Extracellular Vesicles

Chantal Boulanger, Guest Editor

Methodological Guidelines to Study Extracellular Vesicles

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Abstract: **Owing to the relationship between extracellular vesicles (EVs) and physiological and pathological conditions, the interest in EVs is exponentially growing. EVs hold high hopes for novel diagnostic and translational discoveries. This review provides an expert-based update of recent advances in the methods to study EVs and summarizes currently accepted considerations and recommendations from sample collection to isolation, detection, and characterization of EVs. Common misconceptions and methodological pitfalls are highlighted. Although EVs are found in all body fluids, in this review, we will focus on EVs from human blood, not only our most complex but also the most interesting body fluid for cardiovascular research. (*Circ Res*. 2017;120:1632-1648. DOI: 10.1161/CIRCRESAHA.117.309417.)**

Key Words: cardiovascular diseases ■ extracellular vesicles ■ exosomes ■ methods ■ reference standards

Overview

All body fluids contain cell-derived membrane-enclosed vesicles. Such vesicles are shed by prokaryotes and eukaryotic cells and contain messages to the environment. Cell-derived vesicles are thought to contribute to homeostasis, disease development, and progression,[1–9] may provide novel biomarkers,[10,11] and may be suitable for use as therapeutic drug carriers.[12–16]

Various misconceptions and methodological pitfalls have hampered progress in understanding the biological function of these vesicles. First, the independent discovery of vesicles in different fields has led to confusing nomenclature because vesicles were named after their function or biogenesis.[17–21] Because no straightforward criteria exist to distinguish, isolate, and identify (sub)populations of cell-derived vesicles, the term extracellular vesicles (EVs) was introduced by International Society of Extracellular Vesicles (ISEV). We will also use EVs as the common and collective term for the entire population of cell-derived vesicles present in body fluids. The nomenclature used in this review is defined in Table 1. Second, in the emerging field of EV research, many biological effects attributed to EVs could also be caused by the presence of non-EV components in preparations of EVs.[22–24] Third, the scientific community increasingly recognizes the need to standardize methodology and technology[25–27] because standardization is a prerequisite to validate EV-associated biomarkers.[28–30] To improve the reliability and credibility of the reported

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Nonstandard Abbreviations and Acronyms

DC	differential centrifugation
DGC	density gradient centrifugation
EM	electron microscopy
EV	extracellular vesicle
FC	flow cytometry
FXa	coagulation factor Xa
HDL	high-density lipoprotein
IC	immunocapture
ISEV	International Society for Extracellular Vesicles
LDL	low-density lipoprotein
miRNA	micro-RNA
qPCR	quantitative polymerase chain reaction
RPS	resistive pulse sensing
SEC	size exclusion chromatography
TF	tissue factor

findings, ISEV has recommended minimal requirements for definition of EVs, the minimal experimental requirements for definition of extracellular vesicles and their function criteria,[31] a novel EV-TRACK (transparent reporting and centralizing knowledge in extracellular vesicle research) platform has been launched to stimulate the reporting of experimental parameters to interpret and reproduce an experiment,[27] and ISEV, the International Society on Advancement of Cytometry and the International Society on Thrombosis and Haemostasis, have joined forces to standardize detection of EVs by flow cytometry (FC; http://www.evflowcytometry.org/).[32]

Detection of EVs is prone to artifacts partially caused by sample collection and EV isolation (Figure 1). We will discuss the Collection and Handling of Samples, the Isolation and Concentration of EVs, and downstream analysis, including the detection of single EVs (See Methods to Measure Single EV section of this article), and assays to determine EV contents and function (see Measuring the Composition and Function of EV section of this article). We will focus on circulating EVs because blood is easily accessible, routinely isolated, and the most relevant body fluid for cardiovascular

Table 1. Definitions of the Terms

Term	Definition
Circulating EVs	All EVs present in blood; includes EVs from platelets, leukocytes, erythrocytes, endothelial cells, and EVs from tissues
Concentration	Method to increase the number of EVs per unit volume or the number of EVs per unit volume
Downstream analysis	Characterization of EVs after isolation
Isolation	Separation of EVs from non-EV components present within the starting material, including proteins, lipoproteins, etc
Purity	Ratio between EVs and non-EV components
Recovery	Percentage of total EVs preserved after isolation

EV indicates extracellular vesicle.

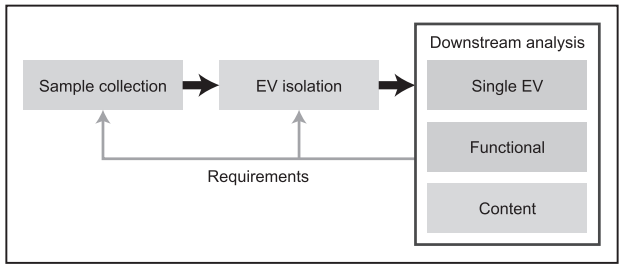

Figure 1. Structure of the review. Sample collection and isolation of extracellular vesicles (EVs) influence the results of subsequent downstream analysis (black large arrows). The requirements of the intended downstream analysis must be considered when designing sample collection and isolation (gray small arrows).

research. Still, most of the considerations and recommendations, summarized in Figures 1, 2, 4, 6, and 7, will also hold true for other body fluids and conditioned culture media and will improve the reliability of results from studies on EVs.

Collection and Handling of Samples

Introduction

The preanalytical phase is an important source of variability and contributes to artifacts. Because blood cells, particularly platelets, become easily activated and release EVs during sample collection and handling, the preanalytical protocol should prevent platelet activation. Both International Society on Thrombosis and Haemostasis and ISEV have provided guidelines,[25,28] but these guidelines may be outdated as they are based on insensitive detection methods.

Blood

Collection of Blood

General recommendations from routine laboratories on blood collection can be applied to EV studies. With regard to the subjects from which blood is collected, variables such as age, circadian cycle, and sex awaits investigation, but when practically feasible overnight fasting is preferred.[24] Plasma is usually the preferred source of EVs because additional EVs are released during the clot formation when preparing serum.[33] Currently, the main application of serum is the study of small RNAs, such as micro-RNAs (miRNA).[34,35]

To prepare plasma, blood requires anticoagulation. Several anticoagulants have been used to collect blood for analysis of EVs, including EDTA, sodium fluoride/potassium oxalate (NaF/KOx), or (trisodium) citrate.[28,36,37] At present, citrate (0.109 mol/L final concentration) is the most commonly used anticoagulant and has been recommended by the International Society on Thrombosis and Haemostasis.[25] Both acid citrate dextrose and citrate, theophylline, adenosine and dipyridamole prevent platelet activation and the release of platelet EVs more efficiently than citrate.[26,38,39] The choice of anticoagulant strongly depends on the downstream analysis, and, for example, EDTA is a suitable anticoagulant for RNA analysis,[40,41] whereas heparin interferes with polymerase chain reaction (PCR).[42] Taken together, both the extent of inhibition of EV release in collected blood samples ex vivo and the intended downstream assays should be taken into account when choosing an anticoagulant.

Considerations and Recommendations

- Collect blood from overnight fasting subjects.The choice of anticoagulant depends on downstream analysis.
- Avoid prolonged use of a tourniquet[43] and use a large diameter, 21-gauge needle.[44–46]
- Discard the first 2 to 3 mL of collected blood[47,48] and collect blood in plastic collection tubes at room temperature (see also Coagulation section of this article).
- Properly fill the tubes to obtain the appropriate blood to anticoagulant ratio and mix gently.[49]
- Keep the blood collection tubes in a vertical position during transport.
- The time interval between blood collection and the first centrifugation step to prepare plasma should be minimized or at least be kept constant between samples, to limit effects on the concentration and functional activity of EVs.[39,50–52]
- Preferably, no measurements of EVs in hemolyzed samples should be done. If hemolyzed samples are included, the obtained results should be interpreted with care[28] and the degree of hemolysis should be measured.[53]

Preparation of Plasma and Serum

Although EV analyses in whole blood have been reported,[54,55] the number of applications is limited because whole blood precludes storage and isolation of EVs. Therefore, we will focus on the preparation of plasma and serum.

To obtain plasma, anticoagulated blood is centrifuged to remove erythrocytes, leukocytes, and platelets.[44] Platelet removal is essential because platelets release EVs on activation and fragment during a freeze–thaw cycle.[50,56] Because a substantial number of platelets persist after a single centrifugation step, a double spin is recommended. Nevertheless, still some residual small platelets and erythrocyte ghosts will remain in the platelet-free plasma.[57]

Considerations and Recommendations

- Centrifuge blood at room temperature.
- Remove platelets by using 2 subsequent centrifugations steps of 2500*g* for 15 minutes as recommended by International Society on Thrombosis and Haemostasis,[25] and use a clean plastic tube for the second centrifugation step.
- To reduce the risk of platelet and leukocyte contamination do not collect the last 0.5 cm of plasma above the buffy coat and set the lowest deceleration on the centrifuge.
- Quantify residual platelets in platelet-free plasma.
- Removal of platelets may also remove large EVs such as apoptotic bodies and oncosomes.
- Apply identical centrifugations conditions, including speed, deceleration, rotor, and temperature, to each sample within a study.
- Plasma is recommended for most applications because serum contains additional vesicles which are released during in vitro clot formation.

Culture Media

EVs can also be isolated from conditioned cell culture media. A main source of contaminating EVs and detectable non-EV components is the serum in the culture media. If the cells cannot be grown in serum-free medium,[58,59] dedicated bioreactors may be an alternative solution.[60]

Considerations and Recommendations

- Remove EVs from the serum by ultracentrifugation before use[61] or purchase EV-free serum and analyze for the presence of EVs.
- Use nonconditioned culture medium as control in downstream analysis.
- The influence of growth factors and other additives on the type and number of EV produced in cell culture is largely unknown. Established protocols for the production of cell culture EV are needed.

Storage

EVs in plasma seem stable during a freeze–thaw cycle and storage.[50,62–65] The effect of additives to protect EVs against freeze–thaw damage, however, awaits detailed investigation.

Considerations and Recommendations

- Use storage vials with a screw lid and rubber ring to reduce freeze-drying artifacts during storage.
- To prevent formation of ice crystals and to reduce cryoprecipitation, snap-freeze aliquots in liquid nitrogen,[36] store aliquots at or below −80°C, and thaw at 37°C.[37,66–68]
- Avoid repeated freeze–thaw cycles.[52,62]
- To which extent EVs expose phosphatidylserine in the circulation is unknown. Likely, in older studies, the presence of residual platelets explain the reported increase in phosphatidylserine exposure of EVs observed after freeze–thawing.

Summary

Because collection, handling, and storage affect the concentration, composition, and function of EVs, the preanalytical phase can have a major impact on downstream analysis. Therefore, an optimal protocol is tailored to the type of (body) fluid, the type and/or cellular origin of the EVs of interest, and the downstream analysis. Please note that the recommendations described in Blood section of this article and summarized in Figure 2 are based on detection methods only sensitive to detect large EVs. These recommendations are probably also valid for smaller EVs, but more research is needed and ongoing to confirm their validity.[69] The relationship between anticoagulant and performance of the downstream implies that a biorepository suitable for different downstream applications requires blood collection in multiple (different) anticoagulants. Clearly, an urgent need exists to establish and validate guidelines for preparation and storage of samples for EV research, because only then reliable and clinically relevant biorepositories can be established.

Isolation of EVs

Introduction

Blood is the most commonly studied body fluid and also the most complex body fluid containing not only EVs but also cells, proteins, lipids, and nucleic acids.[70] To study EVs from blood, the use of isolated EVs is often desirable. Because there is no method that will isolate EVs only, the researcher should be aware of the coisolated non-EV components. Such components include soluble proteins, protein aggregates, lipoproteins (especially high- and low-density lipoproteins [HDL and LDL, respectively]), and other particles including cell organelles and viruses.[24] For example, when studying the presence

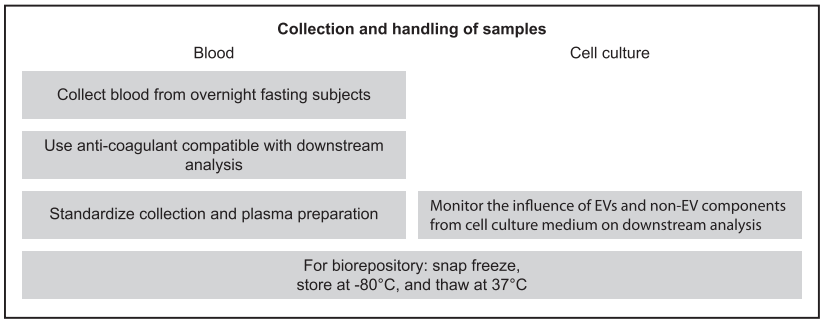

Figure 2. Overview of considerations and recommendations for the collection and handling of samples. EV indicates extracellular vesicle.

of miRNA in EVs isolated from plasma by density gradient centrifugation (DGC), contamination with HDL-associated miRNA should be considered.[71]

At present, the isolation methods have not been compared with each other using a single EV sample and a single detection method. Consequently, quantitative comparisons on recovery and purity of EVs between the various isolation methods are not yet possible.

Methods to Isolate EVs

Different biophysical and biochemical properties can be used to isolate EVs, including size, mass density, shape, charge, and antigen exposure. The principles of the most common EVs isolation methods are presented in Figure 3. Table 2 provides a comparison of these methods including advantages and limitations of each method. All isolation methods affect the concentration of EVs, some methods may be used solely to concentrate EVs, and some methods can be combined.

Considerations and Recommendations

- Isolation is the key determinant of the outcome of any EV measurement; when possible, determines the effect of the isolation or concentration method on size, integrity, morphology, recovery, concentration, and functional properties of EVs, non-EV components, and on the downstream analysis.
- The end product should be characterized for the presence of EVs, for example, by transmission electron microscopy.[31]
- The presence the non-EV components LDL, HDL, and chylomicrons can be quantified by measuring ApoB100, ApoA1, and ApoB48, respectively.
- To quantify the isolation efficiency, the ratio of 3×10^{10} EVs per μg of protein or greater has been proposed as high purity.[89] However, the estimated concentration of EVs is detection method dependent (see Methods to Measure Single EV section of this article); therefore, the EV to protein ratio should be interpreted with caution.
- To ensure methods reporting is adequate for interpretation and experimental reproduction, apply EV-TRACK before publication.[27]

Differential Centrifugation

Differential centrifugation (DC; Figure 3A) isolates EVs based on their size and density by sequentially increasing the centrifugal force to pellet cells and debris (<1500*g*), large EVs (10 000–20 000*g*), and small EVs (100 000–200 000*g*).[72] Although well established and commonly used, DC has major limitations.

First, DC cannot achieve absolute separation of EVs by size alone because the distance to the pellet is not the same for all EVs, and the EV sedimentation rate also depends on the shape and mass density relative to the medium.[72,73] Second, DC may result in clumping of EVs,[69,74] coisolate non-EV components such as protein aggregates[22] and viruses,[75] and damage EVs during the final ultracentrifugation step.[76] Third, the reported recovery of EVs by DC ranges from 2% to 80%, making the study-to-study comparability questionable.[64,90] DC may be applied to concentrate the sample ≈8-fold. DC is not suitable in a clinical setting because DC is laborious, time-consuming, and low throughput.

Considerations and Recommendations

- For viscous fluids such as plasma, dilute the sample at least 2-fold with buffer before centrifugation to enhance the isolation efficiency of EVs.[91] Alternatively, centrifugation speed and time can be increased.[92]
- Non-EV components that copellet with EVs during centrifugation will copellet during identical repeated centrifugation steps.[89]

Density Gradient Centrifugation

DGC (Figure 3B) applies a density gradient to isolate EVs.[93] Isolation depends on the size and mass density (top-down gradient) or mass density only (bottom-up gradient). Sucrose and iodixanol are the most commonly used density media used to isolate EVs.[94] In contrast to sucrose, iodixanol is iso-osmotic, inert, nontoxic, self-forming, and less viscous, thus requiring shorter centrifugation time. Importantly, iso-osmotic has 2 different meanings in this context. First, the osmolarity of the density medium is similar to that of EVs. Second, the gradient layers of the density medium all have similar osmolarity. When the osmolarity is constant throughout the gradient, no changes will occur in the volume and thus in the density of the EVs during centrifugation. Iodixanol-based gradients obtain a better resolution than sucrose.[75]

When EVs are isolated from plasma or serum, the major coisolate is lipoproteins, that is, particles with a comparable density. Although HDL particles have a density comparable to EVs, LDL has a floatation density lower than that of either EVs or HDL, but the reported presence of LDL in density gradient ultracentrifugation-purified EV preparations suggest an interaction of EVs with LDL.[24] Typically, there is no net effect on the sample volume, and EV recovery is 10% to 50% depending on removal of the density medium from the sample. DGC prepares EVs devoid of protein contaminants but is also laborious, time-consuming, and low throughput, which hamper the use in a clinical setting.

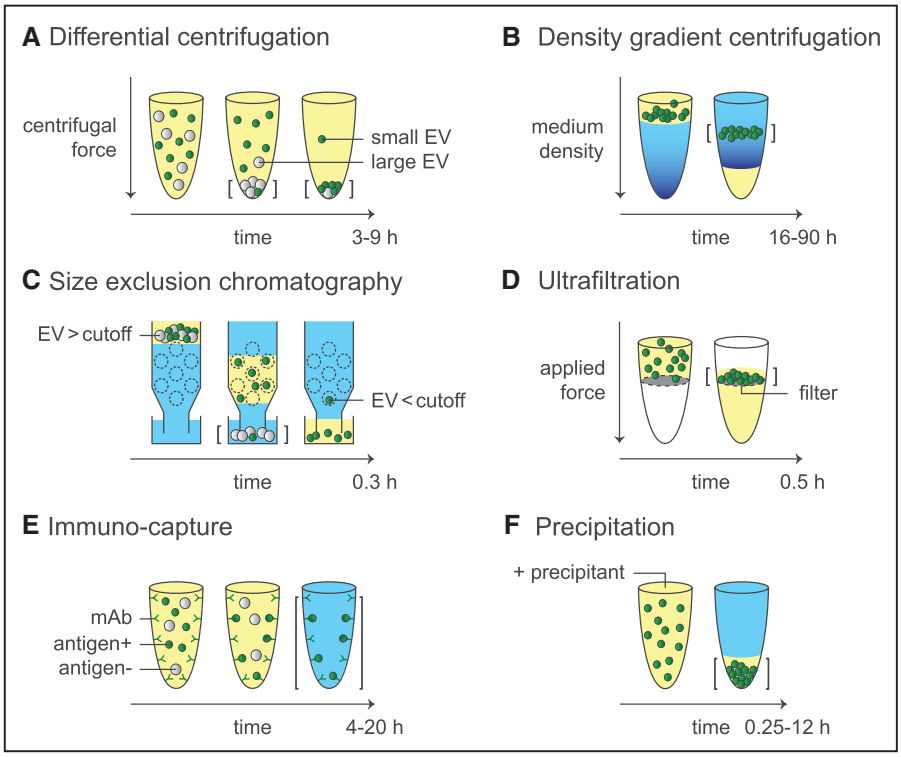

Figure 3. Working principle of common methods to isolate extracellular vesicles (EVs). Separation is based on size, density, and immunophenotype. Straight brackets: isolated EVs; yellow: soluble components; and blue: buffer. **A**, In differential centrifugation, separation is based on size, and large EVs (gray) collect earlier at the bottom of the tube and at lower *g* forces than small EVs (green). The soluble components are not affected by centrifugation, but non-EV particles such as lipoproteins and protein aggregates may copellet with EVs. **B**, In density gradient centrifugation, separation is based on density, and EVs will travel to their equilibrium density. Non-EV particles such as lipoproteins may coelute with EVs because of similar density or interaction. The soluble components with a high density relative to the gradient will collect at the bottom of the tube. **C**, Size exclusion chromatography uses a porous matrix (dotted circles) that separates on size. Soluble components and particles smaller than the size cutoff enter the porous matrix temporarily, whereas EVs and particles larger than the size cutoff do not enter the porous matrix. As a result, EVs and particles larger than the size cutoff elute before the soluble components and particles smaller than the size cut-off. **D**, In ultrafiltration, soluble proteins and particles smaller than the size cutoff (≈10^5 kDa) are pushed through the filter, and the EVs are collected at the filter. **E**, In immunocapture assays, EVs are captured based on their immunophenotype. EVs are captured using an monoclonal antibody (mAb) directed against an antigen exposed on the targeted (green) EVs only. **F**, In precipitation, addition of a precipitating agent induces clumping of EVs, non-EV particles, and soluble proteins. The clumps will sediment, and sedimentation can be accelerated by centrifugation.

Considerations and Recommendations

- Different biofluids require different approaches with regard to the choice of density medium and sample loading approach.[95] Because of the viscosity of plasma, EV may need to be isolated before DGC.[27]
- Measure the densities of collected fractions and determine whether EVs occur in the same fraction between experiments.
- To investigate whether EVs reached the equilibrium density, increase the centrifugation time, and compare top-down with bottom-up loading.[96]
- EVs can be analyzed either directly or after removal of the density media: remove sucrose by dialysis and remove iodixanol by 10- to 20-fold dilution followed by pelleting at 100 000*g*.[77]

Size Exclusion Chromatography

Size exclusion chromatography (SEC; Figure 3C) enables size-based separation on a single column, with the majority of EVs eluting before soluble components such as proteins and HDL.[29] The size cutoff is determined by the choice of the exclusion matrix, for example, Sepharose 2B has a pore size of ≈60 nm. SEC removes 99% of the soluble plasma proteins and >95% of HDL from the purest fraction of EVs,[78] does not induce aggregation of EVs,[79] and retains the integrity and biological activity of EVs.[80] The major coisolated non-EV components are particles above the size cutoff, which may include viruses, protein aggregates, and very large proteins such as von Willebrand factor and chylomicrons, the latter especially present in plasma from nonfasting subjects and LDL.[24,29,78–82] The presence of, for example, von Willebrand factor and LDL are unexpected based on size, possibly they form complexes with or bind to EVs.[24] By using SEC, a reproducible recovery of 40% to 90% of EVs can be attained.[81] SEC is fast, 10 to 20 minutes per sample, and relatively inexpensive,[82] which makes SEC clinically applicable. In essence, SEC exchanges the EV environment with no or minimal detrimental effects on EVs themselves, for example, by exchanging plasma for buffer. Compared with DC, EVs isolated by SEC have a high yield of biophysically intact EVs although at the expense of dilution.[79,97]

Considerations and Recommendations

- SEC performance is determined by the column height, ratio of sample volume to collected volume, the pore size

Table 2. Advantages and Limitations of Methods to Isolate Extracellular Vesicles

	DC	DGC	SEC	UF	IC	Precip.
Isolation						
Major contaminant	Similar-sized particles	Lipoproteins	Same size particles	Same size particles	Soluble proteins	Protein
Major artifact	EV-particle aggregates			EV-particle aggregates		Protein complex, EV-particle aggregates
EVs/μg protein increase (fold)*	1–15	1–20	70–560	1–10	1–50	1–3
Concentration						
Volume reduction (fold)*	0.2–8	≈1	0.2	<240	5	≈50
EVs recovery, %*	2–80	10	40–90	10–80		90
Practical						
Assay time, h	3–9	16–90	0.3	0.5	4–20	0.3–12
Sample volume	mL-L	μL-mL	μL-mL		μL-mL	μL-mL
Clinical applicability	No	No	Yes	No	Yes	Yes
References	22,64,69,72–76	77	29,78–82	27	83–87	77,88

DC indicates differential centrifugation; DGC, density gradient centrifugation; EV, extracellular vesicles; IC, immunocapture; Precip., precipitation; SEC, size exclusion chromatography; and UF, ultrafiltration.

*The values shown are from studies that differ not only in the applied isolation procedure but also in the starting Material and the Method of detection and therefore values should not be compared between the isolation methods.

of SEC media, and the quality of the column stacking. SEC columns are commercially available[81,98] or can be homemade.[80,82]
- Determine which fraction(s) contain the highest concentration of EVs. The fraction number will only be reproducible if the column stacking is constant.
- Combining multiple fractions containing EVs increases the recovery but reduces the purity.[82]
- Non-EV components, including cells, cell-debris, LDL, chylomicrons, and high molecular weight proteins, may coelute with EVs.
- A second SEC using a new column and starting with the EV fractions from the first SEC will further reduce the contamination with soluble components below the size cutoff.
- EVs with a diameter smaller than the size cutoff will elute with the soluble components.

Ultrafiltration

Ultrafiltration (Figure 3D) allows a separation of EVs from soluble components. To pass the soluble components through the filter, a pressure is applied, or the filter is placed in an (ultra)centrifuge. Because of the applied external force deformable particles such as EVs larger than the pore size may pass the filter. Ultrafiltration is more time efficient than DC, taking about 20 minutes to concentrate over a hundred milliliters of sample, compared with 3 to 9 hours required for DC.[99] Ultrafiltration can have a recovery of up to 80%[99] and may concentrate EVs up to 240-fold. This implies that ultrafiltration-based methods are effective to concentrate EVs.

Considerations and Recommendations

- Ultrafiltration may have value over other isolation methods, especially when using large volumes of EV-containing fluids that are less complex in composition than plasma, for example, culture media, but this has not yet been rigorously evaluated or tested.

Immunocapture Assays

Most immunocapture assays (Figure 3E) use monoclonal antibodies immobilized on a surface, for example, a plate, bead,[83,100] or chip[84] to capture EVs that expose a specific ligand. Based on the presence of such ligands, often proteins, immunocapture can isolate subpopulations of EVs.[85] An immunocapture assay can take hours to complete but is readily parallelized in multiwell plates and therefore clinically applicable. Side-by-side comparison of the immunocapture pull down and the flow through of EVs should be performed to evaluate the immunocapture efficacy.[101]

Considerations and Recommendations

- Magnetic beads may capture more efficiently than well plates because of larger contact area, better diffusion characteristics, and magnetic capture.[102]
- Non-EV proteins are recovered in numerous immunocapture assays, and a repository of non-EV proteins is available.[103]
- The antibody panel is the key to the performance of immunocapture. Determine cross-reactivity,[86] nonspecific binding,[101] and be aware that any antibody panel will select a subpopulation of EVs[104]

Precipitation

EV precipitation kits (Figure 3F) are often polyethylene glycol based. Polyethylene glycol is a water-soluble and volume-excluding polymer, which is nontoxic and nondenaturating. In most kits, polyethylene glycol is added to the starting material and incubated at 4°C for 15 minutes to 12 hours. The precipitated EVs and non-EV components are collected in buffer. Although often applied as stand-alone isolation method, precipitation is not suitable for identification of EV-associated

biomarkers because precipitation is primarily a concentration method. EV recovery can be 90%,[88] and a volume reduction of 50-fold is feasible. Precipitation-based isolation is inexpensive, requires no special equipment, and is comparable with both low- and high-sample volumes.

Considerations and Recommendations

– EVs should be isolated before concentration by precipitation.

Summary

None of the discussed isolation methods leads to a perfectly pure sample containing only EVs. DC is easy to use and widely available, yet does not isolate pure EV. DGC isolates highly purified EVs but has a low recovery. SEC removes most soluble components and has a relatively high recovery. Ultrafiltration may be effective to concentrate EV and to remove soluble components. Immunocapture can be used to isolate subpopulations of EVs. Precipitation assays are fast and have high EV yield but are unable to isolate pure EVs. The recommendations applicable for all isolation methods are summarized in Figure 4. Adequate reporting of the isolation method is essential.[27] The impact of the isolation or concentration methods on EV purity, concentration, morphology, size range, and functional activity should be measured whenever possible.

Methods to Measure Single EVs

Introduction

Blood contains EVs originating from a variety of cell types. Ideally, one would like to detect and extract biochemical and physical information from all single EVs, for example, to determine their cellular origin. Furthermore, clinical applications of single EV methods also require standards and calibrators to ensure reproducibility and comparability of measurement results across laboratories and over time.[105,106]

The selection or development of a single EVs detection method requires knowledge on the physical properties of EVs. Platelet-free plasma contains spherical EVs (>95%; 50 nm to 1 µm in diameter), tubular EVs (<5%, 1- to 5-µm long), and membrane fragments (<0.5%, 1–8 µm in diameter).[57,107] About 50% of the EVs are smaller than 400 nm, and the concentration of EVs >200 nm decreases with increasing diameter.[57,107,108]

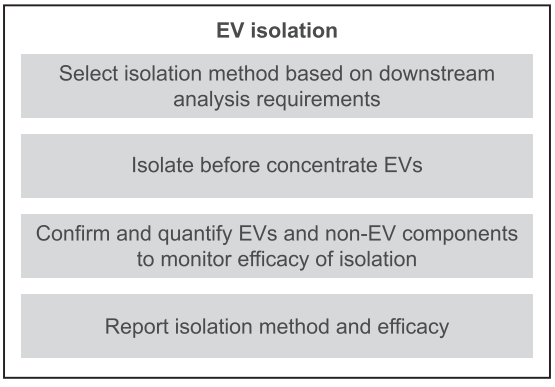

Figure 4. Overview of considerations and recommendations for the isolation of extracellular vesicles (EVs).

Reported concentrations range from 10^4 to 10^{12} EVs/mL plasma,[57,109–112] but are often underestimated or overestimated because of a lack of sensitivity[108] or specificity of the method,[57,107,109] respectively. For healthy individuals, physiological concentrations probably range between 10^7 and 10^9 EVs/mL plasma,[57,111] which is comparable to the concentration of platelets or red blood cells but lower than the concentration of lipoproteins in blood (>10^{12} per mL plasma).[109,113] Besides the size and morphology, EVs can be identified by electric resistance,[114] electrophoretic mobility,[115] fluorescence,[111,116] Raman scattering,[117,118] membrane stiffness,[119] and refractive index.[120,121] Because EVs are small and most signals scale with diameter to the power of 2 up to 6, detection and identification of the smallest EVs are still extremely difficult. For example, compared with platelets, EVs of 80 nm typically scatter >10^5-fold less light, have >10^4-fold less electric resistance, and have 10^3-fold less surface area to expose antigens.[108,111,122]

The physical properties of EVs define the requirements of a single EVs detection method. The ideal method should detect EVs that are 50 nm and larger,[107] have known detection limits for each measured property,[108,123] have a known sample volume to allow EV concentration determination, and be able to determine the immunophenotype of each EV. The immunophenotype can be used to infer the cellular origin and function. Note that in practice most methods cannot detect the smallest EVs and have an unknown detection limit, making the measured EV concentrations difficult to compare and statistical parameters of a size distribution meaningless.[114] Because of marked improvements in the technology to detect EVs, the estimated concentration of EVs in blood has increased ≈100-fold during the past 2 decades.[124]

For rare event analysis, we would like to characterize even the smallest EVs at a count rate >10^4 EVs/s, but such technology does not exist yet. Figure 5 shows the count rate versus the minimum detectable EVs diameter for detection methods of single EVs. Because electron microscopy (EM) can image the smallest EVs and FC has the highest throughput and because both methods are available in most university hospitals, we will focus on EM and FC. We will also briefly discuss nanoparticle tracking analysis, resistive pulse sensing (RPS), and novel methods.

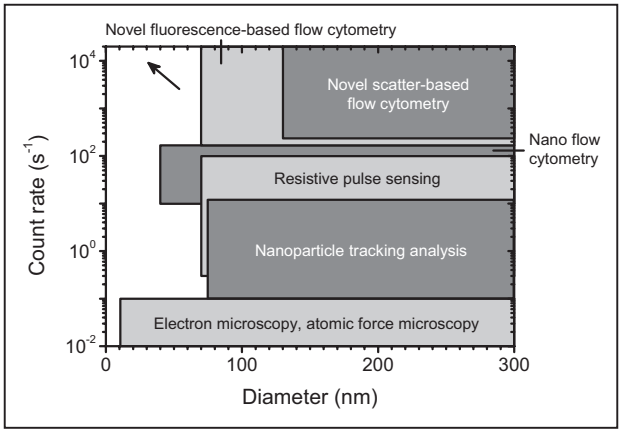

Figure 5. Estimated count rate vs detectable size range of methods used to detect single extracellular vesicles.

Electron Microscopy

EM is the gold standard method for imaging EVs. The resolution of EM images is ≈1 to 3 nm for transmission electron microscopy and ≈5 nm for EVs detection by scanning EM (Marc Schmutz, University of Strasbourg, France, personal communication). Here, we will focus on transmission electron microscopy, which covers most EM studies on EVs.

Depending on the type of sample investigated, a variety of preparation methods can be applied to image EVs. Cells or tissues are usually fixed, embedded in a resin, cut into thin (≈100 nm) sections, and stained before being observed in the EM. Exosomes, present in multivesicular bodies and secreted by cells, were discovered by this classical method.[125,126] To improve preservation of the EVs ultrastructure, high-pressure freezing and resin embedding at low temperature and cryosectioning are applied. Subcellular preparations like plasma or cell culture supernatants are thin enough to be deposited directly onto an EM grid. These specimens can be observed either dried after negative staining or hydrated, unstained, in a thin film of frozen liquid. The latter method is called cryotransmission EM (cryo-EM). Another EM method, called electron tomography, enables to determine the 3-dimensional structure of objects including EVs.[127]

To immunophenotype EVs, the EV-containing sample can be incubated with gold colloidal particles. These gold particles are typically 4 to 40 nm in diameter and are linked to a ligand, such as an antibody directed against a membrane protein or lipid. This approach, called immuno-gold-labeling, can be applied with all types of EM methods. Despite the fundamental role of EM imaging in EV research,[31] EV-EM protocols have not been standardized yet.

Considerations and Recommendations

- Image specimens both at low (≈300×, field of view ≈100 μm) and high magnification (≈30 000×, field of view ≈1 μm).
- Measure the diameter of EVs to determine a size distribution. Beware that different detection methods may find different size distribution of the same population of EVs.[108]
- Use immuno-gold labeling to phenotype EVs. Use distinguishable size gold beads for multiplex labeling.
- Use cryo-EM to identify EVs by their lipid bilayer and to differentiate EVs from nonvesicular particles.
- Use EM to reveal the presence of EVs aggregates or other aggregates.
- The well-known cup-shaped (doughnut) morphology is caused by collapsed EVs. Particles without cup-shape may be intact spherical EVs; by cryo-EM, all EVs <500 nm are spherical.[57,107]
- Because the adsorption processes depositing EVs on an EM grid are complex and poorly controlled, EM cannot be used to measure the concentration of EVs.

Flow Cytometry

FC is a powerful method to analyze EVs in biofluids although this potential has not yet been fully realized.[128] In FC, particles pass one by one through a laser beam, thereby scattering light and emitting fluorescence signals to multiple measurement channels. The detection of a particle is triggered by a signal exceeding a threshold set on ≥1 measurement channels.

EVs detection and standardization using light scatter–based detection has been the subject of numerous studies.[129–132] The light scatter intensities of EVs are often below the background noise. Therefore, it one must either accept many false triggers from irrelevant background noise or limit detection to the very largest EVs, the tip of the iceberg.[133] Relative fluorescence backgrounds are usually lower than scatter backgrounds, making fluorescence-based EV detection attractive.[123] On several widely used FC instruments, the use of specific fluorescent ligands, for example, annexin V, antibodies, or membrane dyes, can enable detection of more EVs compared with light scatter–based detection.[111,116,123,134]

One defining property of EVs is their size. Much confusion has resulted from the incorrect notion that the size of EVs can be determined by calibrating the flow cytometer using polystyrene or silica beads. Light scattering is a complex function of particle diameter and refractive index, illumination wavelength, and angle of light collection.[135,136] Recently, these factors have been integrated into models that enable estimates of particle size and refractive index based on light scattering.[108,121] Alternatively, the intensity of fluorescent membrane probes may be proportional to the EV surface area.[123,137]

A logical approach for immunophenotyping is to measure the presence of surface antigens using fluorescence-labeled antibodies. However, whereas cells expose >1000 surface antigens that can be fluorescently labeled, EVs typically expose <100 surface antigens, meaning that the number of detectable target antigens is at or below the detection limit of most flow cytometers.[128] Because immunofluorescent signals from EVs are dim, flow cytometers vary in EV sensitivity, and data are in arbitrary units, it is crucial to calibrate fluorescence signals of EVs in mean equivalent soluble fluorochrome units,[138] the standard unit of fluorescence, to allow data comparison and facilitate the development of FC dedicated to EV detection.

Finally, EV analysis by FC is susceptible to coincidence (swarm) artifacts, in which an event results from the presence of multiple EVs that are simultaneously present in the laser beam.[139,140] To evaluate the presence of coincidence, a control experiment is required with serial dilutions, where the particle event rate, but not the signal intensities should decrease with dilution.[139] Other confounders are the presence of non-EV particles, including antibody aggregates,[22,141] inorganic microprecipitates,[142] and lipoprotein particles.[24] Taken together, although the principles of FC are well suited to detect, enumerate, and phenotypically analyze EVs, instrument sensitivity improvements are required for full EV phenotyping in biofluids.

Considerations and Recommendations

- Do not analyze EVs with conditions and settings used for cell analysis. Optimize the instrument settings for EV analysis, for example, trigger channel and threshold, detector voltages, and flow rate.
- The fluorescence and scatter sensitivity of FC instrument designs presently applied in EV research have more than an order of magnitude difference. Calibrate the flow rate[130] and the intensities of fluorescence[138] and scatter channels.[121,140]

- Dilute EV samples to exclude coincidence (swarm) artifacts.[139,140]
- Add a detergent to solubilize EVs to confirm that the detected events are indeed EVs.[22,123]
- The diameter of polystyrene or silica beads does not relate to the diameter of EVs due to differences in refractive index.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis determines the size and concentration of submicrometer particles in suspension by tracking their Brownian motion with a dark field microscope. Nanoparticle tracking analysis does not distinguish EVs from non-EV particles. For polydisperse samples, including most biofluids containing EVs, sizing by nanoparticle tracking analysis outperforms dynamic light scattering but is inferior to sizing by RPS.[108,143] Because the detection volume is not exactly known, the concentration of detected particles can only be estimated. Other measureable EV properties are electrophoretic mobility, fluorescence,[109] and refractive index.[120,121] The applications of these options to EVs, however, are still in an early stage of development.

Considerations and Recommendations

- Check the alignment of the laser beam by imaging water at the highest camera level (metves.eu/output/videos).
- Use reference particles for concentration calibration and focus optimization.
- The finite track length adjustment algorithm in some software packages is prone to artifacts.
- Preferably track >4000 particles (minimum 2000) to prevent statistically insignificant peaks in the size distribution. Throughput may be increased through a syringe pump or by acquisition settings (eg, 30 videos of 10 seconds track more unique particles than 10 videos of 30 seconds).
- Do not compare concentrations between samples with different size and refractive index distributions.

Resistive Pulse Sensing

RPS determines the size and concentration of submicrometer particles in suspension by using the Coulter principle,[114] where each particle is detected by passing through a pore. RPS does not distinguish EVs from non-EV particles. Under optimal conditions, a sizing accuracy of <5% is feasible,[108] but this is often not achieved for EV samples. The presence of large EVs and sticky proteins, like fibrinogen or von Willebrand Factor, may clog the pore and make measurements impractical. Pore clogging can be prevented by removing large particles and proteins before measurement.[98] RPS devices compatible with the EV size range exist with fixed pores[144] and tunable pores.[98] The fixed pore device was introduced recently and remains to be evaluated. The tunable pore design is most widely applied, but the size detection limit has limited reproducibility, probably because of the design of the pore.[114] The tunable RPS device can also determine the electrophoretic mobility of particles.

Considerations and Recommendations

- Use filtration and SEC to avoid pore clogging.[82,98] Unclogging of the pore by inversion of voltage and pressure is preferable over pressure pulses delivered by a plunger.To improve reproducibility (1) set a fixed blockade height instead of a fixed stretch and voltage,[114] (2) require the cumulative counts to be linear with time (R^2>0.99), and (3) require the baseline current drift to be <5%.[108]

Novel Methods

Atomic force microscopy can provide information on the topography, elastic properties, and interaction forces of single EVs at supramolecular and submolecular levels.[145,146] However, major pitfalls attributed to the physical properties of EVs demand expertise and explain the limited use of atomic force microscopy in EV studies.[146–148] Three brand-new optical methods, comprising a frequency locked optical whispering evanescent resonator,[149] an interferometric reflectance imaging sensor,[150] and a nanofluidic optical fiber,[151] are capable of detecting single EVs as small as 50 nm. A nanotweezer or a conventional optical tweezer may be able to trap EVs and measure for example their Raman spectrum to obtain label-free chemical information.[117,118,152] At present, further investigation and commercialization is needed before these methods can add value to the EV field.

Summary

To study the contribution of all circulating EVs, we need methods that are capable of characterizing single EVs, but a trade-off between speed and sensitivity must be made, as shown in Figure 5. Considerations and recommendations that apply to all methods are summarized in Figure 6. Whereas flow cytometers are fast and behold great promise for clinical applications, EM provides high-resolution images of EVs and can distinguish EVs from similar-sized non-EV particles.

Measuring the Composition and Function of EVs

Introduction

EVs have emerged as important mediators of communication. The molecules incorporated into EVs are variable and depend on the type and environmental conditions of the parent cells.

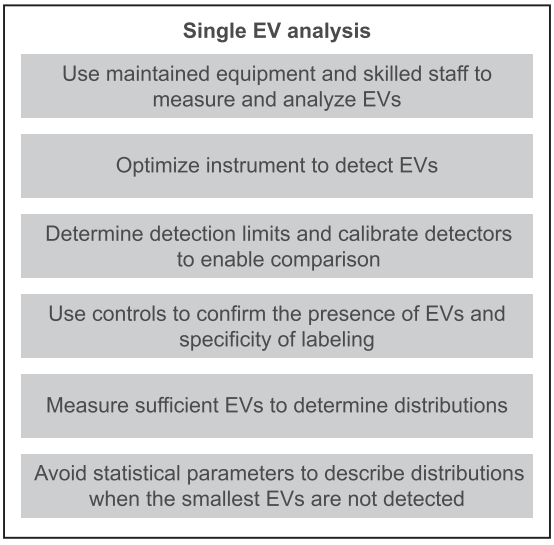

Figure 6. Overview of considerations and recommendations for methods to measure single extracellular vesicles (EV).

Vesicular cargo may be found inside and on the surface of EVs, including RNA, DNA, proteins, lipids, and metabolites. This EV cargo can be transferred to recipient cells, resulting in a pleiotropic response. Insight into the function of EVs can be obtained either by measuring the composition or by assays in which the function can be evaluated. In this section, we will discuss methods to analyze the composition (see Measuring the Content of EVs section of this article) and function of EVs (see Functional Assays section of this article).

Measuring the Content of EVs

RNA

EVs contain a vast diversity of RNA. To study RNA, EVs have to be isolated from the sample. As outlined in Isolation of EVs section of this article, the applied isolation method will affect the results.[40,153,154] For example, miRNA patterns differ when EVs are isolated from serum by either precipitation or DC,[155] and different mRNA expression profiles are found when EVs are isolated from conditioned culture medium using DC, iodixanol DGC, or precipitation-based methods.[77] Of these methods, iodixanol produced the highest number of EVs and the lowest concentrations of non-EV components, indicating that iodixanol may outperform the other examined methods in terms of purity.[77] Next-generation sequencing of RNA isolated from EVs has comprehensively classified all the types of RNA present in EVs,[156–159] and guidelines have been provided by ISEV.[160] Data obtained from RNA sequencing should then be validated by complementary technologies, such as quantitative PCR (qPCR) or Northern blotting.[161] To date, only a few studies attempted to quantify the actual (mi) RNA copy number.[154,162] Because we are far away from having the technical capability to perform RNA sequencing in single EVs, any RNA copy number can only be considered as the average RNA copy number in a large number of EVs. When working with plasma and other biofluids that host a variety of EVs from different tissues and cells, and where isolated EVs may be contaminated with miRNA-carrying (lipo) proteins (see Introduction under Isolation of EVs section of this article),[71] these EV-RNA analyses are often difficult to interpret.

Considerations and Recommendations

- The isolation method of EVs influences RNA measurements.
- Purified EVs are needed for the discovery of sorting mechanisms and proper biological interpretation.[40,163]
- The RNA-extraction method and cDNA synthesis can bias certain RNA types.[164,165]
- Digital droplet PCR is more precise than conventional qPCR for absolute miRNA quantification, and both methods have comparable sensitivity.[166]
- Next-generation sequencing based on adapter labeling has ligation bias that may lead to misrepresentation of transcripts.
- Microarray technology may be applied for expression profiling of the RNA content of EVs. However, this technology is not suitable for discovery of novel sequences and has inferior transcript quantification compared with next-generation sequencing.
- Treatment of intact EVs with RNAse/DNAse, optionally preceded by Prot K-treatment, will degrade externally bound RNAs.[61,167]

DNA

Although evidence that EVs contain DNA is scant in literature when compared with EV-RNA, an increasing number of studies suggests that under stress, cells release EVs containing DNA that differs from DNA present in apoptotic bodies.[168–171] As in RNA analysis, next-generation sequencing, PCR, and other methods can be used to analyze or validate the EV-DNA content. Moreover, a DNAse digestion step of intact EVs is needed to demonstrate the presence of intravesicular DNA.

Considerations and Recommendations

- Remove circulating non-EV DNA by dsDNase digestion before isolation of DNA from EVs.

Proteins

The most widely used methods to demonstrate the presence of a particular protein in EVs are Western blot and ELISA.[102] In this review, we will focus on proteomics because this method provides detailed information on the protein composition of EVs, and thus provides information on the functions and biogenesis pathways of EVs, and proteomics may lead to biomarker discovery. To date, ≈9700 EV-associated proteins have been reported in Vesiclepedia[172] and Exocarta,[173] but <500 of these proteins account for 90% of the total protein content in each individual data set.[79,174]

First, proteome analysis via (liquid chromatography based) mass spectrometry can be stochastic because of real-time sampling of enzymatically digested protein fragments before mass spectrometry. Second, EVs are only a fraction of the entire secretome, and (secreted) soluble proteins can be a major contaminant of EV proteomics. Contamination occurs when EVs are isolated from blood and also when EVs are isolated from serum-containing cell culture media.[61] Even when cells are cultured in serum-free medium,[58,59] or dedicated bioreactors,[60] soluble proteins in culture medium may contribute to artefacts. About the analysis of proteomic data, either the expression levels of selected individual proteins can be compared within an experiment or the identified proteins can be described, classified and grouped using gene ontology terms.[175–179]

Considerations and Recommendations

- Include technical sample replicates in the proteome analysis when quantifying changes of individual EV proteins at different conditions and when using label-free methods.
- Be aware of contamination by non-EV components. The extent of contamination depends on the EV isolation method used, and strategies to lower the albumin contamination could prove beneficial.[180–182]
- Report the nonhuman protein levels in EV samples and controls (eg, medium) because proteins may be conserved between species and incomplete data are available for proteins from most species.
- Clearly define and justify which proteins are included in the sample and reference data sets and be aware that up- or downregulation may be highly subjective and dependent on the experimental conditions.

- Correct data for multiple comparisons by, for example, false discovery rate analysis.
- Depending on the research question, choose an appropriate gene ontology analysis strategy, for example, statistical enrichment analysis or overrepresentation analysis.
- Compare the detected EV proteome with available data in Vesiclepedia,[172] Exocarta,[173] or EVpedia.[183]

Metabolome

EVs carry cytosol-derived small molecules <1500 Da, including metabolites as sugars, amino acids, lipids, and nucleotides. Variations in EV metabolites may reflect the biochemical status of the parent cell, and thus analysis of the metabolic cargo may provide insight into intercellular processes. Metabolomics is a new omics approach, and recently the first metabolomes of EVs have been described.[184,185]

*Considerations and Recommendations*Analyze all samples, including controls of the source material from which EVs have been isolated, simultaneously to minimize artifacts.[186]

Functional Assays

Perhaps the most convincing proofs for EV function have been obtained from functional assays. Each function has dedicated models, and here we will discuss the models for coagulation, fibrinolysis, and angiogenesis.

Coagulation

EVs have a dual role in hemostasis with procoagulant and fibrinolytic properties. Functional assays have been developed to measure these activities with the ultimate goal to evaluate their potential role as biomarker of thrombosis. EVs promote coagulation by exposure of anionic phospholipids, especially phosphatidylserine, and by exposing tissue factor (TF), the trigger of the clotting system.[187] The presence of phosphatidylserine and TF on EVs (EV-phosphatidylserine and EV-TF, respectively) depends on the mechanism of formation, the cellular origin, and the underlying process leading to the release of the EVs.

A variety of functional tests are now utilized to evaluate the coagulant potential of EVs. Several assays measure the amount of coagulant EV-phosphatidylserine in plasma samples. The EV-phosphatidylserine can be quantified when phosphatidylserine is provided only by EVs, and phosphatidylserine is the rate limiting step of the measured coagulation response. For example, by (1) measuring the clotting time of plasma on activation of coagulation factor Xa (FXa)[188] (2) by measuring thrombin generation after capture of EV-phosphatidylserine on annexin V-coated ELISA plates,[44] or (3) on addition of TF and a minimal amount of phospholipids.[189] Other functional assays measure coagulant EV-TF, for example, by measuring generation of thrombin, fibrin,[190] or FXa.[191] In some assays, plasma EVs are concentrated by centrifugation, washed, and resuspended in buffer before measuring the TF-dependent FXa generation.[192,193] Generation of FXa can then be measured in a kinetic assay, in which FVII is added together with synthetic phospholipids,[192] or in an end point assay, in which FVIIa is added without phospholipids.[193] Studies measuring the EV-TF activity in a variety of diseases using both types of FXa tests have been summarized,[194] and a modified version of both assays has been published recently.[195] Finally, the coagulant properties of EVs can also be studied directly in plasma and then the measured generation of fibrin depends on both phosphatidylserine and TF.[196]

Considerations and Recommendations

- To minimize contact activation use plastic blood collection tubes. Be aware that the extent of contact activation differs between collection tubes.[197]
- Include an inhibitor of contact activation.[198]
- Ensure the specificity of antibodies blocking the TF coagulant activity.[199,200]
- Addition of calcium to allow binding of EV-phosphatidylserine to annexin V in plasma or diluted plasma will also trigger coagulation.
- A positive control for human plasma containing coagulant EV-TF can be prepared by incubating fresh human blood with lipopolysaccharide.[201]
- Concentration and isolation of EVs contributes to poor reproducibility of the current functional tests (see Isolation of EVs section of this article).
- Functional EV-TF assays are more sensitive and specific than antigenic assays.

Fibrinolysis

EVs support plasmin generation and thus may contribute to fibrinolysis.[202] Plasmin is generated by incubating plasminogen with EVs and can be monitored with a plasmin-selective chromogenic substrate.[203]

*Considerations and Recommendations*Include controls for specific plasmin generation, for example α_2-antiplasmin or an inhibitory antibody against urokinase

- Need development of standards.

Angiogenesis

The effects of EVs from stem and progenitor cells,[204] cancer cells, platelets, cardiomyocytes, the human pericardial fluid and plasma[205] on angiogenesis have been studied.[206] These effects are commonly measured in vitro, using tube formation assays,[207] migration, and proliferation assays of endothelial cells, and formation of endothelial spheroids and sprouts,[208] and in vivo using Matrigel plug assay,[209] corneal angiogenesis assay,[209] tumor angiogenesis models, and postischemic angiogenesis models.[209] In the past few years, the role of EVs as mediators of proangiogenic communication within and between organs has been in the spotlight. These effects are at least in part mediated by the transfer of several types of miRNAs.[205,210–213] Collectively, these findings have opened up new avenues in cardiovascular stem cell therapeutics and tumor biology.

Considerations and Recommendations

- Growth factors present in or added to culture medium can adhere, bind to, or coisolate with EVs and affect their angiogenic potential. To reduce the risk of artefacts, include appropriate controls, for example, EVs isolated from culture medium not supplemented with growth factors or EVs from a nonangiogenic cell type cultured in the same medium.
- Ensure that the initial endothelial cell numbers for control and EV-treated samples are equal.

Summary

Studies on the composition and function of EVs provide insight in the role of EVs in health and disease. Before a conclusion can be made that a component is truly EV cargo, the presence of non-EV components has to be taken into consideration. With recent improvements in the isolation of EVs (see Isolation of EVs section of this article), progress can be expected. Functional assays provide insight into the putative function of EVs, and such assays may be clinically useful. However, no international standards are available yet, and without standardization, the relevance and comparability of the results from such assays remain insufficiently clear (Figure 7). Together, also the study shown in Measuring the Composition and Function of EVs section of this articles work in progress, but progress is being made and hitherto identified shortcomings will be overcome in the near future.

Concluding Remarks

This review summarizes basic guidelines and experimental parameters that are currently known to affect EV experiments. The outcome of any EV experiment can be biased by choices made in sample collection, storage, and EV isolation. Awareness of the interconnectedness of all steps from sample collection to EV detection will help avoid some common pitfalls in EV research.

The power of science should be the recognition that mistakes are a by-product of progress, but once the mistakes have been identified they should be corrected. The present recommendations are based on current technology and knowledge, and with progress in the field some of our recommendations will become obsolete. Individual discretion should be applied to determine exact experimental conditions, controls, and applicable standardization protocols. Taken together, this review will help to explore the still novel field of EVs and their roles in health and disease.

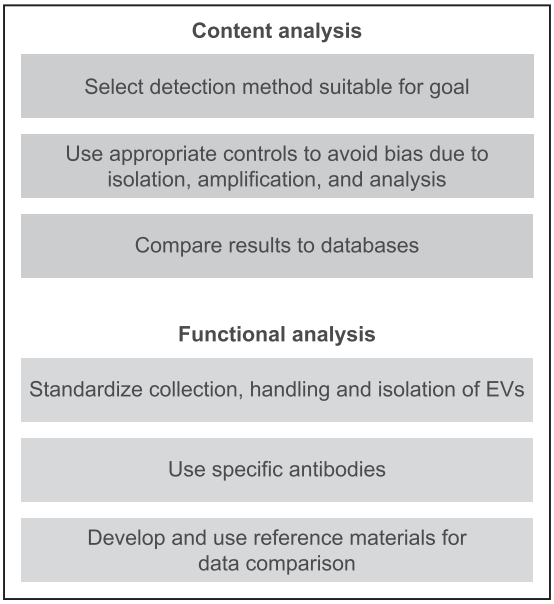

Figure 7. Overview of discussed considerations and recommendations for measuring the composition and function of extracellular vesicles (EV).

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