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INVITED REVIEW

# Modern Techniques for the Isolation of Extracellular Vesicles and Viruses

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## Abstract

Extracellular signaling is pivotal to maintain organismal homeostasis. A quickly emerging field of interest within extracellular signaling is the study of extracellular vesicles (EV), which act as messaging vehicles for nucleic acids, proteins, metabolites, lipids, etc. from donor cells to recipient cells. This transfer of biologically active material within a vesicular body is similar to the infection of a cell through a virus particle, which transfers genetic material from one cell to another to preserve an infection state, and viruses are known to modulate EV. Although considerable heterogeneity exists within EV and viruses, this review focuses on those that are small (< 200 nm in diameter) and of relatively low density (< 1.3 g/mL). A multitude of isolation methods for EV and virus particles exist. In this review, we present an update on methods for their isolation, purification, and phenotypic characterization. We hope that the information we provide will be of use to basic science and clinical investigators, as well as biotechnologists in this emerging field.

**Keywords** Extracellular vesicles · Viruses · Exosomes · Microvesicles

## Introduction

Extracellular vesicles (EV) are secreted from every cell type studied. Several subtypes of EV exist and are classified based on their sizes, cellular origin and surface markers. Apoptotic bodies (800–5000 nm in diameter) and microvesicles (100–800 nm in diameter) bud off at the plasma membrane and are thus enriched for proteins sitting on the cell surface. Exosomes are a class of EV of a small diameter (40–150 nm in diameter) that originate from the inward budding of endosomes into the multivesicular body and contain endosomal trafficking markers such as tumor susceptibility gene 101 (Tsg101), apoptosis-linked gene-2 interacting protein X (Alix), tetraspanins, and flotillins (reviewed in (Crescitelli et al. 2013; Raab-Traub and Dittmer 2017)). EV have received a substantial amount of attention in recent years. They act as an extracellular mailing system, transferring information from one cell to the next, and can contain nucleic acids such as DNA, micro RNAs (miRNA) and non-coding RNAs (ncRNA), mRNA, proteins and enzymes such as histones and esterases, metabolites, and lipids (Chugh et al. 2013; Hurwitz et al. 2016; Meckes et al. 2010, 2013; Wang et al. 2015; Willms et al. 2016; Bukong et al. 2014; Longatti et al. 2015). EV have been proposed to play a critical role in cell differentiation, angiogenesis, metabolic reprogramming, tumor progression, immune modulation, and response to pathogen challenge (Willms et al. 2018; McNamara et al. 2018b; Raab-Traub and Dittmer 2017; Baranyai et al. 2015; Chevillet et al. 2014; M. R. Anderson et al. 2016).

Here, we will review recent updates on the topic of EV isolation using the field of virus isolation for comparison. Virus isolation has been the focus of scientific and engineering studies for well over 100 years, with a particular emphasis on vaccine manufacturing (reviewed in (Effio and Hubbuch 2015). Thus virus isolation represents a validated catalog of tools against which EV isolation techniques can be compared to. Specifically, we will review large-scale methods of ultracentrifugation, precipitation with crowding reagents, crossflow filtration, affinity purification, and nanoscale flow cytometry, and also summarize methods for characterization. Of note, the international society for extracellular vesicles (ISEV) regularly publishes best practices for the field (Théry et al. 2018). These will complement this review.

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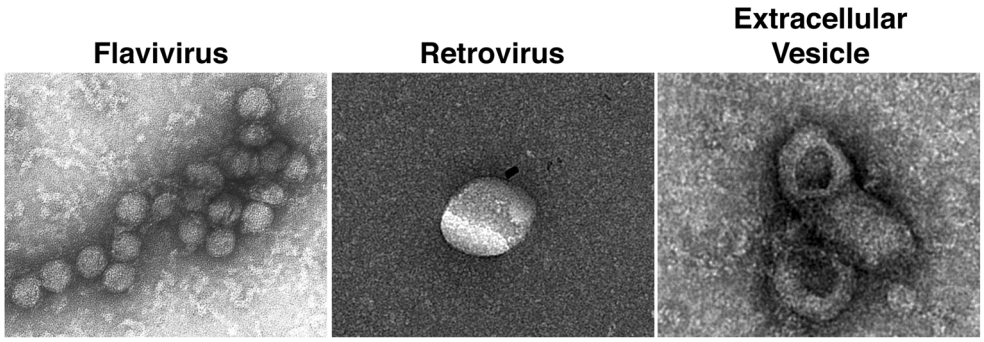
In human and non-human primate plasma, the concentration of EV is believed to be as high as $10^{11}$ particles/mL (McNamara et al. 2018a, b; Chevillet et al. 2014; Baranyai et al. 2015). Even though they are similar in size, these small circulating EV far outnumber even high-titer bloodborne viruses such as West Nile virus, HIV, and Ebola virus during infection. Concentrations of EV < 150 nm in cell culture supernatant also are much higher than in vitro propagated viruses and live-attenuated vaccines or viral like particles (Fig. 1). It has been proposed that every cell type is capable of releasing EV, irrespective of how quickly it divides (Théry et al. 2018; Witwer et al. 2013). To that end, EV have been isolated from various human fluids such as plasma, urine, tumor fluid, effusions, and cerebrospinal fluid. While specific contents can vary depending on cell origin, most EV < 150 nm contain core constituents: (I) tetraspanin proteins, most notably CD9, CD63 and CD81, (II) a lipid membrane rich in phosphatidyl serine, (III) and trafficking proteins such as endosomal sorting complexes required for intracellular transport (ESCRT) machinery (Raab-Traub and Dittmer 2017; Anderson et al. 2016).

Given their abundance and regulatory functions, it is not surprising that viruses have evolved to usurp EV-mediated signaling. Viruses are obligate intracellular parasites and utilize many of the same cellular pathways as EV to facilitate particle uptake, trafficking, and egress. Several viruses have been shown to incorporate viral nucleic acids and/or proteins into EV. Human herpesviruses such as Epstein-Barr virus (EBV, also known as human herpesvirus 4) and Kaposi's Sarcoma-Associated Herpesvirus (KSHV, also known as human herpesvirus 8) incorporate viral proteins and miRNAs into EV (Chugh et al. 2013; Dittmer and Damania 2013; Hurwitz et al. 2017, 2018; Meckes et al. 2010, 2013; Yogev et al. 2017; McNamara et al. 2019; Zhao et al. 2019; Pegtel et al. 2010). Human immunodeficiency virus (HIV), as well as its ancestral simian immunodeficiency virus (SIV) incorporate the protein Nef into CD63/CD81+ EV and can deliver it to naïve and uninfectable cells throughout the course of infection (McNamara et al. 2018b; Raymond et al. 2016; Khan et al. 2016; Lenassi et al. 2010; Sami Saribas et al. 2017; Aqil et al. 2014; Mukhamedova et al. 2019; Muratori et al. 2009). In addition to Nef, the HIV-encoded TAR RNA has been detected in EV (Narayanan et al. 2013; Sampey et al. 2016). An even more overt example of virus hijacking the EV biogenesis pathway is incorporation of the full length viral genomic mRNA or the entire viral particle into EV upon infection with hepatitis C virus (HCV) or Hepatitis A virus (HAV), respectively (Feng et al. 2013; McKnight et al. 2017; Rivera-Serrano et al. 2019; Longatti et al. 2015; Bukong et al. 2014; Ramakrishnaiah et al. 2013). Given the propensity of evolutionarily distinct viruses to usurp the EV pathways, it has been proposed the viruses utilize EV as a "Trojan horse" to deliver functional materials for full disease pathogenesis (Gould et al. 2003). Taken together, studies in the field of virology have contributed to our knowledge of EV biogenesis, packaging and delivery, and vice versa.

The isolation of EV or virus particles to high concentration and purity is critical to their study. The separation of these vesicles based on physical parameters alone, such as size, markers, and from contaminants such as protein aggregates has proven to be a non-trivial aspect of their purification. Methods of their purification, as well as distinctive qualities




| Example: West Nile Virus | Example: Simian Immunodeficiency Virus | Example: CD63+ EV |
|---|---|---|
| Capsid | Capsid | No Capsid |
| No Envelope Membrane | Envelope Membrane | Envelope Membrane |
| Nucleic Acids: Viral Genome | Nucleic Acids: Viral Genome, tRNA Lys | Nucleic Acids: miRNAs, lncRNA, tRNAs |
| 40-70 nm Diameter | 120-150 nm Diameter | 40-120 nm Diameter |
| Density: 1.03-1.24 g/cm$^3$ | Density: 1.15-1.20 g/cm$^3$ | Density: 1.06-1.11 g/cm$^3$ |
| Peak [Plasma]: ~10$^8$ | Peak [Plasma]: ~10$^8$ | Peak [Plasma]: ~10$^{11}$ |

**Fig. 1 Comparison of non-enveloped viruses, enveloped viruses, and EV.** Transmission electron micrographs of a non-enveloped virus (West Nile virus), an enveloped virus (Simian immunodeficiency virus), and EV are shown. Compositions of the particles, such as capsids, envelopes, and nucleic acids are listed below each image. Also, biophysical properties such as size and density are shown, as are peak concentrations of the particles found in blood plasma.

of subclasses of EV have been discussed previously (Théry et al. 2006; Lotvall et al. 2014; Witwer et al. 2013; Reiner et al. 2017; Peterson et al. 2015; Théry et al. 2018; Théry et al. 2002; Stoorvogel et al. 2002). The most prominent methods applicable to both EV and virus isolation include, but are not limited to: (I) ultracentrifugation, (II) precipitation with crowding reagents, (III) crossflow filtration (IV), column chromatography and high performance liquid chromatography (HPLC), (V) immunoprecipitation/affinity capture, and (VI) nanoscale flow cytometry. Additionally, assays that can quantitate the phenotype of cells exposed to EV from sources such as an infected cell or a tumor representa developing field (Raab-Traub and Dittmer 2017; M. Anderson et al. 2018). In this review, we will compare purification strategies of viruses and EV, and recent efforts to characterize the functions they play in cellular homeostasis. Of note, this review is focused on manufacturing and lab-based assays; the emerging field of EV in diagnostics is not covered.

## Ultracentrifugation

Given that viruses and EV are low-density particles, only high-speed centrifugation can pellet them directly from solution. For decades, ultracentrifugation (>100,000 * g) has been used to isolate eukaryotic viruses such as influenza, and bacteriophages (Kutner et al. 2009; Reimer et al. 1966, 1967; Sugita et al. 2011; Bachrach and Friedmann 1971). Since virus particles and EV are similar in size and density (Table 1), ultracentrifugation became a frequently employed technique for EV isolation. Given that ultracentrifugation is widely used in routine molecular biology and biochemistry, it presents itself as a rapid and reliable isolation technique for virus and EV isolation. Viruses and EV isolated through ultracentrifugation retain functional capacities such as infectivity and esterase enzymatic activity, respectively. A lingering question, and target for optimization, is the exact amount of biological activity that is maintained throughout purification and which assays are used to define "biological activity" vis-à-vis non-purified biological fluids.

The majority of ultracentrifugation instruments are meant for smaller volume inputs (~20–50 mL), but there are some that can hold larger volumes. For this reason, ultracentrifugation is frequently employed as a quick method for the isolation of particles like lentiviruses and EV from these volumes (though exceptions exist, further reviewed in (Li et al. 2017)). Many protocols include creating sucrose or iodixanol gradients to separate the heavy viruses and EV away from protein aggregates, nucleic acid bodies, low-density lipoproteins (LDLs), etc. (Willms et al. 2016, 2018; Iwai et al. 2016; Onódi et al. 2018; Lemon et al. 1985). Repeated ultracentrifugation steps have been shown to decrease EV product yields (Lobb et al. 2015; McNamara et al. 2018a), perhaps due to the tremendous g-force exerted on the vesicles.

In terms of gradients, there are two principles: (I) density gradients used as a cushion, and (II) floatation designs. When used as a cushion, an EV prep is placed at the top of a density gradient (such as iodixanol or sucrose), and ultracentrifugation allows for the sedimentation of macromolecules based on their density. A detailed protocol regarding this approach was published by Théry's group (Théry et al. 2006). In a floatation design, the mixture is placed at the bottom of a gradient tube, and molecules "float" up to their densities. Denser particles, such as nucleic acid and protein aggregates collect at the bottom, while smaller non-EV associated proteins collect above the EV band. This has allowed researchers to not only separate EV from contaminates, but identify subpopulations within the EV mixture (Bobrie et al. 2012; Jeppesen et al. 2014; Willms et al. 2018, 2016). In herpesvirology, gradient centrifugation has matured to the point were distinct packaging intermediates (A, B and C capsids) can be differentiated and isolated in high enough concentrations to perform biochemical assays and structural analysis (Trus et al. 2001; Deng et al. 2008)

While ultracentrifugation is a durable and time-tested method for the isolation of viruses and EV, there are some caveats to its employment. Isolation using ultracentrifugation results in abnormal size distribution profiles and flattening/lysing of EV (McNamara et al. 2018a; Sugita et al. 2011). Without carefully prepared cushions, viruses and EV will co-sediment with similarly weighted macromolecules such as protein aggregates and LDLs after prolonged spin cycles (Cvjetkovic et al. 2014). Of importance to our discussion, some viruses exhibit overlapping densities with EV such as exosomes and microvesicles, demonstrating that even carefully prepared sucrose/iodixanol/cesium chloride gradients cannot be used to conclusively separate them (Table 1) (Chu and Ng 2004; Feng et al. 2013; Lemon et al. 1985; McKnight et al. 2017; Ivanova et al. 2017; Ettelaie et al. 2014). Therefore, while gradients can be prepared to separate some more dense viruses from EV, co-contamination is expected for lower density families of viruses such as the Flaviviruses.

To conclude, ultracentrifugation is an efficient tool to concentrate viruses and EV from fluids such as tissue culture supernatant for analytical and small-scale experimental biochemistry. Typically, the input requires in excess of 5 mL and becomes impractical if more than 50 mL of input need to be processed. The method is not ideally suited for the separation of EV and viruses, particularly from infected cultures and/or body fluids and it is difficult to adopt ultracentrifugation to large-scale production environments or to environments that present biosafety concerns.

## Precipitation with Crowding Reagents

Crowding reagents such as polyethylene glycol (PEG) have been used for many years to precipitate macromolecules and complex protein structures, such as viruse particles, out of

**Table 1** Macromolecules such as viruses and extracellular vesicle subclasses are listed, along with their approximate densities and diameters. * For Hepatitis A Virus, the non-enveloped and cell-derived envelope data are merged (see (Feng et al. 2013; McKnight et al. 2017).

| Macromolecule | Density | Approx. Diameter (in body fluid) |
|---|---|---|
| Influenza virus | 1.25 g/cm$^3$ | 60–200 nm |
| HIV | 1.15–1.20 g/cm$^3$ | 120–150 nm |
| Hepatitis A Virus* | 1.06–1.28 g/cm$^3$ | 30–60 nm |
| West Nile Virus | 1.03–1.14 g/cm$^3$ | 40–70 nm |
| Exosome | 1.06–1.11 g/cm$^3$ | 40–150 nm |
| Microvesicles | 1.03–1.08 g/cm$^3$ | 100–800 nm |
| Apoptotic Bodies | 1.16–1.28 g/cm$^3$ | 800–5000 nm |
| LDL | 1.02–1.03 g/cm$^3$ | 20–28 nm |

solution. This precipitation of viruses and EV allows for lower speed centrifugation. Instruments using both larger (3000 mL) and smaller (0.2 mL) input volumes than ultracentrifugation can be used. PEG is the primary active ingredient in various exosome precipitation reagents that are sold commerically.

Similar to ultracentrifugation, precipitation is thoroughly reliable technique for the concentration of viruses out of solution. Successful precipitation protocols for diverse viruses such as herpesviruses, retroviruses, and bacteriophages exist with PEG and other crowding reagents (Adams 1973; Friedmann and Haas 1970; Orlando et al. 2000; Kutner et al. 2009). Given that viruses and smaller EV such as microvesicles and exosomes have similar biophysical properties, PEG precipitation has become a widely adapted procedure for EV isolation from fluids such as tissue culture supernatant and plasma. The introduction of PEG into a solution like plasma or tissue culture supernatant can be thought of as acting like a molecular fishing net: grabbing larger molecules (depending on the molecular weight of the PEG) into a dense cluster while allowing smaller molecules to be left in the solution. This allows for low-speed centrifugation (< 2000 * g) to pellet the virus and/or EV aggregates (Peterson et al. 2015; Hurwitz et al. 2016; Rider et al. 2016; Hurwitz et al. 2017; McNamara et al. 2018b).

A substantial advantage for crowding reagent precipitation is that it allows for large input volumes. While many ultracentrifuge units are limited based on the volumes that ultracentrifuge-compatible tubes can hold, precipitating using crowding reagents can be done at lower speeds, usually allowing for much larger input volumes as most desktop centrifuges can hold larger volumes (100 mL – 1 L) than ultracentrifuge units. Desktop centrifuge units are much cheaper than ultracentrifuges, and many institutions have an abundance/surplus of the former and a deficiency of the latter. From a cost-of-production standpoint, precipitation with crowding reagents enjoys a wide advantage compared to other methods.

Since PEG precipitation is able to pellet vesicular bodies such as viruses and EV, it suffers from the same caveat as ultracentrifugation: the inability to separate them. PEG precipitation is usually a more time-extended isolation method for EV, requiring hours of precipitation prior to any centrifugation. Like all biological molecules, there exist half-lives for viruses (infectability) and EV (enzymatic and endocytic activity). Therefore, a slight loss in biological activity should be expected. Additionally, electron microscopy (EM) mounting techniques often require the elimination of PEG and other crowding reagents prior to visualization. Crowding reagents also precipitate protein aggregates and non-EV associated extracellular nucleic acids. To that end, additional steps to remove the PEG are advisable prior to any functional assays or imaging (McNamara et al. 2018b; Rider et al. 2016; Chugh et al. 2013; McNamara et al. 2019; Soares Martins et al. 2018).

In sum, precipitation with crowding reagents is another highly successful method for the concentration of extracellular vesicular bodies such as viruses and EV. The ability to take EV or viruses out from a large volume of fluid without high-speed and damaging centrifugations presents itself as a great strength. Conversely, precipitations can take many hours. Precipitation with crowding reagents does more to concentrate viruses and EV rather than separate them away from contaminants.

## Crossflow Filtration

An emerging technology for the isolation of viruses and EV from solution is crossflow filtration (also referred to as tangential flow filtration). Crossflow filtration has been used for many years for the bulk production of biologically active molecules such antibiotics and antibodies, particularly in the biotechnology sector where adherence to good manufacturing practices (GMP) is paramount (Lebreton et al. 2008). In this method of filtration, solutions are passed tangentially across a membrane instead of head on. Molecules smaller than the weight cut-off filter are trapped inside the membrane and eventually removed into a discard (permeate) chamber. The solution retaining molecules not captured by the membrane return to the crossflow chamber (retentate) and are continuously pumped through the system as the volume decreases. This results in a concentration of filtered solution in the crossflow chamber. In addition, most crossflow filtration units have an equilibration tank to exchange buffers and wash the filtered solution as it is being concentrated (Fig. 2).

Multiple independent groups have proposed using crossflow filtration as a first step in virus and EV isolation (Grzenia et al. 2008; McNamara et al. 2018a; Wickramasinghe et al. 2005; Corso et al. 2017; Castro-Mejía et al. 2015; Busatto et al. 2018; Lamparski et al. 2002). This can be accomplished through the use of high molecular weight membranes, which allow for the retention of macromolecules such as viruses and EV while smaller molecules are lost to the permeate. There are several distinct advantages to using crossflow filtration: (I) industrial-scale input volumes can be used as many crossflow filtration units hold liter-level volumes; (II) buffer exchanges and real-time washing during concentration; (III) a lack of potentially damaging high-speed centrifugation steps.

Since crossflow filtration is an emerging field of concentration and purification of viruses and EV, it is likely that studies and innovations in the coming years will improve upon this technique. As it stands, there are a few pitfalls to employing crossflow that need to be addressed. Like the other methods, crossflow filtration does not allow for the separation of viruses and EV. While hollow-fiber cartridges can vary in their pore size (from purification of antibodies to large virus isolation), viruses and EV have too similar a size to be separated by this method. Therefore, regardless of the conditions used, the separation of viruses from EV is not feasible by exclusively using simple crossflow filtration (McNamara et al. 2018a). Another caveat to using crossflow is that the technology was designed for large input volumes. Using crossflow on a small input volume, such as a few mL of human plasma, but across many samples, would require a new/modified instrument design. Therefore, while crossflow-based isolation of EV and viruses could have major upside in the biotechnology sector for mass production under GMP, its use for analytics and biomarker explorations in smaller clinical specimens is limited.

By contrast to ultracentrifugation and crowding reagent precipitation, crossflow filtration is designed to purify molecules away from contaminants *while* concentrating them. In that regard, it can be thought of as a hybrid of concentration and purification strategies. While it cannot separate viruses from EV due to overlapping sizes and biophysical properties, it can remove non-virus/EV associated molecules such as extracellular Ago-RNA complexes and albumin, increasing the particle:protein ratio. Albumin removal, in particular, has been the subject of detailed studies as albumin represents the major protein compound of plasma (Arroyo et al. 2011; Webber and Clayton 2013; McNamara et al. 2018a; Welton et al. 2015; Busatto et al. 2018; Grzenia et al. 2008; Wickramasinghe et al. 2005). Similar to column chromatography, crossflow does not exert extreme biophysical forces onto the EV.

## Column Chromatography

Column chromatography has been employed for the separation of molecules based on their sizes for decades. There are two main types: exclusion chromatography based on size and chromatography based on charge, hydrophobicity, or another affinity measure. There have been a number of modifications to column chromatography for the enhancement of final molecule purity, biological activity, and binding capacity. New columns have been developed recently to improve the isolation of viruses and removal of albumin (Baranyai et al. 2015):

(A) Capto Core resin distributed by General Electric, was specifically designed to remove poultry albumin contaminants in the flu vaccine development pipeline (Blom et al. 2014). This column resin was developed to improve upon other flu isolation and purification strategies such as zonal ultracentrifugation. In recent years, Capto Core has been adapted for the purification of EV. Capto Core resin has a very high capacity, and eluted material retains biological activity – whether it is EV or viruses (James et al. 2016; McNamara et al. 2018a; Reiter et al. 2018; Corso et al. 2017).
(B) Another recently developed chromatography-based isolation method is the qEV column from Izon. Independent groups have shown that the qEV columns outperform other methods such as ultracentrifugation in removing excess albumin from EV preparations, increasing the particle:protein ratio and removing non-EV associated contaminants. Importantly, several of these observations were in the context of biological fluids, which are rich in non-EV associated contaminants (Stranska et al. 2018; Lobb et al. 2015; Smith and Daniel 2016; Davis et al. 2019).

As exemplified above, column chromatography for the purification of live-virus and active EV is a technology that has seen considerable advancement in the past few years; however, column chromatography still suffers from a few drawbacks. Perhaps the most consequential drawback to using column chromatography is that it limits the input volume size. This is in proportion to bed volume of the beads. For this reason, several groups have proposed using a concentration step prior to use of specialized columns (McNamara et al. 2018a; Corso et al. 2017; McNamara et al. 2019; Davis et al. 2019). In the case of size exclusion chromatography, EV cannot be separated from viruses given their similar sizes.

Column chromatography has been widely used in academic and industrial settings for decades given its reliability. It is used to elute proteins and complexes into fractions based on their biophysical properties. Because of this, column chromatography is viewed more as a purification strategy as opposed to a concentration strategy. While fractions containing EV will likely be more concentrated than the input solution, chromatography is meant to be used more for the separation of contaminants.

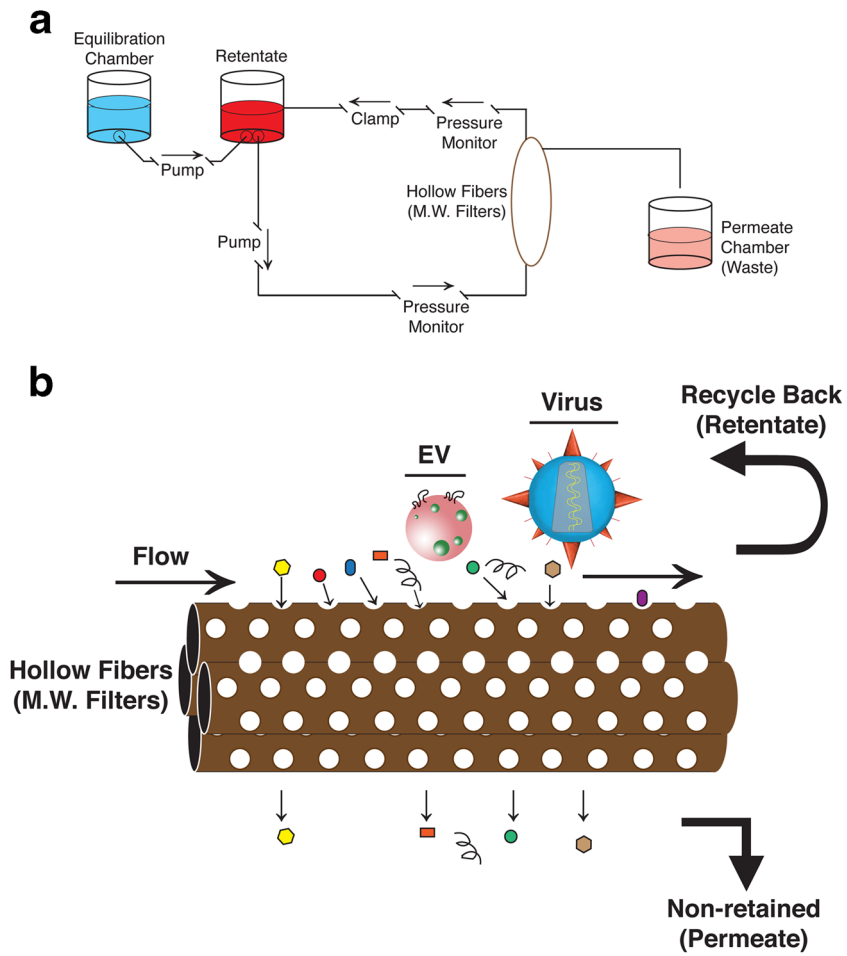



**Fig. 2 Diagram of crossflow filtration. a** Design of a basic crossflow filtration unit. A retentate tank is loaded with input solution and processed through. Molecules not meeting the molecular weight cutoff are lost to the permeate while molecules meeting the cutoff are returned to the retentate. A separate tank containing equilibration solution can also be pumped into the retentate tank to equilibrate/wash the filtered and concentrated solution. **b** Zoomed in representation of hollow-fibers used in crossflow filtration.

## Affinity Purification

Affinity purification, which can involve antibody or specific-protein based capture systems such as streptavidin, is a manner to biochemically purify a discreet population from a heterogeneous mixture. Therefore, this method can be used for separation of viruses and EV given the differences in membrane/capsid composition.

Several antibody-based affinity-capture beads are available for the purification of EV. Most of these capture beads take advantage of the tetraspanin proteins present on the surface of EV. Tetraspanins are incorporated onto the EV membrane during trafficking through the endosomal recycling pathway. The tetraspanin-coated EV (most likely exosomes), are biologically active post capture/elution from the beads, and are competent for uptake by recipient cells (McNamara et al. 2018b; Chugh et al. 2013; McNamara et al. 2019). Moreover, affinity beads can be used to analyze biophysical properties of the immobilized EV through microscopy, sequencing, and flow cytometry analyses (Smith and Daniel 2016; Mukherjee et al. 2016; Peterson et al. 2015; Raab-Traub and Dittmer 2017; Théry et al. 2006).

A similar approach can be taken to isolate viruses based on their surface-exposed proteins. Purification of virus particles from solutions such as blood plasma using antibody-coated beads has been extensively used over the years, with a particular emphasis on vaccine development (Davenport et al. 2011; Sellhorn et al. 2009; Ceglarek et al. 2013). The antibody-mediated capture premise is the same as a typical enzyme-linked immunosorbance assay (ELISA), with the exception that ELISAs are meant to quantitate virus particle concentration and not purify a functional product. Moreover, polymers with a natural affinity for virus epitopes have been used to increase binding capacity (Sakudo et al. 2009, 2016; Patramool et al. 2013; Sakudo and Onodera 2012). Similar to EV, immobilized virus particles can be used for flow cytometry analysis. This premise can be expanded upon to analyze for differences/abundance of certain viral particle subspecies, such as HIV-1 particles containing functional trimeric Env spikes (Arakelyan et al. 2013, 2017).

When it comes to affinity selection of EV and/or viruses, the approaches are limited by the binding capacity of the beads and the ability to elute off a functional product. The binding capacity can be attributable to: (I) affinity of the antibody/polymer for its antigen; (II) accessibility of antigens

to antibodies/polymers through steric hindrance on the bead; (III) stability of the epitope on the EV or virus surface. In regard to elution, many protocols utilize either proprietary buffers or acidic glycine to remove EV or viruses from the beads (McNamara et al. 2018b; Jørgensen et al. 2013; McNamara et al. 2019; Tauro et al. 2012). Prolonged presence in an acidic solution denatures proteins, causing a loss in virus infectability and/or EV functionality. Therefore, the use of affinity purification can result in a substantial loss of biological integrity and functionality.

Affinity purification remains the Gold Standard for the isolation of a homogenous entity from a heterogeneous input, including the successful separation of virus particles from EV and vice versa. It maximizes specificity at the cost of sensitivity/yield and functional integrity.

## NanoFACS and Flow Virometry

Another methodology to separate viruses from EV that has been explored and improved in recent years is a nanoscale flow cytometry approach. Similar to fluorescence assisted cell sorting (FACS), nanoscale flow cytometry and nanoFACS (also called vesicle flow cytometry) are meant to identify and sort EV subpopulations based on a heterogeneous input population (van der Pol et al. 2018; Nolan and Duggan 2018; Lippé 2018). Limitations to this technology are largely based on the inability of small particles (i.e. EV < 200 nm and both enveloped and non-enveloped viruses) to scatter significant amounts of light. The addition of antigen/substrate-specific fluorophores to the EV/virus mixture, coupled with size exclusion chromatography to remove unbound fluorophores, allows for the particles to scatter enough light for specialized cytometers to differentiate above background (Morales-Kastresana et al. 2017; Tang et al. 2017; Musich et al. 2017; Arakelyan et al. 2013; El Bilali et al. 2017; Loret et al. 2012; Tang et al. 2016). A similar approach has been utilized for endogenously labeled viruses, such as fusion of a GFP to HIV-1 gag protein (Bonar and Tilton 2017; Dale et al. 2011; Hübner et al. 2009).

A powerful advantage to nanoFACS is that it can be employed to rapidly characterize heterogeneous input mixtures without the need to concentrate them first. This is owed to the high concentration of EV released during physiological conditions ($10^9$–$10^{11}$ particles/mL) (Mathieu et al. 2019; Thery et al. 2001, 2018; Raab-Traub and Dittmer 2017), or the high concentration of virus particles released during pathogen challenge (up to $10^9$ infectious units/mL depending on the virus) (Bonar and Tilton 2017; Morales-Kastresana et al. 2017; Tang et al. 2016). Moreover, sorting of EV and viruses based on their fluorescence allows for the recovery of a functional product, similar to cell-based FACS, and the sorted fraction has unsurpassed purity and retains biological activity.

A caveat to nanoFACS and flow virometry is that it sorts the small particles into larger volumes of flow-capture solution. These need to be concentrated to visualize by techniques such as immunoblot. Few standards exist to accommodate for the use of different cytometers by different groups; however nanoFACS presents itself as an incredibly powerful technique to separate viruses and EV from contaminating material.

## Phenotypic Characterization

In the isolation of EV and viruses it is critical to properly characterize their biological function and activity in addition to ensuring biophysical homogeneity. While viruses exert easily discernable phenotypes upon infection of a cell, the effects of EV on recipient cell physiology are more diverse, subtler, and less understood. EV have been shown to exert a bevy of effects in recipient cells (Kalamvoki et al. 2014; Bridgeman et al. 2015; Kitai et al. 2017; Baglio et al. 2016; Raab-Traub and Dittmer 2017; Chugh et al. 2013; Barclay et al. 2017; Hurwitz et al. 2017, 2018; Meckes et al. 2010, 2013). Therefore, experimental design must be considered carefully, with all necessary controls accounted for.

When it comes to understanding the phenotype of EV on recipient cells, one tool that has become widely used for their

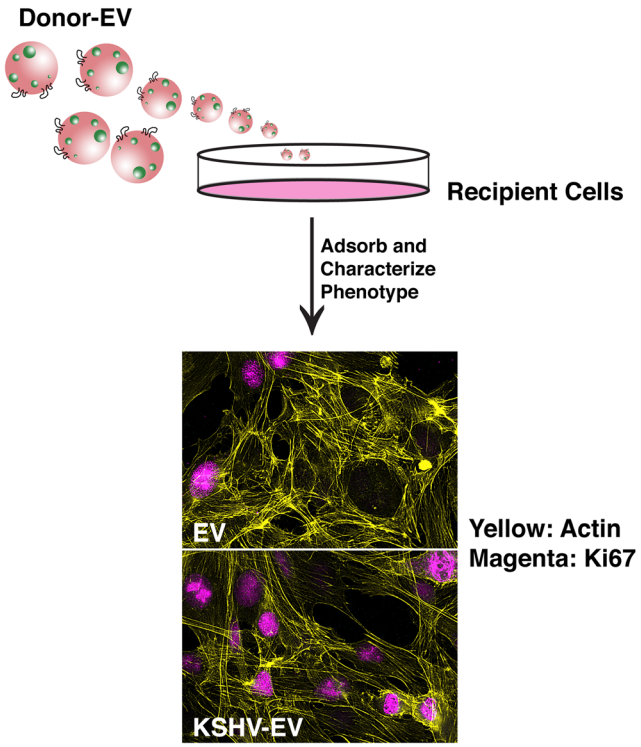



**Fig. 3 Representative image of a phenotypic characterization of EV treatment.** Purified EV are added to cells and any number of phenotypic consequences can be monitored. In this case, EV taken from healthy donor (EV) or from KSHV-infected cells (KSHV-EV) were added to cells and Ki67 staining was monitored. An increase in Ki67 positive cells was observed in the KSHV-EV group (also see (McNamara et al. 2019)).

**Table 2** List of employed techniques for the isolation of EV and viruses, along with two pros and cons of each method.

| Technique | Pros | Cons |
|---|---|---|
| Ultracentrifugation | • Rapid isolation from fluids such as tissue culture supernatant or body fluids<br>• Can be modified with density gradients | • Overlapping densities between viruses and EV can cause co-contamination<br>• Centrifuges limited by volume capacity |
| PEG Precipitation | • High input volume<br>• Low speed centrifugation steps to prevent high *g*-force | • Precipitates larger macromolecules, so virus/EV co-contamination is high,<br>• Higher contamination with protein aggregates |
| Crossflow Filtration | • Can concentrate large volumes while removing contaminating protein aggregates<br>• Allows for buffer exchange | • Viruses and EV are similar in size and molecular weight, and will co-contaminate preparations<br>• Only useful when starting with large volumes |
| Column Chromatography | • Separates viruses/EV from contaminating material<br>• Cost efficient | • Not per se a concentration method<br>• Viruses and EV will fractionate similarly |
| Affinity Purification | • Epitope-specific purification that allows for separation of viruses from EV<br>• Can select for sub-populations of EV and viruses | • Saturation of epitope-binding beads can lead to product loss<br>• Can require initial concentration step. |
| nanoFACS | • Can separate EV from viruses based on indirect fluorescence labeling or de novo labeled proteins (such as GFP-Gag, GFP-HSV-1 fusion proteins, etc.)<br>• Does not require initial concentration step | • Only certain flow cytometers calibrated for nanoFACS<br>• Sample usually needs to be diluted prior nanoFACS and may be further diluted post sorting. |

study are infections with viruses. A number of evolutionarily distinct viruses have been shown to usurp EV-mediated signaling (M. Anderson et al. 2018; 2016; Raab-Traub and Dittmer 2017). Therefore a virology-based approach to characterize how EV modulates local homeostasis great promise. In order to properly ascribe an observed phenotype as a consequence of EV addition, experimental groups must account for the ability to separate EV from virus particles in their experimental setup. This can be complicated by the incorporation of viral factors into secreted EV from infected cells such as viral proteins (such as EBV LMP1, HIV/SIV Nef) and nucleic acids (such as HCV RNA, EBV/KSHV miRNAs) (Hurwitz et al. 2017, 2018; Meckes et al. 2010, 2013; Verweij et al. 2011; Yogev et al. 2017; Khan et al. 2016; Kirchhoff et al. 2008; Lee et al. 2016; Lenassi et al. 2010; McNamara et al. 2018b; Narayanan et al. 2013; Pereira and daSilva 2016; Sami Saribas et al. 2017; Mukhamedova et al. 2019; Chugh et al. 2013; McNamara et al. 2018a, 2019; Bukong et al. 2014; Longatti et al. 2015; Ramakrishnaiah et al. 2013). In the case of HAV, in which a fully infectious virion can be incorporated into an EV (Feng et al. 2013; Lemon et al. 1985; McKnight et al. 2017; Rivera-Serrano et al. 2019). Multiple experiments need to be coupled to conclusively show that any viral components detected in an EV preparation are not due to the presence of contaminating virus particles. Such assays include, but are not limited to: (I) viral genome and miRNA-specific q(RT)-PCR, (II) high-sensitivity antigen detection assays, (III) virus propagation assays (plaque assays, viral genome reproduction, etc.) post EV treatment.

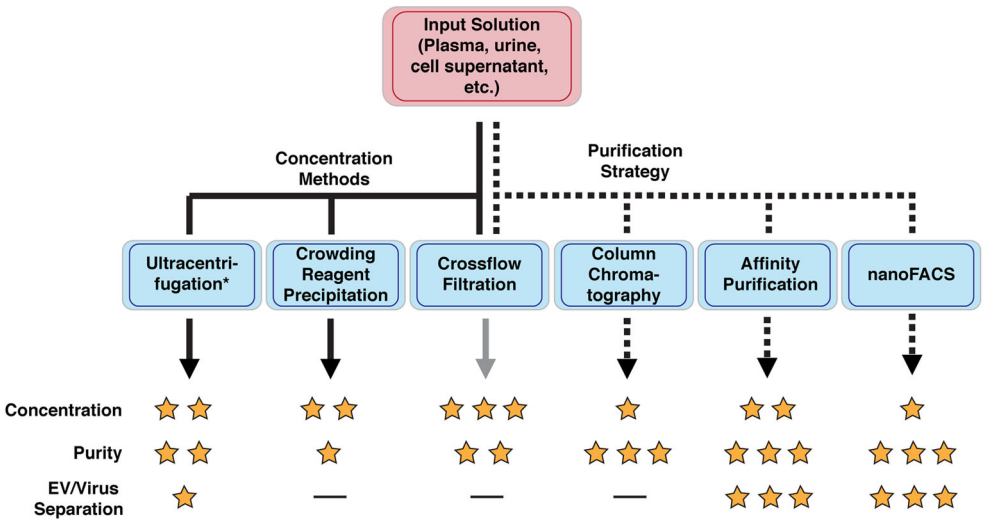



**Fig. 4 Summary of virus/EV isolation strategies.** The overviewed methods are summarized here. Based on cited literature, estimates were made to judge each procedure on their ability to concentrate viruses/EV, the relative purity of the product, and the ability to separate viruses and EV. The asterisks by ultracentrifugation is to represent that this encompasses gradients as well (through gradients, some viruses and EV can be separated).

Several assays have been utilized to show the functional consequence of EV-uptake, particularly in the context of pathogen challenge. Such functional assays include, but are certainly not limited to: gene expression reprogramming (Hurwitz et al. 2017; Barclay et al. 2017; McNamara et al. 2019), cellular adhesion/migration (Chugh et al. 2013; Koumangoye et al. 2011), signaling pathway activation (Chugh et al. 2013; Meckes et al. 2010; Kalamvoki et al. 2014), cell proliferation (Keller et al. 2009; Koumangoye et al. 2011; Ahsan et al. 2016), cell differentiation (Chowdhury et al. 2015; Webber et al. 2015), and apoptosis (Lenassi et al. 2010; Ren et al. 2011) (see also reviews by (Anderson et al. 2016; 2018; Raab-Traub and Dittmer 2017). Mechanisms by which EV elicit these phenotypes are still being deduced but are likely directly tied to the modified cargo (Fig. 3).

It is important to differentiate functional or phenotypic characterization and biochemical characterization. Characterizations of EV and viruses by approaches such as quantitative polymerase chain reaction (qPCR), nanoparticle tracking analyses (NTA), and protein-specific immunoblotting are not usually phenotypic characterizations. While biochemical assays such as these are critical for proper characterizations of the input EV/viral pool (Théry et al. 2018), the consequence of adding them to cells is of great importance as these fields continue to progress.

## Future Directions and Concluding Remarks

The purification of EV and virus particles is a rapidly evolving field. This review was meant to introduce and comment on commonly employed techniques, as well as newly developed technologies or modifications to existing ones (Table 2). This includes the emerging field of nanoFACS and flow virometry. Additionally, this review was composed to cover the basic science, manufacturing and lab-based assays as related to virus and EV purification and characterization; the diagnostic arena was not our focus, and we would refer the interested reader to other reviews (Rodrigues et al. 2018; Wu et al. 2019; Xu et al. 2018).

As previously mentioned, one aspect of the EV field of study that is garnering significant attention are mechanisms by which they exert a phenotypic response from recipient cells. Activation of signaling pathways has been observed in cells treated with EV; however, the discreet mechanisms by which EV exert pathway specific activation are still being deduced. The transfer of small molecule agonists and metabolites, for instance as produced by virally infected cells, through EV has been described (Kalamvoki et al. 2014; Zhao et al. 2016). This further supports the model that EV act to maintain equilibrium in their close environment.

Virus particles and EV contain many similarities such as densities, radii, maturation, and the ability to serve as packaging systems for nucleic acids (Bousse et al. 2013; Chu and Ng 2004; Feng et al. 2013; Lemon et al. 1985; Théry et al. 2018; Willms et al. 2016; Vader et al. 2014; Anderson et al. 2018; 2016). Given these many overlapping properties, it is not surprising that differentiating between the two in fluids such as plasma and tissue culture supernatant has proven challenging. Their functions, however, are quite different. Viruses usurp a number of cellular processes for the sole reason of propagation. The emerging picture of EV is that they serve to transmit information locally, maintaining an equilibrium. Given this role, it is not surprising that many viruses have evolved to manipulate EV cargo.

In conclusion, there exist multiple ways that have been developed to isolate, purify and concentrate viruses, and these can easily be applied to EV. At the same time, it is very difficult to separate EV from viruses by these methods for all the aforementioned reasons (Fig. 4). When dealing with mixed populations of EV and viruses, it is imperative that these two entities are separated to avoid misinterpretations of data. Major advances when it comes to isolation of pure and functional EV are needed and are bound to drive the field forward.

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