



# Elevate Your Extracellular Vesicle (EV) Research – An Introduction to EVs

## Introduction

Extracellular vesicles (EVs) are secreted by eukaryotic cells, archaea, and gram-negative bacteria [25] and are used for intercellular communication. Due to this, EVs are currently being investigated for their utility in diagnostics and are increasingly recognized for their therapeutic potential in the delivery of biocompatible molecules and their diagnostic potential. Their application in regenerative medicine holds promise for safer and more effective treatments [16]. However, challenges related to the isolation and purification of EVs and difficulties in scalability and standardization continue to impede their clinical translation. Ultracentrifugation remains the primary method of choice for EV isolation; it provides a reliable approach with the ability to apply several different purification methods, explored below, allowing researchers to find a balance between purity and yield [16].

Extracellular vesicles are heterogeneous lipid bilayer vesicles that differ in size and content, are incapable of replication, and lack a nucleus (Figure 1) [2]. Released by various cell types, including epithelial, endothelial, B cells and neural cells, they are present in extracellular spaces and body fluids such as blood, urine, cerebrospinal fluid and breast milk [5, 6, 17, 20]. EV subtypes are classified by size, density, molecular composition, or cellular origin [17]. They include exosomes (50–150 nm, from the endocytic pathway), microvesicles (200–800 nm, from plasma membrane budding) and apoptotic bodies (>1  $\mu\text{m}$ , from apoptotic or migratory cells) [20].



**Figure 1:** Illustration of the variety of size and composition of EVs. The size of EVs varies from approximately 30 nm to 500 nm. Additionally, the markers expressed on the surface of EVs as well as the internal composition is very diverse. This is influenced by the cell of origin and its activation status.

EVs and exosomes are formed with nucleic acids (DNA, RNA, miRNAs, lncRNAs), lipids (cholesterol, sphingomyelin), proteins, and MHC molecules [20]. An example of some of the proteins found in small EVs includes:

- Cytoskeletal proteins (e.g., actin, gelsolin, myosin and tubulin)
- Heat shock proteins (e.g., HSP70 and HSP90)
- Biogenesis-related proteins (e.g., ALIX and TSG101)
- Membrane proteins (e.g., tetraspanins)
- Enzymes (e.g., catalase, GAPDH, GTPase, nitric oxide synthase).

Additionally, they can contain membrane transport proteins, including Rab GTPases, flotillins and multivesicular body (MVB) production proteins. The composition of proteins and nucleic acids in small EVs is highly dependent on their parent cells (e.g., their health); they also carry specific markers reflecting their endosomal origin [5, 9, 20].

**Functions:** Cells release EVs into the extracellular space to perform various biological functions, highlighting their importance in pathophysiological conditions [20].

- **Physiological functions:** Under normal conditions, EVs are crucial for intercellular signaling, regulating functions such as angiogenesis, cell migration, and immune regulation [5, 20]. For instance, exosomes can influence angiogenesis by regulating specific miRNAs, and promote cell migration and immunomodulation. Notably, placental-exosome miRNA C19MC provides antiviral protection to the fetus by inhibiting viral proliferation [20, 22].
- **Pathological effects:** In disease states, EVs can facilitate tumor cell invasion, cardiovascular diseases, and pathogen infections. Tumor-derived exosomes serve as signaling messengers that enhance intercellular communication, promoting tumor growth and metastasis. For example, in colorectal cancer, miR-25-3p transferred via exosomes aids in metastasis, while in breast cancer, microRNA-92 increases PD-L1 expression, leading to T cell apoptosis [20].
- **Clinical relevance:** EVs play roles in hemostasis and thrombosis, affecting clot formation and resolution [23]. Urinary cell-free miRNAs are associated with kidney disease, [11] and neurodegenerative conditions may be influenced by exosomes containing interferon and TNF, which disrupt the blood-brain barrier and trigger white blood cell migration [3, 20].

**Applications:** EVs have diverse applications in disease biomarkers, drug delivery, tissue repair, vaccines, genetic engineering and gene therapy.

- **Biomarkers:** EVs in body fluids reflect host cell states and can serve as disease biomarkers. They demonstrate high sensitivity (87%) and specificity (84%) for diagnosing glioma, with EGFRvIII mRNA in serum microvesicles as a potential glioblastoma marker. Their advantages include high concentrations in body fluids, membrane stability, reflection of parental cell status and ease of extraction for clinical use [19].
- **Tissue repair and regeneration:** EVs facilitate intercellular communication supporting cell expansion and division [19], with examples including placental MSC-derived exosomes promoting angiogenesis, lung-derived EVs reprogramming marrow cells for lung engineering, and MSC-derived exosomes enhancing CNS repair and cartilage regeneration. They are also being explored for treating joint conditions like rheumatoid arthritis [4, 6].
- **Drug delivery:** EVs can cross physiological barriers and serve as a method to protect RNA drugs, such as miRNAs and siRNAs—which are used to treat diabetic wounds, tumors, and neurodegenerative diseases—from degradation in the body, making them a promising drug delivery vector. Strategies to reduce EV immunogenicity involve combining EVs with liposomes or protein nanocages [19].

- **Vaccines:** Exosomes are promising clinical delivery vehicles due to their stability and low toxicity. Exosomes derived from virus-infected cells are shown to have the capacity to elicit an antiviral interferon response, positioning them as promising candidates for the development of cancer vaccines [18].
- **Gene therapy:** EVs can be engineered for improved stability and targeting. Transfection methods introduce RNA into exosomes for enhanced delivery efficiency, resulting in genetic modifications of target cells. This approach is exemplified by the use of CAR-T cell-derived exosomes in tumor therapy, which do not trigger cytokine release syndrome [20, 22].
- **Metabolic engineering:** This approach modifies EV parental cells by adding substances like amino acids, lipids, and polysaccharides to the growth medium, which are integrated into the EVs' proteome, glycoproteins, and liposomes [20, 22].

**EV purification:** Isolating EVs with high purity is essential for assessing their properties, as they vary in size, function and content. Common isolation methods include size-based isolation, polymer precipitation, immunoaffinity chromatography, microfluidics, and centrifugation—with centrifugation being the most widely used method for isolating EVs.

## Centrifugation-based techniques

Centrifugation results in high-purity vesicles with consistent particle size, and minimizes batch-to-batch variance, aiding in EV engineering and enhancing therapeutic stability and scalability [22]. Centrifugation offers matrix-free and gentle separation, reproducibility, documentability, separation based on either particle density or sedimentation, and reduces the risk of artifacts, product loss, vesicle lysis and cargo release.

Ultracentrifugation is considered the “gold standard” for EV isolation, as it relies on differences in size, shape and density of particles for separation. Ultracentrifuges operate at very high speeds, up to 150,000 rpm, and include refrigeration systems to help maintain sample integrity. They are used for both sample preparation and particle characterization and are capable of removing unwanted contaminants and separating different particle populations. Several different centrifugation methods can be applied to reach the ideal purity level, many of which are described here.

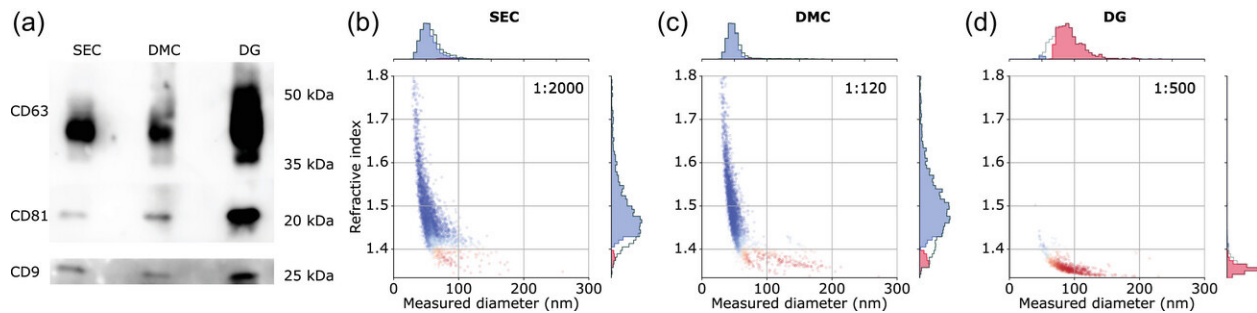
**Differential centrifugation:** Also known as differential pelleting, this method separates particles based on their sedimentation coefficients, with larger and denser particles sedimenting faster under centrifugal force. Samples undergo multiple low-speed spins to remove larger particles before high-speed centrifugation isolates the EVs [22]. However, due to the heterogeneity of biological particles, this method often results in the isolation of several EV subpopulations. A sucrose cushion can be used in the final centrifugation step to improve purity and prevent EVs from forming a hard pellet at the bottom of the tube [17]. Additionally it is important to consider that the quality of EVs can be affected by centrifugation duration and force [15, 22].

**Density gradient ultracentrifugation (DGUC):** This alternative centrifugation method can be used if increased purity or resolution are required. There are three different types of DGUC –isopycnic, equilibrium zonal, and rate zonal- that can be used, which are separated based on fundamentally different principles; however, they all separate particles in solutions using density gradients, such as cesium chloride (CsCl) and iodixanol (IDX). Below are several centrifugation purification methods that use DGUC.

- **Isopycnic centrifugation:** In isopycnic or density separation, particles are separated within a density gradient based on their density; therefore, the maximal density of the gradient medium must exceed that of the particles. During centrifugation, particles migrate until their density matches the surrounding medium, achieving equilibrium. Thus, they form distinct bands separating components with different densities. It relies on the principle of density equilibrium rather than the size or shape of the particles, making it highly effective for separating complex mixtures.

- **Equilibrium-zonal centrifugation:** This technique is similar to isopycnic centrifugation, as it uses density gradients to separate particles based on their densities. The difference between the two is that equilibrium-zonal centrifugation uses a step gradient rather than a linear gradient, which allows for a reduction in the overall required centrifugation time. It is typically used for primary purification, as the overall yield of particles is generally higher, although the purification efficiency may be lower.

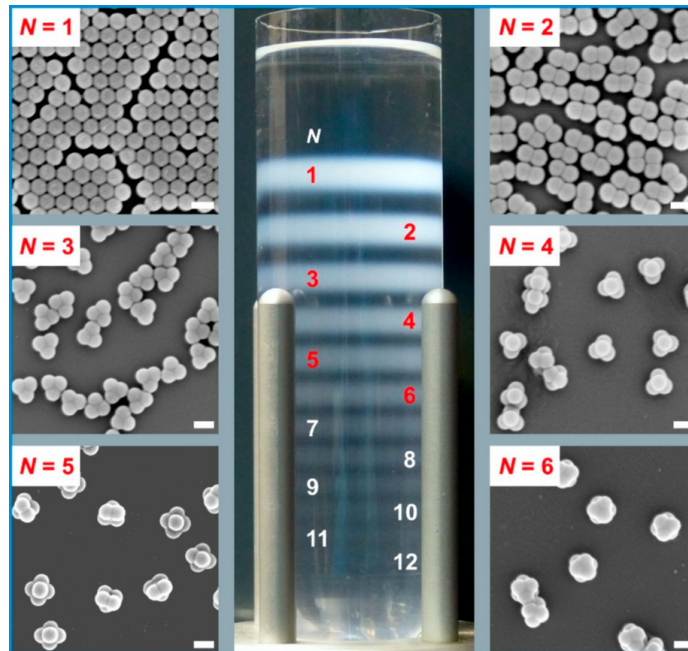
Kashkanova *et al.* [1] tested several different EV purification methods, including size exclusion chromatography (SEC), dual-mode chromatography (DMC), and density gradient ultracentrifugation (DGUC). For the DGUC, they performed equilibrium zonal centrifugation using an iodixanol step gradient, created by sequentially layering 40%, 20%, 10%, and 5% solutions and overlaying 500  $\mu$ L of sample on top of the gradient. After purification, western blots and interferometric nanoparticle tracking analysis (iNTA) were used to measure the EVs derived from lipid-rich samples. The western blot and iNTA confirmed the presence of EVs from all three purification methods (Figure 2). iNTA detected EVs and lipoproteins (LPs) in both the SEC and DMC purified samples, while in the DGUC-purified sample iNTA only detected an EV population with no background of large LPs (Figure 2).



**Figure 2:** iNTA of EV samples enriched from lipemic melanoma patient plasma by SEC, DMC or DGUC. (a) Western blot of EV-associated tetraspanins. Equal volumes of EV sample were loaded, with DGUC (6 mL) having a 12 $\times$  higher plasma sample input than SEC and DMC (0.5 mL). Size-RI plots of EVs enriched by (b) SEC; (c) DMC, arrowheads indicate the EV population; (d) DGUC. Numbers in the upper right corner indicate sample dilution factor by PBS prior to iNTA measurements. Figure taken from Kashkanova *et al.*, 2023. The image was not altered <https://creativecommons.org/licenses/by/4.0/>

- **Rate-zonal centrifugation:** This DGUC technique separates particles based on their size and mass. Under centrifugal force, particles move through the gradient at different rates depending on sedimentation coefficients, i.e., size, shape, and density. However, centrifugation is ended before the particles pellet, and due to the density gradient, the particles with different sedimentation coefficients are separated throughout the tube at the end of the experiment (Figure 3). This minimizes cross-contamination between particles by ensuring that faster sedimenting particles are not mixed with slower ones.

In a study by Plüsch *et al.* [14], a mixed suspension of 12 nanoparticles with varying sedimentation coefficients were layered on a sucrose gradient ranging from 2% to 8% (Figure 3). Centrifugation was carried out using an Optima XPN-90 Ultracentrifuge from Beckman Coulter Life Sciences equipped with an SW 32 Ti swinging-bucket rotor. Nanoparticle clusters comprising more than 12 particles were resolved into discrete bands, enabling precise identification of distinct species.


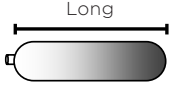
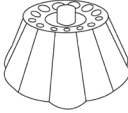

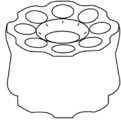

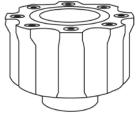



**Figure 3:** Centrifugal separation of colloidal clusters according to their sedimentation coefficients in a sucrose density gradient ranging from 2% (m/m) to 8% (m/m). The fractionation was carried out in an SW 32Ti rotor at 24,000 rpm. Cluster populations of up to 12 constituent particles were isolated as individual zones that could be harvested by using a self-built fraction recovery unit. FESEM micrographs of fractions of particle monomers ( $N = 1$ ), dimers ( $N = 2$ ), trimers ( $N = 3$ ), tetramers ( $N = 4$ ), pentamers ( $N = 5$ ), and hexamers ( $N = 6$ ) are grouped around the centrifuge tube hosting the gradient and the particle zones. The scale bars represent 200 nm. Figure taken from Plüsch, C.S. et al. 2021. The image was not altered <https://creativecommons.org/licenses/by/4.0/>

## Centrifuge Rotors

Several different types of centrifugation rotors are used for macromolecule and vesicle purification. Each type of rotor has a different angle of rotation, which affects the pathlength over which the particle sediments, making each optimized for different applications, purity levels and run times (Table 1). Four main types of rotors, and the applications they are suited for, are listed below:

1. Fixed-angle rotors - hold sample tubes at a 20-30° angle to the rotor axis, allowing particles to slide down to the bottom, suitable for pelleting.
2. Swinging-bucket rotors - tubes are held at a 90° angle, swinging them into a horizontal position during operation, making them suitable for rate zonal, isopycnic, equilibrium zonal and differential centrifugation. With differential centrifugation, the pellet forms at the very bottom of the tube, improving visibility and recovery of the pellet.
3. Vertical rotors - position tubes vertically, providing the shortest pathlength and fastest run time. They are ideal for isopycnic and density gradient centrifugation, as they minimize run time and maximize output.
4. Near-vertical rotors - hold tubes at a 7-10° angle, ideal for density gradients with less pure samples, and effective for isopycnic and equilibrium zonal centrifugation.

	Use cases	Angle	Example	Pathlength (at speed)
Swinging-bucket (SW)	With the longest pathlength, SW rotors are the best option available for rate zonal separations. SW rotors are also beneficial for pelleting very small sample masses to maximize visibility and pellet stability.	90°		
Fixed-angle (FA)	Highly versatile, FA rotors are applicable for all purification methods except rate zonal. FA rotors are preferable for larger-scale pelleting, especially when there is sufficient sample to allow for visualization.	20-30°		
Near-vertical (NVT)	NVT rotors are preferred for density-based separation with less pure samples that may have a small amount of floating or sedimented contaminants.	7-10°		
Vertical (VT)	VT rotors are the most preferable option for density gradient formation and high-resolution density-based separations.	0°		

**Table 1:** Rotors for EV purification

## Characterization of EVs

Currently, EV analysis is challenging. Deciphering the specific functions and mechanisms of action of EVs in different contexts is difficult. The functional heterogeneity of EVs requires more comprehensive characterization and standardized functional assays. Due to these challenges, it is beneficial to analyze EV samples at both the single-particle level and the bulk characterization level, where you look at the entire sample to characterize different populations, aggregation and contaminants that might be present.

**Analytical ultracentrifugation (AUC):** AUC is a versatile technique for analyzing biophysical properties of analytes in solution. Developed by Theodor Svedberg, whose work on molecular weights earned him the Nobel Prize in Chemistry in 1926, AUC has a rich history, including Meselson and Stahl's use of it to confirm the semiconservative model of DNA replication.

AUC separates analytes in solution based on mass, density, and anisotropy, while characterizing them, and can be used to investigate various properties, including:

- Sedimentation coefficient (s)
- Diffusion coefficient (D)
- Frictional ratio (f/f<sub>0</sub>)
- Molar mass
- Particle loading
- Heterogeneity
- Sample purity
- Aggregation
- Stoichiometry
- Reversible and irreversible binding interactions
- Equilibrium constants

The Optima AUC analytical ultracentrifuge integrates ultracentrifugation for particle sedimentation with optical detection modules, such as absorbance (ABS) and Rayleigh interference (INT), for real-time monitoring. Although AUC is a well-established technique for the detailed characterization of proteins and viral particles, its application in the study of EVs remains relatively underexplored. This presents a significant opportunity to harness the full potential of AUC in advancing the understanding and characterization of EVs.

**Heterogeneity, composition, and conformation of EVs by AUC:** The Optima AUC provides information on the presence of different EV subpopulations, as well as low and high molecular weight contaminants and aggregates. Additionally, the utility of multiwavelength capabilities means different macromolecules loaded into the EVs can start to be identified [12, 13, 24].

## Functional heterogeneity of EVs using a CytoFLEX nano Flow Cytometer

Novel single-vesicle analysis methods have been developed to improve subclassification and characterization of EVs. [7, 8]

The CytoFLEX nano Flow Cytometer has been specifically designed for nanoparticle analysis, enabling characterization of EVs at least as small as 40 nm (based on polystyrene beads), while simultaneously offering up to 6 separate fluorescent channels of detection and 5 side scatter channels. It offers superior resolution, particularly in the lower nanometer range, enabling the identification of populations that traditional flow cytometry may fail to detect. Additionally, the CytoFLEX nano Flow Cytometer can resolve eight-peak multicolor multi-intensity beads, demonstrating its sensitivity across a wide dynamic range. Its multicolor phenotyping capabilities contribute to improved data quality and analytical precision for single EV analysis.

### Conclusion

The exploration of EVs presents significant opportunities and challenges in therapeutic applications, particularly in regenerative medicine. While EVs demonstrate potential in delivering biocompatible molecules and facilitating intercellular communication, obstacles such as isolation, purification, and scalability must be addressed to enable clinical translation. The insights underscore the importance of advanced techniques, such as ultracentrifugation and analyzers like the Optima AUC and the CytoFLEX nano Flow Cytometer, for characterizing heterogeneity and functionality of EVs. By embracing these innovative approaches and tools, researchers can enhance their understanding of EVs and harness their therapeutic potential, paving the way for more effective treatments in various medical fields.

*This whitepaper summarizes a Beckman Coulter Life Sciences webinar, titled "Elevate Your Extracellular Vesicle Research," presented by Dr. Luca Musante and Dr. Amy Henrickson. Dr. Musante presented an elaborate review of EVs including their biogenesis, functions, and characterization applications, drawing on his research. Dr. Henrickson detailed the principles of centrifugation for EV isolation, and characterization using analytical ultracentrifugation, emphasizing key instruments used in EV research, including flow cytometry tools.*

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