30:317–327.

Cvjetkovic A, Lötvall J, Lässer C. 2014. The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. Journal of Extracellular Vesicles. 3:23111.

de Marco A. 2011. Biotechnological applications of recombinant single-domain antibody fragments. Microbial Cell Factories. 10:44.

Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. 2005. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood. 106:1604–1611.

Dimuccio V, Ranghino A, Barbato LP, Fop F, Biancone L, Camussi G, Bussolati B. 2014. Urinary CD133(+) Extracellular Vesicles Are Decreased in Kidney Transplanted Patients with Slow Graft Function and Vascular Damage. PLOS ONE. 9(8):e104490.

Escudier B, Dorval T, Chaput N, Andre F, Caby MP, Novault S, Flament C, Leboulaire C, Borg C, Amigorena S, et al. 2005. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. Journal of Translational Medicine. 3(1):10.

Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, Camussi G. 2011. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. Nephrology Dialysis Transplantation. 26:1474–83.

Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. 2015. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods in Molecular Biology. 1295:179–209.

Guescini M, Genedani S, Stocchi V, Agnati LF. 2010. Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. Journal of Neural Transmission (Vienna) 117:1–4.

Hong CS, Muller L, Boyiadzis M, Whiteside TL. 2014. Isolation and Characterization of CD34+Blast-Derived Exosomes in Acute Myeloid Leukemia. PLOS ONE. 9(8):e103310.

Hosseini HM, Fooladi AA, Nourani MR, Ghanezadeh F. 2013. The role of exosomes in infectious diseases. Inflammation & Allergy-Drug Targets. 12:29–37.

Kalra H, Adda CG, Liem M, Ang CS, Mechler A, Simpson RJ,

Hulett MD, Mathivanan S. 2013. Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. Proteomics. 13:3354–3364.

Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, Bond VC, Borras FE, Breakefield X, Budnik V, et al. 2012. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biology. 10:e1001450.

Kim DK, Kang B, Kim OY, Choi DS, Lee J, Kim SR, Go G, Yoon YJ, Kim JH, Jang SC, et al. 2013. EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. Journal of Extracellular Vesicles. 2.

Kim DK, Nishida H, An SY, Shetty AK, Bartosh TJ, Prockop DJ. 2016. Chromatographically isolated CD63(+) CD81(+) extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI. Proceedings of the National Academy of Sciences of the United States of America. 113:170–175.

Koga K, Matsumoto K, Akiyoshi T, Kubo M, Yamanaka N, Tasaki A, Nakashima H, Nakamura M, Kurok S, Tanaka M, et al. 2005. Purification, characterization and biological significance of tumor-derived exosomes. Anticancer Research. 25:3703–3707.

Lai RC, Chen TS, Lim SK. 2011. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. Regenerative Medicine. 6:481-492.

Lee Y, El Andaloussi S, Wood MJ. 2012. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. Human Molecular Genetics. 21:R125–134.

Li P, Kaslan M, Lee SH, Yao J, Gao Z. 2017. Progress in Exosome Isolation Techniques. Theranostics. 7:789–804.

Lotvall J, Hill AF, Hochberg F, Buzas EI, Di Vizio D, Gardiner C, Gho YS, Kurochkin IV, Mathivanan S, Quesenberry P, et al. 2014. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. Journal of Extracellular Vesicles. 3:26913.

Momen-Heravi F, Balaj L, Alian S, Mantel PY, Halleck AE, Trachtenberg AJ, Soria CE, Oquin S, Bonebreak CM, Saracoglu E, Skog J, Kuo WP. 2013. Current methods for the isolation of extracellular vesicles. Biological Chemistry. 394:1253–1262.

Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, Kuo WP. 2012. Impact of Biofluid Viscosity on Size and Sedimentation Efficiency of the Isolated Microvesicles. Frontiers in Physiology. 3:162.

Moutel S, Bery N, Bernard V, Keller L, Lemesre E, de Marco A, Ligat L, Rain JC, Favre G, Olichon A, et al. 2016. NaLi-H1: A universal synthetic library of humanized nanobodies providing highly functional antibodies and intrabodies. eLife. 5:e16228.

Pan BT, Johnstone RM. 1983. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell. 33:967–78.

Popovic M, Mazzega E, Toffoletto B, de Marco A. 2018. Isolation of anti-extra-cellular vesicle single-domain antibodies by direct panning on vesicle-enriched fractions. Microbial Cell Factories. 17(1):6.

Popović M, de Marco A. 2017. Canonical and selective approaches in exosome purification and their implications for diagnostic accuracy. Translational Cancer Research. 7(Suppl 2):S209-S225.

Quintana JF, Makepeace BL, Babayan SA, Ivens A, Pfarr KM, Blaxter M, Debrah A, Wanji S, Ngangyung HF, Bah GS, et al. 2015. Extracellular Onchocerca-derived small RNAs in host nodules and blood. Parasit Vectors. 8:58.

Rak J, Guha A. 2012. Extracellular vesicles--vehicles that spread cancer genes. Bioessays. 34: 489–497.

Revenfeld AL, Baek R, Nielsen MH, Stensballe A, Varming K, Jorgensen M. 2014. Diagnostic and prognostic potential of extracellular vesicles in peripheral blood. Clinical Therapeutics. 36:830–846.

Simpson RJ, Kalra H, Mathivanan S. 2012. ExoCarta as a resource for exosomal research. Journal of Extracellular Vesicles. 1:10.3402/jev.v1i0.18374.

Sodar BW, Kittel A, Paloczi K, Vukman KV, Osteikoetxea X, Szabo-Taylor K, Nemeth A, Sperlagh B, Baranyai T, Giricz Z, et al. 2016. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. Scientific Reports 6:24316.

Steppert P, Burgstaller D, Klausberger M, Berger E, Aguilar PP, Schneider TA, Kramberger P, Tover A, Nobauer K, Razzazi-Fazeli E, et al. 2016. Purification of HIV-1 gag virus-like particles and separation of other extracellular particles. Journal of Chromatography A. 1455:93–101.

Tauro BJ, Greening DW, Mathias RA, Ji H, Mathivanan S, Scott AM, Simpson RJ. 2012. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. Methods. 56:293–304.

Tauro BJ, Greening DW, Mathias RA, Mathivanan S, Ji H, Simpson RJ. 2013. Two Distinct Populations of Exosomes Are Released from LIM1863 Colon Carcinoma Cell-derived Organoids. Molecular & Cellular Proteomics. 12:587–598.

Taylor DD, Homesley HD, Doellgast GJ. 1980. Binding of specific peroxidase-labeled antibody to placental-type phosphatase on tumor-derived membrane fragments. Cancer Research. 40:4064–4069.

Taylor DD, Shah S. 2015. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. Methods. 87:3–10.

Thery C, Ostrowski M, Segura E. 2009. Membrane vesicles as conveyors of immune responses. Nature Reviews Immunology. 9:581–593.

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology. 9:654–659.

van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. 2012. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev,* 64: 676-705.

Van Deun J, Mestdagh P, Agostinis P, Akay O, Anand S, Anckaert J, Martinez ZA, Baetens T, Beghein E, Bertier L, et al. 2017. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. Nature Methods. 14:228–232.

Vergauwen G, Dhondt B, Van Deun J, De Smedt E, Berx G, Timmerman E, Gevaert K, Miinalainen I, Cocquyt V, Braems G, et al. 2017. Confounding factors of ultrafiltration and protein analysis in extracellular vesicle research. Scientific Reports. 7:2704.

Wahlgren J, Karlson TDL, Glader P, Telemo E, Valadi H. 2012. Activated Human T Cells Secrete Exosomes That Participate in IL-2 Mediated Immune Response Signaling. PLOS ONE. 7:e49723.

Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, Nolte-'t Hoen EN, Piper MG, Sivaraman S, Skog J, et al. 2013. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. Journal of Extracellular Vesicles. 2:20360.

Wubbolts R, Leckie RS, Veenhuizen PTM, Schwarzmann G, Mobius W, Hoernschemeyer J, Slot JW, Geuze HJ, Stoorvogel W. 2003. Proteomic and biochemical analyses of human B cell-derived exosomes - Potential implications for their function and multivesicular body formation. Journal of Biological Chemistry. 278:10963–10972.

Yuana Y, Levels J, Grootemaat A, Sturk A, Nieuwland R. 2014. Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation. Journal of Extracellular Vesicles. 3:10.3402/jev.v3.23262.

Zarovni N, Corrado A, Guazzi P, Zocco D, Lari E, Radano G, Muhhina J, Fondelli C, Gavrilova J, Chiesi A. 2015. Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. Methods. 87:46–58.