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.
Routine and novel methods for isolation of extracellular vesicles

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 characterization 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
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 as 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-

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

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<th>Purification method</th>
<th>Principle</th>
<th>Shortcomings</th>
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| 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 |
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 used 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

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**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.

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