Chapter 9

Extracellular Vesicles as An Alternative Modality to Stem Cell-Based Therapies

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Abstract

The increase in stem cell transplantation in different pathologies has led to progress in the control and healing of patients in the clinical setting. The isolation and expansion of stem cells need various *in vitro* technologies before grafting into the injured tissues. Therefore, finding suitable substitutes with a therapeutic potential similar to the whole stem cells is highly recommended. In most circumstances, allogeneic stem cell sources

are used for regenerative purposes, which can increase the possibility of rejection by allo-reactive immune cells. The discovery of EVs[1] with relatively similar regenerative potential to the parent stem cells has led to enhanced regenerative outcomes and a reduction of immune system rejection. Here, in this chapter, we discussed details about the biology and characteristics of EVs in biomedicine and regenerative medicine.

Keywords: Extracellular Vesicles; Exosomes; Biogenesis; Theranostics.

1. Biology of EVs

EVs are a collection of different vesicle types, Exos, ectosomes/MVs[2], migrasomes, apoptotic bodies, exophers, and large oncosomes with an active role in intercellular communication (**Figure 1**) [1, 2]. Among different subsets of EVs, Exos[3] with an average size of 40–150 nm are released into the ECM[4] and involved in paracrine interaction between the normal and cancer cells (**Table 1**) [3]. Exos are produced by endosomal systems having a relatively spherical morphology and a lipid bilayer membrane [4]. Based on various studies, signaling molecules are sequestrated into the lumen and membrane of Exos during the biological phenomenon known as exobiogenesis (**Figure 2**) [5, 6]. For example, the external surface of the Exo membrane harbors biomarkers such as flotillins, tetraspanins (CD9, 63, 81, and 82), Alix[5], TSG101[6], RABs[7] , and ARF[8], and various HSPs[9] (HSP60, 70, and 90) [7, 8].

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1. Extracellular vesicles
2. Microvesicles
3. Exosomes
4. Extracellular matrix
5. ALG-2 interacting protein X
6. Tumor susceptibility gene 101
7. Ras superfamily of small G proteins
8. ADP-ribosylation factor
9. Heat shock proteins

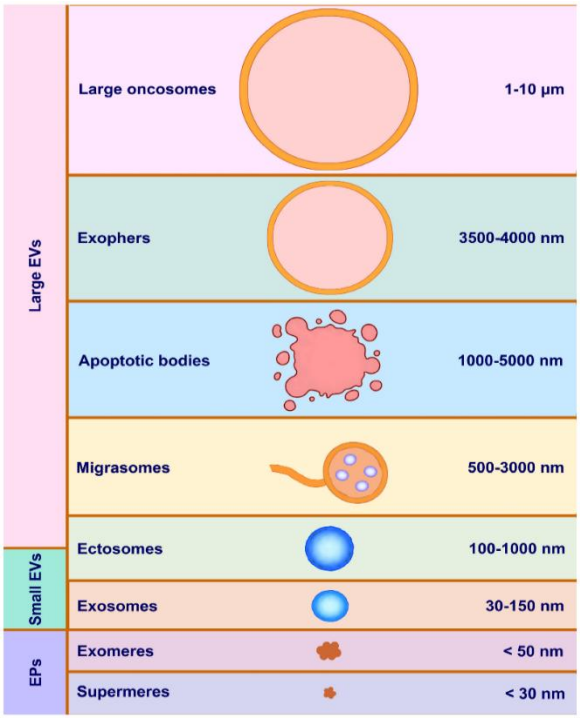

Figure 1. Various EV types. Cells can produce small-sized EP[1], namely exosomers and supremeres. Copyright. [1]. International journal of molecular sciences. 2024.

[1]. Extracellular particles

Table 1. Features and possible use of normal and cancer cell Exos in biomedicine

Feature / Application	Normal cell Exos	Cancer cell Exos	Research Applications
Proteins	-Structural proteins: CD9, CD63, CD81, Alix, TSG101 - Heat shock proteins (HSP70, HSP90) - Common cellular antigens	- Overexpressed or mutated proteins: EGFRvIII, HER2 - Metalloproteinases (MMPs) - Tumor-promoting proteins such as survivin	Identification of disease-specific protein biomarkers (cancer, neurodegenerative diseases)
RNAs	- Natural miRNAs and lncRNAs maintain cellular homeostasis - Repair-related mRNAs	- Oncogenic miRNAs: miR-21 (growth promotion), miR-210 (hypoxia-induced angiogenesis), miR-155 (immune evasion) - Altered RNAs	Genetic signature analysis of exosomes for early cancer detection and treatment monitoring
Lipids	- Cholesterol, sphingomyelin, phosphatidylserine (for membrane stability)	- Increased ceramide, externalized phosphatidylserine, promoting uptake by target cells	Targeting lipid composition alterations as novel therapeutic or diagnostic strategies
Natural Function	- Tissue repair - Regulation of immune responses - Modulation of cell differentiation	- Induction of metastasis and invasion (through microenvironmental remodeling) - Transmission of drug resistance (e.g., MDR1-containing exosomes) - Immune suppression	Studying cancer cell interactions with their TME
Release into Body Fluids	- Stable and regulated release into blood, saliva, urine, CSF	- Elevated exosome levels in blood/urine/pleural effusions - Carrying cancer-specific markers	Development of non-invasive liquid biopsy platforms
Effects on the Immune System	- Stimulation of T and B cell responses - Antigen presentation to dendritic cells	- Inhibition of T-cell activation via exosomal PD-L1 - Induction of regulatory T cells (Tregs) - Suppression of NK cell activity	Exploring immune suppression mechanisms for designing advanced immunotherapies (e.g., exosomal PD-L1 inhibitors)
Natural Therapeutic Potential	- Aiding cardiac and brain tissue repair - Regulation of inflammation	- Potential use of engineered tumor-derived exosomes for therapeutic RNA or drug delivery	Designing exosome-based drug delivery systems and vaccines for cancer and infectious diseases
Role in EMT and Metastasis	- -	- Activation of EMT transcription factors (Snail, Twist) via cancer-derived	Discovering EMT regulatory pathways and developing anti-metastatic therapies

		exosomes - Promotion of cell migration and invasion	
Biomarker Discovery	- -	- Altered protein and RNA patterns in cancer-derived exosomes compared to healthy ones	Development of microfluidic platforms for exosome isolation and analysis
Study of Tumor Microenvironment (TME)	- -	- Inducing changes in fibroblasts (CAF activation), endothelial cells (angiogenesis), and macrophages (M2 polarization)	Investigating exosome-mediated TME remodeling for anti-angiogenesis and anti-CAF therapy strategies
Genetic and Gene Therapy Applications	- -	- Delivery of siRNA, shRNA, and miRNA to target cells using engineered exosomes	Developing safe and non-immunogenic gene therapy methods without viral vectors
Bioengineering and Biomimetics	- -	- Production of synthetic exosome mimetics with customized size and composition	Creating smart nanocarriers for targeted drug delivery or cellular vaccines
Research in Non-Cancer Diseases	- -	- Studying exosomal effects in Alzheimer's (Tau and β-amyloid accumulation), Parkinson's (α-synuclein aggregation), type 2 diabetes, and renal failure	Identification of new therapeutic targets in degenerative and metabolic diseases

Additionally, the Exo surface is decorated with lipid substances such as ceramides, sphingomyelin, cholesterol, and ganglioside GM3 [9, 10]. These substances enable the Exo natural shuttles to transport biomolecules across cells in a paracrine manner. Exo biogenesis is initiated inside the cytosol along with the formation of early endosomes via membrane invagination. Early endosomes can further develop into late endosomes and MVBs[1] containing numerous ILVs[2]. Upon the release of ILVs into the ECM, these particles are hereafter named Exos [11, 12]. Several types of molecular machinery control Exo biogenesis within the endosomal system [13]. ESCRT[3] machinery with four complexes (ESCRT-0, I, II, and III) and some accessory proteins, such as AAA ATPase Vps4 stimulate cargo sorting and ILV formation. Of note, certain effectors such as ALIX, TSG101, and HRS[4] collaborate with ESCRT subunits in Exobiogenesis [14, 15].

[1]. Multivesicular bodies
[2]. Intraluminal vesicles
[3]. Endosomal sorting complex required for transport
[4]. Hepatocyte growth factor-regulated tyrosine kinase substrate

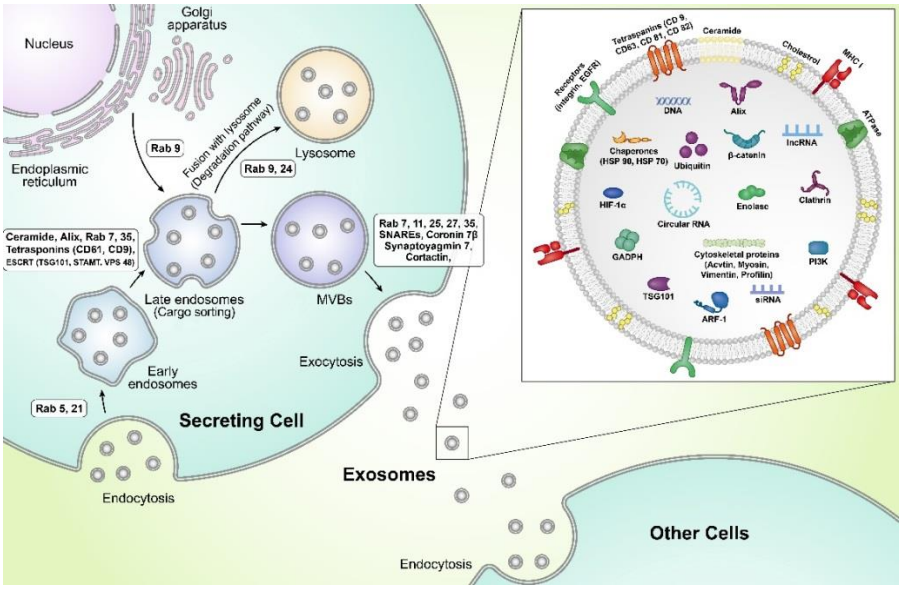

Figure 2. Mechanisms related to Exobiogenesis. Copyright [16]. Cell Communication and Signaling. 2024.

To control Exo biogenesis, the complex of ALIX and ESCRT-III provokes the ATG12–ATG3 combination for vesicle trafficking inside the cells. The Inhibition of the ATG12–ATG3 complex can influence MVB trafficking and morphology [17]. To be specific, ESCRT III functions in membrane reshaping, the fission procedure to generate ILVs. Of ESCRT subsets, ESCRT-0, I, and II with ubiquitin-binding motifs are involved in cargo sorting into the forming ILVs [18, 19]. It is suggested that AAA ATPase Vps4 can help the ESCRT-III in narrowing the release of forming ILVS into the endosome lumen. This process can be accelerated via the collaboration of ESCRT-independent effector factors such as CD63 and ceramides [20]. After the formation of MVBs, these vesicles have two destinies. First, they can fuse with the plasma membrane to release their contents into the ECM and be directed toward lysosomal degradation [11, 21]. For example, Rabs like Rab35, 11, and 27 can help MVB-plasma

membrane fusion with the activity of SNARE[1] proteins [22]. Exos enter the target cells via different mechanisms. Direct fusion occurs when the plasma membrane of the cell merges with the Exo membrane [23, 24]. Other methods include ligand-receptor interaction, which persuades the endocytosis-based internalization, like caveolin-, and clathrin-dependent endocytosis, macropinocytosis, micropinocytosis, and lipid raft dependence [25].

2. Exo isolation and enrichment methods

Currently, Exos are isolated from biological systems using different methods with inherent pros and cons [26]. Exos range from 40 to 250 nm with a specific gradient density of 1.13–1.19 g/ml. In general, the existence of cell debris and various protein types can influence Exo isolation and purification [27]. The conventional and most available isolation methods are polymer-based precipitation, SEC[2], immunoaffinity-based separation, ultrafiltration, microfluidics, and ultracentrifugation [26]. Among them, the ultracentrifugation method is frequently used for EV isolation purposes. To eliminate minute bioparticles such as cell debris, organelles, and dead cells during Exo isolation, sequential centrifugation cycles with different forces, including 300, 2000, and 10,000 g, are used. After the completion of these steps, Exos are pelleted using centrifugation forces between 100,000 and 120,000 g (**Figure 3**) [28]. However, the existence of protein aggregates, viral particles, and tiny subcellular compartments can reduce the purity of isolated Exos. It has been indicated that Exos isolated by ultracentrifugation exhibit morphological deformities [29]. Despite the existence of some disadvantages,

[1]. Soluble N-ethylmaleimide sensitive fusion protein (NSF) attachment receptor
[2]. Size exclusion chromatography

ultracentrifugation is a gold standard method in the isolation of Exos and other EV types from the biofluids [30].

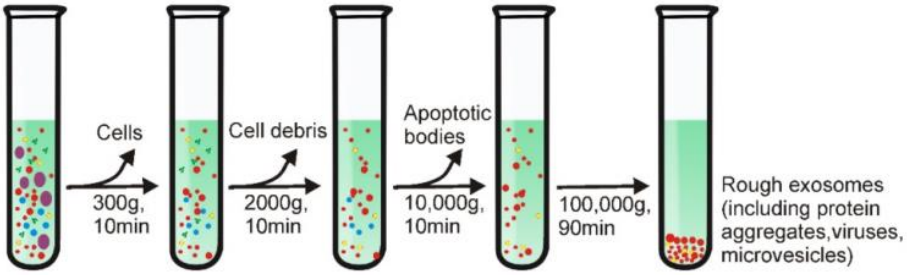

Figure 3. Illustration of differential ultracentrifugation methods for the isolation of Exos using multiple forces ranging from 300 to 100,000 g. Each step is essential to eliminate nano-sized particles, floating cells, cell debris, and apoptotic bodies in biofluids. In the last centrifugation step (100,000 g), the Exos formed pellets. It is recommended to perform these steps at 4°C. Copyright. [31]. Theranostics 2020.

The isolation of Exos by ultrafiltration is done by passing the biofluids through the membranes with specific MWCO[1] and hole diameters less than 100 nm (**Figure 4**) [32]. It is thought that undesirable nano-sized particles can pass the membranes with larger pore diameters. Compared to ultracentrifugation, ultrafiltration is fast and easy to use. The exposure to mechanical pressure and shear stress during the ultrafiltration may damage the Exos and contribute to aggregation and membrane blockage [33]. To date, various forms of ultrafiltration techniques, such as sequential, centrifugal, tandem, and tangential flow filtration, are used for the purification of Exos [34]. The bypassing of Exos through membranes is commonly done via electric stimulation, centrifugation, and compression [35].

[1]. Molecular weight cut-offs

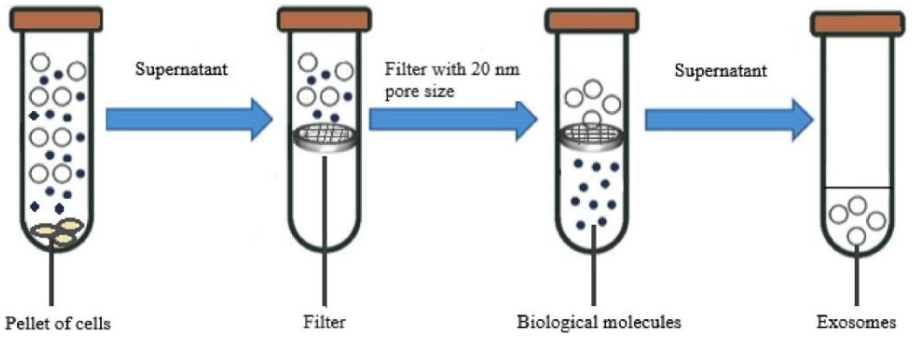

Figure 4. Ultrafiltration for the isolation of Exos from biofluids. Copyright. [32]. Translational Oncology. 2024.

It is also claimed that the isolation of Exos via ultracentrifugation is not suitable for large-scale Exo yielding in the clinical setting [36, 37]. Using SEC, samples are introduced into columns containing numerous porous beads for trapping nano-sized particles such as Exos. This method can isolate Exo with high purity, similar to reduced protein contamination, using the ultrafiltration technique (**Figure 5**) [38]. This approach allows particles of different sizes to elute due to gravity flow [39]. In this regard, cross-linked Sepharose materials are frequently used for the isolation of EVs [39]. Despite these advantages, possible contamination with protein fraction and loss of Exo integrity can influence the efficiency of this approach. Besides, the process of SEC is laborious, multistep, and time-consuming, and needs several diluents [38]. As above-mentioned, immunoaffinity capture-based approaches are other available modalities for the isolation of Exos [40]. Exo isolation using magnetic beads is done in two separate steps. To this end, Exos are primarily pelleted via ultracentrifugation followed by a purification step with magnetic beads [40]. The close interaction between the surface tetraspanins and bead-bearing antibodies leads to the sedimentation and isolation of Exos in biofluids (**Figure 6**) [41, 42].

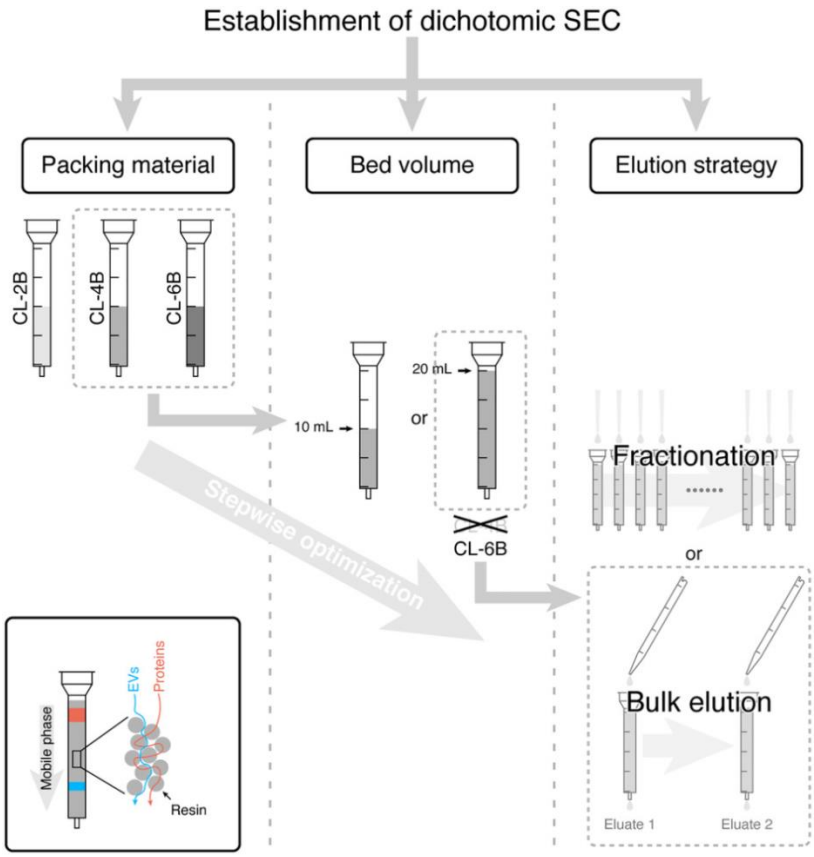

Figure 5. Application of SEC for the isolation of Exos. Copyright. [39]. Journal of Extracellular Vesicles. 2021.

Despite the purity of isolated Exos, the Immunoaffinity approaches are applicable for larger sample volumes, while this process is laborious and time-consuming [43]. The application of antibodies with suitable affinities is at the center of debate and can influence the quality of Exo separation. The incubation of Exos with high-affinity antibodies can make the release of captured Exos problematic and cause further injuries to their structures. It is thought that a simultaneous load of moderate-affinity antibodies can contribute to optimal conditions for capturing and releasing Exos [43].

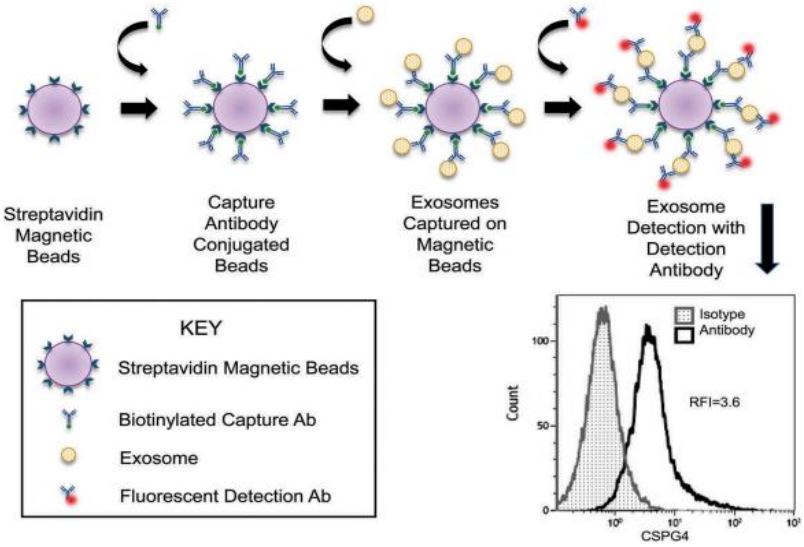

Figure 6. Bead-immunocaptured Exos can be analyzed using different assays, such as flow cytometry. The levels of relative fluorescence intensities can be monitored using fluorochrome-conjugated antibodies. Copyright [44]. Journal of Extracellular Vesicles. 2018.

The microfluidic method has been extensively applied for the rapid and high-throughput isolation of Exos from the biofluids. Indeed, this method is label-free and used for the isolation and analysis of Exos in the aqueous phases (**Figure 7**) [45]. This technique is based on the isolation of Exos from small sample volumes during the passing through micron-sized channels. Of note, microfluidics can reduce the necessity for biological samples and reagents with high sensitivity and specificity. During the last decades, two microfluidic-based isolation systems, including physical and immunoaffinity-based systems, have been used for Exo isolation [45]. It is suggested that excessive shear stress damages the structural integrity of Exos. Besides, microfluidic platforms are difficult to design need well-experienced personnel and high technology, and only yield Exos in nano-scales [46].

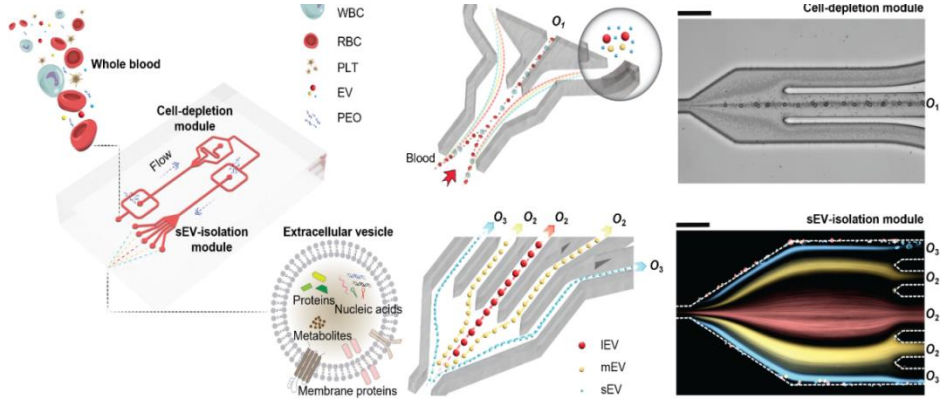

Figure 7. The microfluidic platform consists of cell-depletion and EV isolation modules. In this platform, large-sized particles (X>1 μm) such as blood cells are eliminated using outlet O1. The cell-depleted fraction is then directed to outlet O2 to collect the circulating EVs. Image of EV isolation by the microfluidic system. Copyright. [47]. Science Advances. 2023.

3. Exo cargoes

A bilayer lipid membrane construction makes up Exos to harbor a variety of ligands and receptors. In addition to surface monocular signature, different macromolecules like proteins, carbohydrates, and nucleic acids are present in their lumens [48]. Since exosomal cargoes can help us better understand the intricate underlying mechanisms in the biogenesis and metabolic status of parent cells, new isolation and purification methods with minimal damage have been developed to monitor the molecular profile of Exos. In this regard, Exos are used as early-stage diagnostics for the detection of various pathological conditions [49, 50]. A piece of evidence points to the fact that many Exos have relatively similar biomolecular signatures isolated from different sources. For example, in the biogenesis steps, Exos are loaded with endosomal markers such as Alix and TSG101 molecules by the endocytic route. Along with these molecules, tetraspanins (CD9, 63, and 81), cytoskeletal proteins (tubulin, actin, and annexins), membrane molecules

(integrins, ICAM-1[1]), and trafficking effectors (Rab-GTPase family and SNAREs) are detectable in nearly all Exos [51]. Lipids such as PE[2], DAGs[3], ceramides, PS[4], cholesterol, and sphingomyelin are relatively abundant in Exos [52]. In Exos from certain cell types like mast cells, the remnants of nucleic acids, and other nucleic acids like mRNAs and miRNAs, lncRNA[5] , and circRNA[6] have been confirmed [53]. Instead of being an accidental process, the exosomal loading procedure is done by a sophisticated sorting apparatus. Notably, cells use intricate processes to sequestrate certain nucleic acids into the ILVs in a preferred manner [54, 55]. Notably, the exosomal profile is fundamentally changed in response to the alteration of metabolic status or the occurrence of various pathologies compared to healthy conditions. Commensurate with these descriptions, monitoring the molecular signature of Exos can reflect the real-time metabolic status of parental cells [56]. The exposure of cells to hypoxia and irradiation can alter the exosomal content and Exo abscission [57, 58]. In this regard, Exocarta, the worldwide exosomal folder, is a useful resource that introduced 1,639 mRNAs, 4764 miRNAs, 563 proteins, and 194 lipids according to species and origin. Similarly, Vesiclepedia is a catalog devoted to Exos that has compiled a wealth of information on Exos payloads from several research groups. About 349988 proteins, 38146 different RNAs, and 639 lipids have been classified by this website, which has been gathered from 1254 EV studies. Taken together, these data demonstrate that Exos function as bio-containers that transport a wide variety of biomolecules and that discrete cells release diverse types of Exos with dissimilar contents [59].

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1. Intercellular adhesion molecule 1
2. Phosphatidylethanolamine
3. Diacylglycerols
4. Phosphatidyl-serine
5. Long non-coding RNAs
6. Circular RNAs

4. Characterization and confirmation methods

To date, Several techniques have been used for the characterization of isolated EVs and Exos based on physical features such as size, external electrical net charge, and density. While the biological specifications, such as the type of luminal cargoes, and surface molecular signature, can also be monitored for confirmation of Exo entity, source, and the existence of certain pathological conditions [60]. Using NTA[1] and DLS[2], it is possible to measure the hydrodynamic diameter of Exos in the aqueous phase. Using TEM[3] and SEM[4] imaging, the actual size and morphology of purified Exos can be achieved (**Figures 8** and **9**). While the application of labeling approaches and genetic manipulations can facilitate the load of certain factors and monitor the destination of Exos in the target cells/tissues [60]. NTA can detect features such as the size of NPs[5]. This approach can monitor speed, Brownian motion, the concentration of NPs, and their size using a video system, allowing for the diffusion coefficient. Compared to the NTA, DLS can monitor the changes in the intensity of scatter lights and hydrodynamic radius generated by the Brownian motion of Exos. Compared to DLS, NTA is more specific and sensitive in the detection of small-sized particles. DLS is applicable for solutions containing particles at ranges between 1 nm and 6 mm and generates valid data when the solution is homogenous [61]. To be specific, DLS does not provide data about the concentration or number of particles because this technique analyzes all particles in solution simultaneously [62].

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1. Nanoparticle tracking analysis
2. Dynamic light scattering
3. Transmission electron microscopy
4. Scanning electron microscopy
5. Nanoparticles

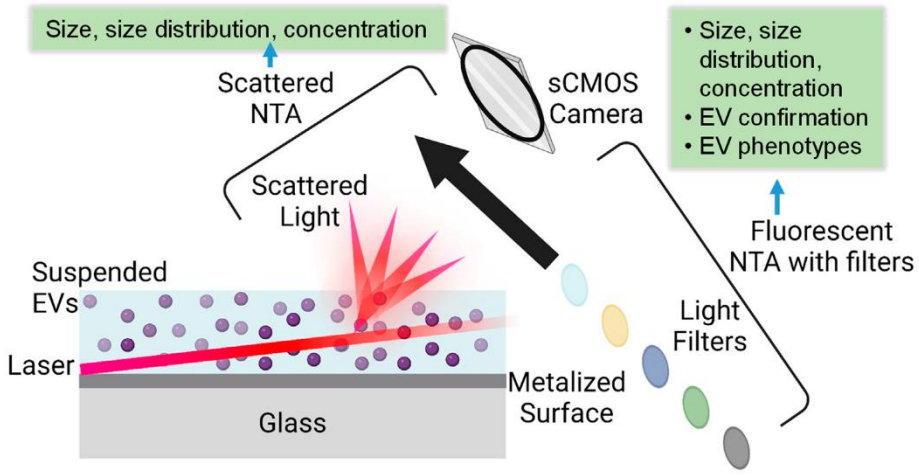

Figure 8. NTA technology for the monitoring of EV features in biological samples in scattering and fluorescent modes. The existence of various colored particles shows the different wavelengths emitted and collected by suitable filters. Copyright. [62]. Molecules 2024.

Using fluorescence microscopy, Exos can be monitored directly inside the cells in *in vitro* and *in vivo* conditions (**Figure 10**) [63]. To this end, several fluorochrome agents are used to label other subcellular components for better resolution [64]. The labeling of Exos is done directly via direct staining surface exosomal proteins via fluorescent agents, the introduction of fluorescent-tagged proteins into the cytosol of parent cells, or the expression of tagged proteins in parent cells via genetic manipulation [65]. Compared to direct Exo staining, it has been shown that the transfection of parent cells with fluorescent proteins or the production of fluorescent-tagged proteins can yield constant and accurate signals for Exo tracking in biological systems [65]. The recently developed ability to provide ultra-resolution images has helped the deep comprehensiveness

of Exo studies. Systems such as TIRF[1], SMLM[2], PALM[3], and STORM[4] are exemplified as high-resolution imaging techniques [66, 67]. Along with techniques, the application of AFM[5] has been extended to the characterization of isolated Exos in recent years. Besides high-resolution images, this technology helps provide data associated with Exo morphology and surface features such as size, shape, and stiffness [68].

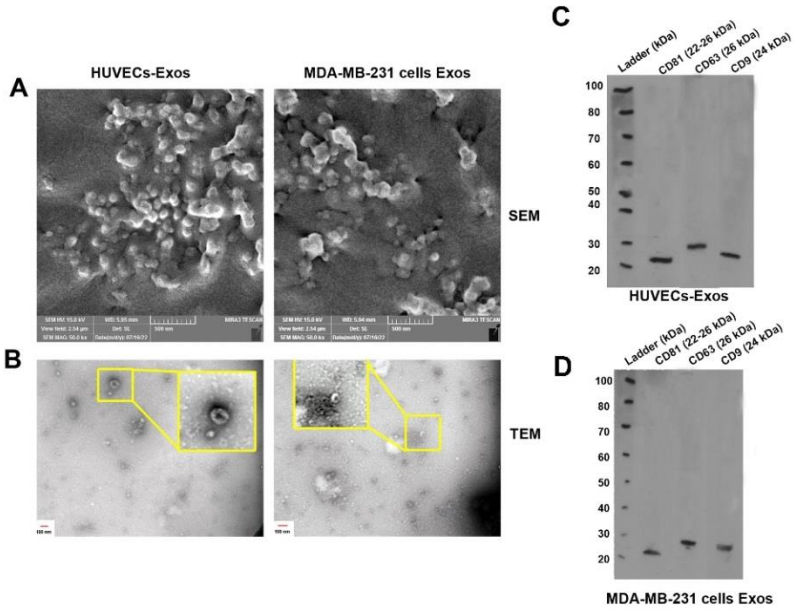

Figure 9. Monitoring the morphology and size of Exos isolated from human endothelial cells (HUVECs) and breast cancer MDA-MB-231 cells by SEM (A) and TEM (B) images. Using western blotting, the existence of tetraspanins such as CD9, CD63, and CD81 was identified in isolated Exos. Copyright. [28]. Advanced Pharmaceutical Bulletin. 2024.

[1]. Internal reflection fluorescence microscopy
[2]. Single-molecule localization microscopy
[3]. Photoactivation localization microscopy
[4]. Stochastic optical reconstruction microscopy
[5]. Atomic force microscopy

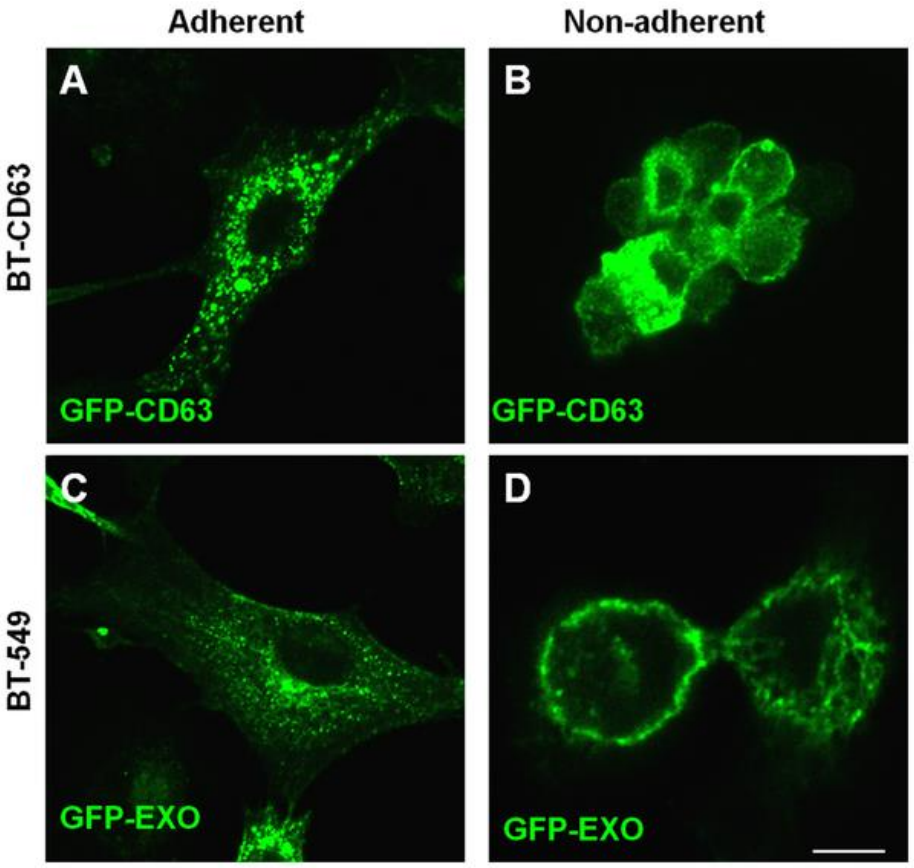

Figure 10. Monitoring the intracellular distribution of GFP⁺ CD63 Exos in adherent and non-adherent BT-CD63 cells (Adherent cells: A, and Non-adherent cells: B). The exposure of cells to EDTA led to the detachment of cells and accumulation of GFP⁺ CD63 Exos on the cell surface. In the adherent cells or cells cultured on polyHEMA[1] surface, a conditioned medium containing GFP⁺ CD63 vesicles was added to the culture system and kept for 3 hours (Scale bar: 10 μm). Copyright. [69]. PLOS ONE. 2021.

5. Small-sized Exo subset (Exosmeres)

Researchers have recently acknowledged that cells secrete an EV subtype, namely exomeres, with an average below 50 nm [70]. Compared to other EV types, exomeres are devoid of a lipid bilayer membrane and

[1]. Poly (2-hydroxyethyl methacrylate)

have non-membranous entities [71]. Of note, exomeres lack ESCRT components and contain various cargoes, such as proteins, lipids, and nucleic acids [70, 72]. It has been indicated that exomeres harbor high levels of enzymes and signaling proteins that are involved in the regulation of coagulation, hypoxia, and metabolic pathways, including glycolysis and the mTOR axis [70, 73]. Observations have indicated that exomers can bind proteins located in mitochondria, endoplasmic reticulum, and microtubules, proposing a possible role in the biogenesis and abscission of these particles [73]. Exosomes were first identified in *Saccharomyces Cerevisiae* to transport certain cargoes, such as Chs3p, Fus1p, and Pin2p, from the late TGN[1] to PM[2] [74]. The phenomenon of cargo sorting in TGN is solely associated with the metabolic status and cell cycle status [75]. Although several adaptor proteins can orchestrate the phenomenon of exosomers transport from TGN to PM, the exact underlying mechanisms remain unknown [76]. It is thought that integral membrane proteins are related to PM, small buds, and microtubule plus-end[3] [77]. Chs3 is the most well-known factor involved in the transport of exomeres between the TGN and PM. The suppression of this factor can disrupt the trafficking process, resulting in impaired chitin production [77]. Interestingly, while Pin2 promotes prion formation, Fus1 is necessary for cell fusion during mating [78]. Previously, the function and exact roles of these proteins have been addressed in the structure of the TGN. It is conceived that these factors are eligible cargoes inside the exomeres [78]. Specifically, exomers are constituted by scaffolding subunit Chs5p, and cargo-binding paralogs known as Chs5 and Arf1 binding proteins (ChAPs), including Bud7, Bch1,

[1]. Trans-Golgi network
[2]. Plasma membrane
[3]. Shmoo tip

Bch2, and Chs6 [74]. During cargo sorting, Chs6 and Bch2 attach to specific cargo proteins. After that, Bch1 and Bud7 are involved in bending membranes through interaction with the GTPase Arf1, leading to the budding of vesicles [77, 79]. Like Exos, several approaches have been employed to identify pure EV subpopulations so far, such as immuno-affinity capture, ultrafiltration, polymer-based precipitation, size-exclusion chromatography, and microfluidics [80-83]. Despite this, there have been few advances in the field of EVs due to the lack of simple methods for isolating secreted vesicles from non-vesicular constituents [84]. Thus, further improvement is required in the purity, yield, integrity, labor, and time-efficiency of isolated EVs [85]. To isolate exomers, two main protocols have been applied: ultracentrifugation and AF4[1] [86]. This approach is relatively new and utilizes only one semipermeable membrane [84]. This technique was developed by Zhang and Lyden for the separation and characterization of EVs, including Exos and exomers [85]. AF4 is capable of separating ENPs[2] at high resolution, as well as providing a wide range of dynamic separations based on size and hydrodynamic properties [84]. Additionally, several benefits have been mentioned for AF4 separation, such as label-free, gentle, highly reproducible, and efficient recovery of analytes [85]. A further advantage of this procedure is the short processing time (about 1 hour) in comparison with ultracentrifugation, which allows numerous further characterizations to be carried out to gain insight into the abundance and purity of the particles [87, 88]. However, there is a concern regarding loading capacity due to the requirement of large starting volumes to produce sufficient material for downstream processing [87]. Furthermore, AF4 requires sophisticated instruments and a high level of

[1]. Asymmetric-flow field-flow fractionation
[2]. Extracellular nanoparticles

user competence; therefore, it is preferable to use a much simpler method, such as ultracentrifugation [89].

6. Clinical trials and limitations

Emerging data have shown an increase in clinical studies related to the theranostic properties of Exos (**Table 2**). Since Exos are released actively in both pathological and physiological conditions, these particles can reflect the real-time metabolic status of parent cells, especially in cardiovascular diseases [90]. In this regard, circulating Exos in the blood is a valid theranostic tool for various pathologies in clinical settings. Despite these advantages, several bottlenecks and limitations restrict the clinical application of Exos [60]. It seems that the lack of off-the-shelf Exo sources is a big challenge in the clinical setting. Based on the previous data, storage temperature, and the possibility of pH fluctuations can exert detrimental effects on Exo structure [91]. The exposure of pooled Exos to multiple freeze-thaw cycles can influence the physicochemical properties as well [91]. It has not been well-addressed that the metabolic status of donor cells to what extent affects the quality of isolated Exos. For instance, various insulting conditions and inflammation can alter the molecular signature of Exos compared to the physiological conditions [92]. The lack of standard and GMP-grade protocols for the isolation of Exos from biological systems can lead to inconsistencies in clinical outcomes [93, 94]. Sterility and the possibility of transmission of infectious particles should not be neglected in terms of Exo application in the clinical setting. Recent data have confirmed the existence of common shared effectors between viral replication and Exo biogenesis, leading to the distribution of viruses via circulating EVs [30]. Some side effects, like thrombosis and hemostatic perturbations, can make the Exo therapy problematic [95]. The

activity of immune cells and alloreactive T cell responses reduces the Exo retention time inside the body [30].

Table 2. Some clinical studies related to the application of EVs in humans based on a search at URL: https://clinicaltrials.gov/

NCT Number	Study URL	Conditions	Study Type
NCT04127591	https://clinicaltrials.gov/study/NCT04127591	Myocardial Infarction	Observational
NCT05669144	https://clinicaltrials.gov/study/NCT05669144	Myocardial Infarction	Interventional
NCT03384433	https://clinicaltrials.gov/study/NCT03384433	Cerebrovascular Disorders	Interventional
NCT05370105	https://clinicaltrials.gov/study/NCT05370105	Stroke Rehabilitation	Observational
NCT05326724	https://clinicaltrials.gov/study/NCT05326724	Post-stroke Dementia| Acupuncture	Interventional
NCT06319742	https://clinicaltrials.gov/study/NCT06319742	Stroke, Stroke Biomarkers	Observational

7. Conclusions

Stem cell-free Exo therapy is one of the promising alternatives in regenerative medicine. Due to their theranostic properties, the application of Exos can circumvent the problems associated with direct stem cell transplantation and invasive methods used for the diagnosis of certain pathological conditions. The Exo market has been increased via different investigations from pharmaceutical companies, indicating that different Exo sources are possibly a future medication for the delivery and therapy of pathologies with minimal side effects [96]. More attentionally, the problematic side of Exo therapy and research is related to purification and isolation. Therefore, future studies should focus on addressing these challenges. Development of standard and safe protocols for the isolation

of Exos from different biofluids, such as blood, urine, and serum, can help to yield consistent outcomes in the clinical setting. Additionally, it should be noted that Exo biogenesis machinery has intricate and shared common effectors with the other signaling pathways, such as autophagy (**Figure 11**) [8]. These properties can help us in the modulation of other intracellular pathways via Exos with fewer side effects.

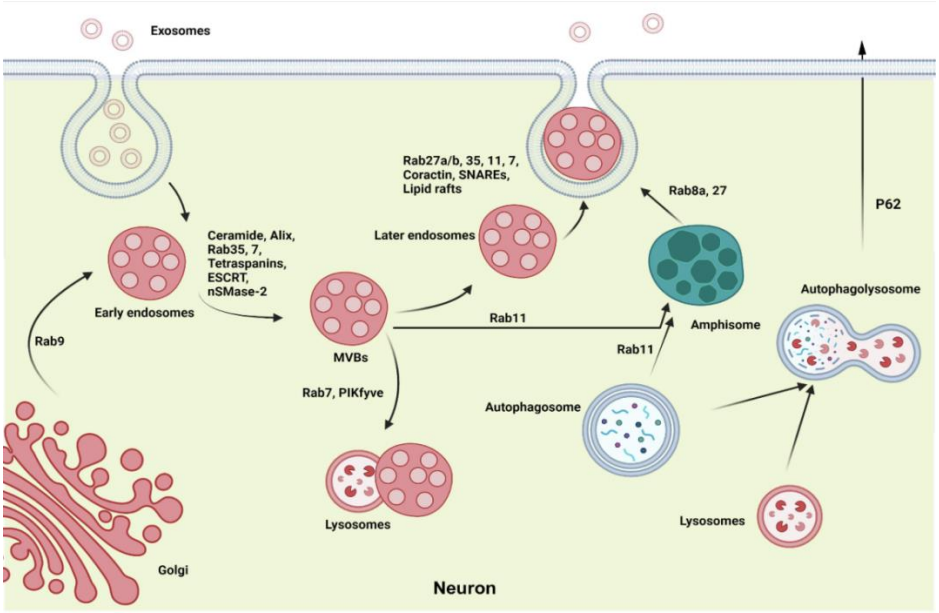

Figure 11. Exo biogenesis machinery has several shared effectors with other signaling pathways, such as autophagy. Late endosomes and especially MVBs may fuse with autophagic autophagosomes to generate the amphisomes. In the next step, amphisomes can excrete their contents via the plasma membrane. These data show the close autophagy-Exo signaling pathway interplay. Copyright. [8]. Neurobiology of Pain. 2022.

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