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REVIEW

Extracellular Vesicles in Drug Delivery: From Quality Assurance to Therapeutic Application

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Abstract: Developing optimal drug delivery carriers to enhance the pharmacokinetics of therapeutic agents and mitigate toxicity to normal cells remains a pivotal focus in medical research. Extracellular vesicles (EVs) have emerged as a highly promising platform for drug delivery, owing to their unique biological properties. Through intrinsic biogenesis pathways, EVs can selectively encapsulate genetic material, proteins, cytokines, and other bioactive components from donor cells. They subsequently mediate intercellular communication and regulate target cell behavior via humoral transport, surface protein interactions, membrane fusion, and other mechanisms—biological features that lay the foundation for their potential in therapeutic delivery. In recent years, EVs have attracted tremendous research interest due to their excellent biocompatibility, nanoscale size, low immunogenicity, facile modifiability, and versatile capacity to load various therapeutic agents. In this review, we analyse strategies for improving the quality control of drug-loaded EVs across multiple dimensions, specifically including the selection of EVs sources, control of isolation and purification, control of drug-loading strategies, and evaluation strategies after EVs drug loading, we also report the latest preclinical and clinical studies on the use of EVs as drug delivery systems for small-molecule drugs, nucleic acids, and proteins in disease treatment.

Keywords: extracellular vesicles, exosome, drug delivery, nucleic acid, quality control

Introduction

Over the past decades, various materials including liposomes, micelles, metal nanoparticles, and dendritic polymers have been successfully developed as drug delivery vehicles.[1] These advancements aim to improve the efficacy of therapeutic drugs and to reduce drug toxicity and off-target side effects.[2] Studies have reported that the tissue distribution profiles and clearance rates of therapeutic drugs depend primarily on the carrier platform, rather than the physicochemical properties of the drug molecule itself.[3–5] While various nanoparticles have shown promising results in drug delivery, obstacles such as rapid clearance by the body's reticuloendothelial system, low biocompatibility, acute hypersensitivity reactions, and in vivo deposition leading to other pathologies have hindered their clinical translation.[6–8] Therefore, there is an imminent need for the development of novel and superior performance drug delivery platforms.

In recent years, researchers have shown significant interest in the secretion of lipid bilayer EVs enclosed by almost all cell types as potential drug delivery carriers. Initially considered as waste products, EVs are recognized for their ability to transfer membrane proteins and cytoplasmic contents such as RNA and DNA to receptor cells, facilitating cellular communication and regulating biological behavior.[9,10] The biocompatibility, low immunogenicity, low toxicity, and targeting capabilities of EVs, coupled with the diverse loading methods for various drug payloads, have positioned them as promising drug delivery vehicles.[11–14] Furthermore, the non-replicative and non-mutagenic nature of EVs minimizes regulatory concerns related to adverse reactions and tumor formation. This paper provides an overview of recent advancements in the use of EVs as drug delivery systems, with a focus on quality control, cargo loading methods, and clinical applications for delivering therapeutic cargoes.

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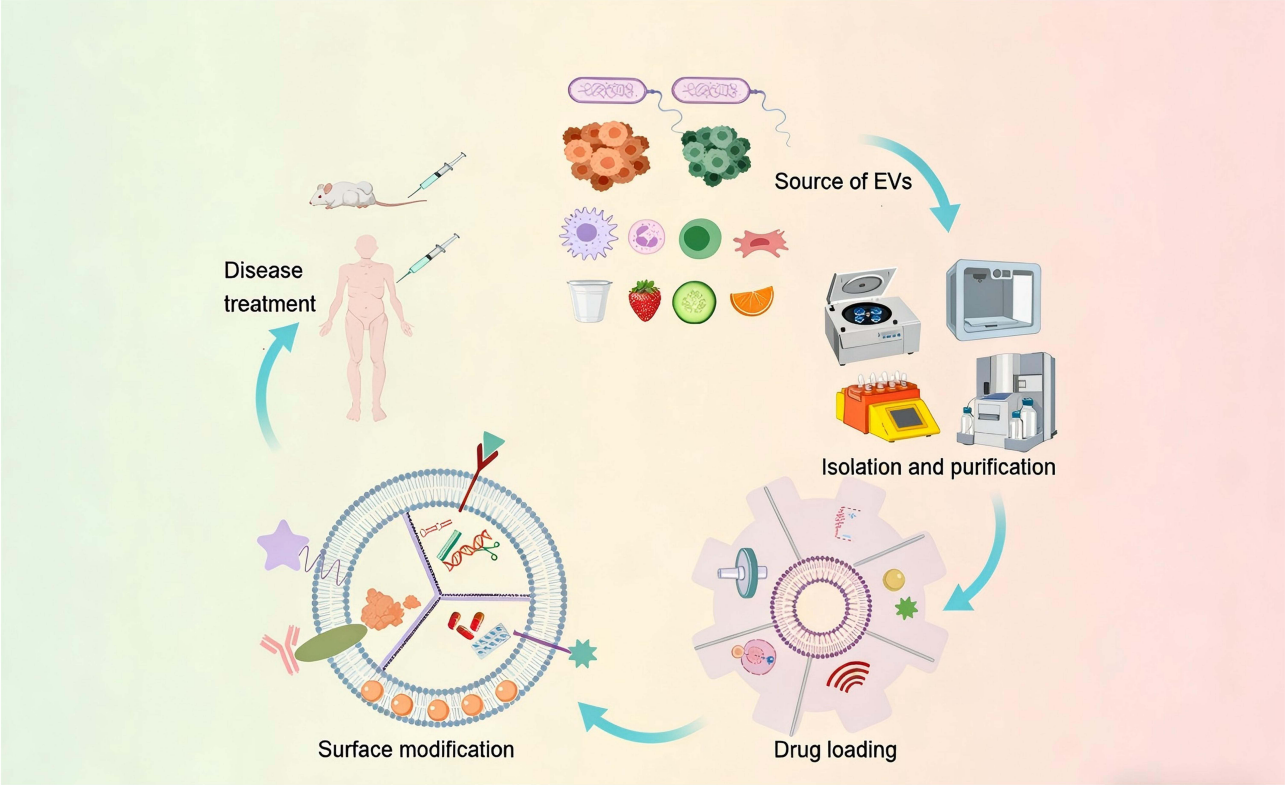
Graphical Abstract



Biogenesis of EVs and Targeting Receptor Cells

EVs are traditionally classified into exosomes (30–150nm), microvesicles (MVs) (100nm-1μm), and apoptotic bodies (50nm-5μm) based on their biogenesis and size.[15,16] Understanding the biogenesis of EVs is crucial for elucidating the mechanisms by which they target receptor cells.

Exosomes Biogenesis

Exosomes biogenesis involves the invagination of the plasma membrane and the formation of intracellular multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). The process begins with the plasma membrane engulfing cellular and soluble proteins from the extracellular environment, leading to the maturation of early sorting endosomes into late endosomes. Within the late endosomes, inward depressions result in the formation of multiple ILVs within the MVBs. Subsequently, the MVBs undergo degradation by fusing with lysosomes or autophagosomes, leading to the release of ILVs (ie., exosomes) with diameters of 30–120 nm into the extracellular milieu by fusion with the plasma membrane.[17,18] The endosomal sorting complex required for the transport of specific components in this process consists of four essential proteins. ESCRT-0, responsible for cargo recruitment, works alongside ESCRT-I and ESCRT-II, which are responsible for specific cargo encapsulation during plasma membrane endocytosis. ESCRT-III primarily facilitates the formation of ILVs in the MVBs.[19] In cases where ESCRT is inhibited, the ESCRT-independent pathway, involving lipids, tetratransmembrane proteins, and HSP, can also participate in the biogenesis of ILVs and MVBs.[20]

MVs Biogenesis

MVs typically originate from specific plasma membrane structures rich in lipids, cytoskeleton, and signaling proteins.[21] The biogenesis of MVsinvolves distinct stages: budding, cargo sorting, and shedding. Budding Stage: This stage involves two key steps. First, the localized accumulation of actin and actin fibers on the cell surface, and second, the release of intracellular calcium particles that alter the bioactivity of flippase and scramblase, causing the flipping of negatively charged phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. This change in net charge on the outer surface of the cell enhances membrane curvature effects, inducing MVs outgrowth. Cargo Sorting Stage: Protein and RNA cargoes can be selectively loaded into MVs through mechanisms such as glycosylphosphatidylinositol anchors and base complementary pairing.[22,23] Shedding Phase: ADP-ribosylation factor 6 induces actin-myosin contraction by triggering signal-induced phosphorylation of myosin light chain kinase (MLCK). This contraction leads to MVs neck or bottom shearing, followed by shedding into the extracellular space.[24]

Apoptotic Bodies Biogenesis

Apoptotic bodies are derived from various types of apoptotic cells, characterized by nuclear fragmentation, coalescence, and breakage of the genetic material chromatin and nucleosome DNA.[25] The main steps involved in apoptotic body formation can be summarized as follows: (1) Membrane vesicle formation: rupture of the plasma membrane of the apoptotic cytoskeleton, its decreasing size, and the contractile effect of actinoglobulin cause the dense accumulation of cellular contents and an increase in hydrostatic pressure, inducing the formation of plasma membrane blisters. (2) Pseudopod formation: subsequently, diverse pseudopods, such as spiking, apoptotic, and beaded apoptotic pseudopods are formed, beaded apoptotic pseudopods are considered the predominant form of apoptotic bodies.[26] (3) Nuclear vesicle fragmentation: the nucleus invaginates and breaks into fragments, ultimately forming apoptotic bodies.[27] Notably, compared to MVs biogenesis, apoptotic bodies are not autonomously selective for cargo sorting, and components such as cytoplasm, organelles, and nuclear contents are randomly assigned to apoptotic bodies, resulting in diverse compositions within apoptotic bodies from the same cell.[28] Externalized PS on the membrane surface of apoptotic bodies acts as an "eat-me signal," facilitating their clearance by macrophages. PS can be bridged to macrophage surface TAM receptors, αvβ3 and αvβ5 integrins, via specific proteins, indirectly inducing their clearance. Additionally, PS can directly bind to macrophage receptors, facilitating phagocytosis and contributing to the relative stability of the organism.[29–32] It is important to note that externalized PS, once thought to be specific to apoptotic bodies, has been found on other types of EVs surfaces, leading to controversy regarding its role as a marker for distinguishing apoptotic bodies from exosomes and microvesicles.[33]

EVs Targeting to Receptor Cells

The targeting of therapeutic EVs to receptor cells encompasses diverse modalities, including endocytosis, protein interactions between EVs and receptor cells, and direct fusion of EVs membranes with receptor cell surface membranes to deliver therapeutic cargoes to the cytoplasmic lysosomes or membrane surfaces of the receptor cells, subsequently influencing downstream signaling effects.[34–37] The vast majority of receptor cells for EVs uptake mainly utilize clathrin-dependent endocytosis pathways, giant cytotoxicity, phagocytosis, and other energy-dependent endocytosis mechanisms. They achieve this through fusion with endosomes or plasma membrane fusion to regulate the biological behavior of receptor cells.[38–40] Regulators such as cytochalasin D, chlorpromazine, phosphatidylinositol-3-kinase (PI3K), and Dynamin2 have been reported to modulate endocytosis uptake in EVs.[41–45] Specific protein-protein interactions mediate EVs attachment and uptake into cells. High-abundance EVs surface tetratransmembrane proteins (eg., CD63, CD9, CD81) target receptor cells through adhesion, while antagonism of the receptor cell surface integrin proteins CD51 and CD61 reduces EVs uptake by dendritic cells (DCs).[46] Direct membrane contact fusion involves the formation of semi-fused stems by lipid outer leaflets upon EVs membrane-receptor cell contact in an aqueous environment. This process includes stem expansion to produce semi-fused septa, subsequently leading to the mixing of hydrophobic cores through fused pores into a single structure, allowing the exchange of material information

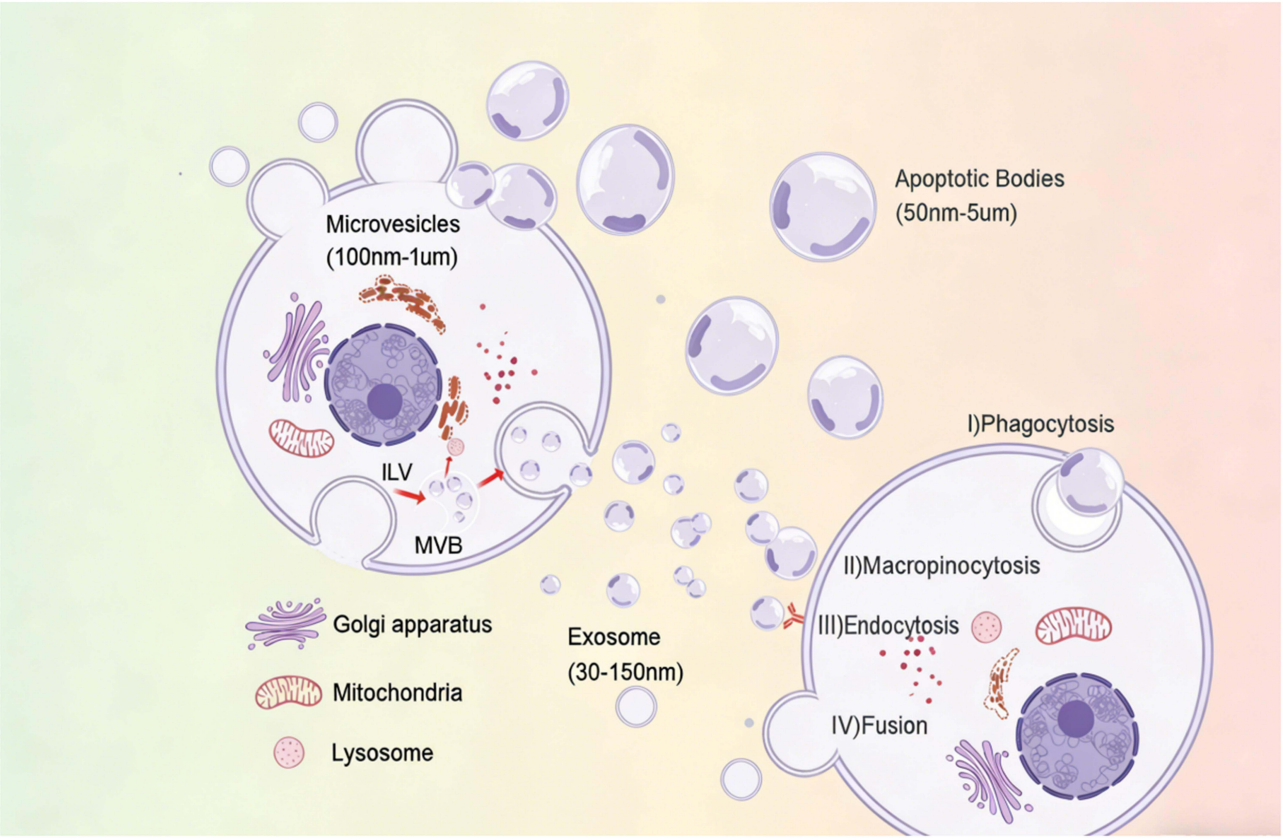


Figure 1 Biogenesis, Secretion, and Cell Entry of EVs This diagram illustrates the biogenesis, secretion processes of EVs, and their uptake mechanisms by recipient cells. EVs are categorized into three main subtypes (consistent with the visualization), Exosomes (30–150nm): Their biogenesis starts with the inward budding of early endosomes to form intraluminal vesicles (ILVs); ILVs accumulate within the endosome to assemble into multivesicular bodies (MVBs). MVBs then undergo two main fates: fusion with the plasma membrane to release ILVs as exosomes, or fusion with lysosomes for content degradation. Microvesicles (MVs,100nm–1μm): Generated by direct outward budding from the plasma membrane of the donor cell. Apoptotic Bodies (50nm–5μm): Produced by cells undergoing apoptosis (accompanied by nuclear fragmentation). Golgi apparatus: Participates in the sorting and transport of cellular components, providing support for the trafficking of EV-related substances. Mitochondria: Supplies energy for the biological processes involved in EV biogenesis and secretion. Lysosome: Mediates the degradation of unreleased EVs via fusion with MVBs. Four uptake mechanisms (mediating EV internalization by recipient cells and subsequent cargo delivery) are shown: Phagocytosis, Micropinocytosis, Endocytosis, Fusion.

between EVs and receptor cells.[47,48] In the field of drug delivery, all three types of EVs can deliver signaling molecules and therapeutic substances to receptor cells to exert biological effects (Figure 1). Therefore, in this review, we will consider them as a collective entity for study rather than discussing their individual roles in drug delivery separately.

Carrier EVs Source Selection

Human Origin

In the human body, most cells, tissues, and body fluids can produce EVs, although not all are suitable for use as drug carriers in disease treatment. For example, tumour cell-derived EVs were initially considered ideal delivery vehicles for tumour-targeted delivery of anticancer drugs, owing to their unique tumour "homing effect" and their rich content of MHC-I molecules, HSP70, and antigens that can enhance the body's immune response against tumours.[49,50] However, subsequent studies have further found that tumour-derived EVs possess specific oncogenic capabilities. They contain components such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs, such as MMP-2, MMP-9), transforming growth factor β (TGF-β), programmed death-ligand 1 (PD-L1), as well as miR-21, miR-10b, which can promote tumor growth through various mechanisms including promoting angiogenesis, degrading the extracellular matrix, and inhibiting T cell proliferation, relevant work has been mainly carried out in preclinical studies.[51–53] Therefore, the suitability of tumor-derived EVs as drug delivery vehicles for targeting tumor therapy

requires further examination, taking into consideration factors such as the stability of the cell line, culture difficulty, EVs yield, and the safety of secreted EVs. At present, EVs derived from mesenchymal stem cells (MSCs) and DCs are considered good choices as drug delivery vehicles. MSCs are known for their application as cell-free strategies for tissue repair and regeneration, and a study comparing the differences in the number of EVs obtained by various cell lines found that MSCs produced 81-fold the number of EVs compared to the other cell lines under equivalent treatment conditions.[54,55] Interventions such as C-MYC gene transfection and exposure to hydrogen peroxide and lipopolysaccharide can further enhance MSC-EVs secretion.[56,57] On the other hand, DC-derived EVs retain the basic immunostimulatory capacity of DCs and possess multiple surface membrane proteins that enhance their ability to target receptor cells.[58,59] It is indeed exciting to note the progress in developing EVs from sources such as T cells, macrophages, and platelets as potential drug delivery vectors for treating diseases.[60] This expansion of EVs donor sources holds significant promise for advancing the field of drug delivery and underscores the potential for utilizing a diverse array of EVs in therapeutic applications.

Non-Human Origin

In addition to the human body, various sources such as plants, microorganisms, and animals are currently undergoing extensive study as donors for EVs for potential use in drug delivery. Research on plant-derived extracellular vesicles (PDEVs) is thriving, with ongoing efforts to elucidate the biogenesis mechanism. Possible pathways include the MVBs pathway and the vesicular pathway, and it is clear that PDEVs can traverse biological barriers to reach target tissues, enabling intercellular exchange of material information.[61,62] For example, Zhang et al developed a folic acid (FA)-modified ginger-PDEVs drug delivery system that efficiently loaded Doxorubicin (Dox) for colon cancer therapy. The study showed that unlike free Dox, which enters cells via passive diffusion, Dox-PDEVs can mediate direct targeting of the drug to the cell nucleus, thereby exerting antitumor effects. This drug delivery system exhibits time-dependent drug release and significantly enhances apoptosis in colon cancer cell lines, Colon-26 and HT-29, compared with free Dox.[63] These features make PDEVs an attractive option for drug delivery, and successful extraction has been achieved from various plants such as grape, grapefruit, ginger, and lemon.[64] The core bottleneck in the large-scale development of PDEVs as drug delivery carriers lies in the significant differences between plant and mammalian cell membrane components. Plant-specific lipids such as phytosterols, polysaccharides, and proteins can be recognised by the human immune system as foreign antigens, triggering non-specific immune responses. Additionally, due to limitations in purification processes, toxic components of plant origin, such as alkaloids and terpenes, can easily remain and enter the body with the carrier, causing hepatotoxicity, nephrotoxicity, and cytotoxicity, thereby threatening drug safety.[65] Apart from plant-derived EVs, outer membrane vesicles (OMVs) produced by Gram-negative bacteria have garnered significant attention as delivery vectors in recent years. Originating from the outer membrane and periplasm of the parental bacteria, OMVs are rich in genetic material, protein molecules, virulence factors, and other components.[66] With antimicrobial properties, OMVs contain various components that can inhibit the growth of fungi and bacteria. As drug delivery carriers, OMVs meet several prerequisites such as good drug loading, targeting ability, thermal stability, and modifiability.[67] For instance, a study demonstrated that small interfering RNAs (siRNAs) targeting the kinesin spindle protein (KSP) were loaded into attenuated Escherichia coli-derived OMVs, leading to significant inhibition of tumor growth in an animal model.[68] However, despite the progress in researching non-human sources of EVs as drug delivery systems, there is a current lack of comparative studies with human-derived EVs, and their safety and practicality require further investigation.

Quality Control of EVs Isolation Strategies

Achieving scalable isolation, high-purity enrichment, and preservation of structural integrity of EVs is crucial for their clinical translation and application as drug delivery carriers. Currently, there are various techniques for isolating and purifying EVs from biological fluids, mainly including ultracentrifugation (UC), ultrafiltration (UF), size-exclusion chromatography (SEC), precipitation, and ion exchange. The specific technique choice needs to be determined based on a comprehensive assessment of the EVs' size distribution, density characteristics, and surface protein markers.[69–73] Among them, ultracentrifugation is the most widely used traditional separation technique. Its core principle is to achieve

separation and purification by exploiting differences in sedimentation coefficient between EVs and impurity particles in a centrifugal field. This method has notable advantages such as a simple operation process, cost-effectiveness for long-term use, and the ability to handle large sample volumes. However, it also has inherent drawbacks, including the potential to damage EVs membranes and induce impurity aggregation.[74] As an improved ultracentrifugation technique, density gradient centrifugation effectively mitigates the damage traditional ultracentrifugation causes to the structural integrity of EVs. It constructs a density gradient through gradient media and achieves precise separation based on the density differences between EVs and impurities. Sucrose, caesium chloride, and iodixanol are currently commonly used gradient media. This method can significantly improve the purity of EVs separation, but it is time-consuming and yields relatively low final product amounts.[75] SEC is a gentle, efficient chromatographic separation technique suitable for processing various biological fluids, such as plasma and serum. A single separation cycle only takes 10–20 minutes, offering the significant advantage of rapid processing. Its core mechanism relies on a porous stationary phase constructed from polymers, separating EVs based on differences in particle size: small molecules, lipoproteins, plasma proteins, and other impurities can enter the pores of the stationary phase, migrating slowly or being retained, while larger EVs cannot enter the pores and can be quickly eluted. This technique can stably remove impurities while maximally maintaining the structural integrity of EVs; however, insufficient purity remains its main limitation, restricting its use in high-purity EVs preparation scenarios.[76] Tangential flow filtration (TFF) is also a widely used technique for separating and enriching EVs. Its principle is to utilise the selective permeability of biological membranes to biological fluid components, allowing small molecules and impurities to pass through the membrane as permeate. At the same time, larger EVs are retained in the original solution, thereby achieving preliminary separation and enrichment.[77] Immunoaffinity technology, on the other hand, uses specific antibodies (such as those targeting EVs surface markers like CD9, CD63, and CD81) to efficiently capture target EVs from complex biological fluids via antigen-antibody interactions. It is characterised by strong targeting and high separation efficiency.[78]

Microfluidic technology, as a new method for EVs separation, offers key advantages in controllable separation efficiency and strong structural preservation. This technology can integrate multiple external forces, such as hydrodynamic, electric, or acoustic fields, to work synergistically.[79] It can achieve precise separation based on the physical properties of EVs (such as size, charge, and mass) and also enable targeted capture by combining surface markers, adapting to separation needs in different scenarios. Moreover, the separation process is gentle; compared to traditional ultracentrifugation, it minimizes damage to EVs membranes and maintains their biological activity, meeting the requirements of clinical applications for preserving the functional integrity of EVs.[80,81] At the same time, microfluidic devices integrate core components such as micromixers, microvalves, microchannels, and micropumps, offering features such as high surface area-to-volume ratio, low sample consumption, short analysis cycles, excellent laminar flow characteristics, and ease of operation, making them highly promising for large-scale EVs separation applications.[82] However, this technology also has obvious limitations: First, the technological threshold is relatively high, with complex device design and manufacturing processes, requiring stringent microfabrication techniques, and making the development and mass production of core components complex, which restricts widespread adoption; second, the capability for handling large-volume samples is limited, as existing devices are mostly suited for small-volume specimens, resulting in insufficient processing efficiency for large-volume biological fluids such as plasma and serum; third, achieving high-purity preparation is constrained, as a single microfluidic technique cannot completely remove small molecule impurities, lipoproteins, and other contaminants, often necessitating combination with techniques like immunoaffinity to meet high-purity requirements.[83] For example, the Exodisc system developed by Woo et al consists of a disc body and two nanofilters, using 600 nm and 20 nm nanofilters in sequence. By spinning at a low speed (<500 g), EVs with diameters between 20 and 600 nm are captured between the two filter layers and then collected through a microchannel. This system can achieve a recovery rate of up to 95% for EVs from biological fluids. In addition, microfluidic devices based on charge interactions can exploit electrostatic attraction between the device's positive charges and the EV's negative charges to enrich EVs in designated areas.[84] Other studies have developed a centrifugal microfluidic disc system combined with functionalized membranes, achieving 82.54% efficient separation of EVs in biological fluids by adsorbing the negative charges on EVs surfaces to the positive charges on the surface of renewable cellulose membranes.[85] The EXID system developed by Lu et al features high sensitivity, ease of operation, and low sample consumption. Its

Table 1 Comparison of Different Isolation Methods for Extracellular Vesicles (EVs)

Isolation Strategy	Principle	Advantages & Disadvantages	Optimization Strategies	References
Ultracentrifugation	Separate EVs by density/size differences via gradient centrifugation (100,000×g or higher) to enrich EVs precipitation.	Advantages: Mature, widely used, suitable for basic research. Disadvantages: Severe damage to EVs, low efficiency, time-consuming, high equipment requirements.	Use density gradient centrifugation; control speed/time; pre-treat samples with ultrafiltration to shorten centrifugation time.	[87]
Immunoaffinity Isolation	Capture target EVs via specific binding between EVs surface markers (CD9/CD63/CD81) and antibodies fixed on carriers.	Advantages: High specificity/purity, mild conditions, less EVs damage. Disadvantages: High cost, low throughput, antibody interference on EVs function.	Use multi-marker antibodies; optimize antibody immobilization; combine with microfluidics to improve throughput.	[78,81]
Microfluidic Technology	Separate EVs via microchannel structure and external forces (hydrodynamics/electric field), combined with immunoaffinity for high specificity.	Advantages: Mild conditions, high efficiency/specificity, small sample volume, miniaturizable. Disadvantages: High technical threshold, low large-sample handling capacity.	Optimize microchannel design; integrate multiple forces; develop large-scale equipment; simplify chip manufacturing.	[79–81]
Combined Isolation Strategies	Integrate two+ single strategies (eg., low-speed+ultracentrifugation, microfluidics +immunoaffinity) for high efficiency/purity.	Advantages: Higher purity/efficiency than single strategy, multi-demand adaptable Disadvantages: Complex operation, high cost, need for step optimization.	Optimize step parameters; select optimal combination; establish standardized procedures.	[87]
Ultrafiltration+size-exclusion chromatography (SEC)	Ultrafiltration: Sieving via membrane; SEC: Molecular sieve effect to separate EVs from impurities (auxiliary purification).	Advantages: Simple/fast (ultrafiltration), mild/no EVs damage (SEC). Disadvantages: Membrane clogging (ultrafiltration), low throughput (SEC).	Select suitable membrane/pore size; control pressure; optimize SEC column and elution conditions.	[86]

optimized design, with serpentine channels and incubation chambers, ensures sufficient contact between EVs and CD9-coated magnetic beads, enabling efficient capture of target EVs.[82]

Currently, no research has confirmed that a single isolation strategy can be applied to the complete range of EVs isolation in biological fluids. However, the existing literature indicates that combining multiple isolation methods can significantly improve the efficiency and purity of EVs isolation. Common combined strategies include combining low-speed and high-speed ultracentrifugation: first, low-speed centrifugation at 10,000×g is used to remove large contaminants, such as impurities and drug aggregates, from biological fluids, followed by high-speed ultracentrifugation at 100,000×g or higher to enrich EV particles.[86] In addition, the combination of ultrafiltration and ultracentrifugation is also a widely used efficient isolation method[87] (Table 1).

EVs Drug Loading Strategy

As endogenous intercellular information carriers, EVs can mediate the systemic delivery of various bioactive substances. At present, EVs drug-loading strategies mainly fall into two categories: the first involves pretreating donor cells to achieve drug loading before EVs secretion; the second involves loading drugs after EVs isolation and purification via in vitro methods. Pre-secretion loading technology is based on the natural property of cells to select and integrate molecules or genetic material into EVs during biosynthesis. In contrast, post-isolation loading technology, also known as exogenous loading, uses external forces to create reversible openings in the EV membrane, allowing the target drug to be loaded through these openings. Next, we will specifically describe existing EVs drug-loading strategies and further explore how to optimize them to improve the controllability of EVs quality.

Pre-EV Isolation Drug Loading Strategy

Co-Incubation

The core principle of the co-incubation loading strategy is to co-culture donor cells with the target drug (or functional biomolecules) in vitro. Through the cells' active uptake, metabolism, and vesicle sorting mechanisms, drug molecules are promoted to enter the cytoplasm and be incorporated into newly formed EVs. Drug-loaded EVs are obtained by collecting the cell culture supernatant and isolating and purifying them.[88] Relevant studies have confirmed that after co-incubating MSCs with 2000 ng/mL paclitaxel for 24 hours, the amount of paclitaxel enriched in the EVs isolated from the cell supernatant can reach 11.68 ng/mg, and such drug-loaded EVs exhibit significant inhibitory effects on the proliferation and invasion of pancreatic adenocarcinoma cells.[89] This strategy has advantages such as ease of operation and the ability to preserve the natural structure and biological activity of EVs maximally, but it has limitations, including low drug loading efficiency and significant raw material waste; blindly increasing the drug concentration to enhance loading capacity can also cause additional damage to the physiological activity of donor cells, thereby affecting the yield and quality of EVs.[90] Therefore, in the co-incubation loading mode, donor cells in the logarithmic growth phase with good growth status are selected (to ensure EVs secretion and loading) and incubated in serum-free medium containing the target drug (to avoid interference from miscellaneous proteins and exogenous vesicles). At the same time, a drug concentration gradient is established, and the optimal range is screened through preliminary experiments (to balance drug loading efficiency and cytotoxicity, ensuring EVs yield and characteristics), thereby improving the quality of drug-loaded EVs.

Transfection

Transfection is the technical process of introducing exogenous nucleic acids into cells to obtain genetically modified cells. Based on this technology, EVs carrying therapeutic molecules can be isolated from the culture supernatant of transfected cells through methods such as ultracentrifugation, and this has become a conventional technical approach for constructing pre-secretion drug-loaded EVs.[91] For transfection techniques, high efficiency with low toxicity, minimal interference with cell physiological status, ease of operation, and strong reproducibility are core indicators of their application value.[92] At present, cell transfection technologies can be divided into three major categories based on their working principles: virus-mediated transfection utilizes the infective capability of viral vectors to deliver nucleic acids; chemical transfection relies on the electrostatic adsorption of positively charged nucleic acid–chemical complexes to negatively charged cell membranes for transport; and physical transfection techniques, such as microinjection, bioparticle delivery, electroporation, and laser-mediated transfection, directly alter cell membrane properties through physical means to achieve nucleic acid delivery.[93] In terms of optimizing transfection efficiency, numerous studies have shown that the appropriate use of cations or cationic polymers can effectively enhance transfection outcomes, and cations such as calcium and magnesium have also been confirmed to have similar enhancing effects.[94] The research by Jordan J. Green and others further supports this conclusion. They found that using a divalent cation buffer with a pH of 5.0 (such as magnesium or calcium acetate buffer) as the transfection buffer can also significantly increase transfection efficiency.[91] However, this technology still faces two key challenges in the practical application of drug-loaded EVs: First, when therapeutic mRNA, siRNA, and miRNA are expressed in donor cells, they simultaneously promote the loading of both therapeutic and non-essential proteins into EVs. This phenomenon suggests that during the preparation of therapeutic EVs, precise selection of cargo mRNA is necessary to reduce the co-delivery of non-essential proteins, ensuring the therapeutic specificity and safety of EVs. Second, contamination control during the transfection process is crucial. Studies have indicated that contaminating transfection complexes can mimic small EVs, thereby hindering the effective delivery of target RNA.[95]

Drug Loading Strategies for EVs After Isolation

Electroporation

Electroporation is a common technique for loading hydrophilic small-molecule drugs such as miRNA, siRNA, 5-FU, and Dox into EVs[96] During the procedure, according to research needs, the target drug molecules are mixed with EVs in electroporation buffer at a specific ratio. The mixture is then usually treated using a Bio-Rad Gene Pulser Xcell

electroporation system at 250V and 100μF.[97,98] Under the influence of the electric field, the lipid bilayer of the EVs temporarily ruptures, increasing membrane permeability and allowing small molecule drugs to be loaded into the EVs.[98] After electroporation is completed, the mixture is incubated at 37°C for 30 minutes, which helps restore the membrane integrity of EVs.[99] The voltage intensity, discharge parameters (such as capacitance/pulse number), the mixing ratio of EVs to drug molecules, the physicochemical properties of the buffer, and the electrode materials are all considered important factors influencing the drug-loading efficiency of EVs.[100] The main advantages of this method are its ease of operation, strong controllability of parameters, and stable drug-loading efficiency.[16] The primary drawback is that high-voltage electrical pulses may cause irreversible physical damage to the membranes of EVs, leading to reduced EV integrity, leakage of contents, and potentially affecting their biological activity and targeting function.[101] In addition, for large nucleic acids (such as certain siRNAs/miRNAs), there may be issues of aggregation, degradation, or uneven loading. Therefore, optimizing parameters such as voltage, capacitance, and pulse number during practical application can effectively improve the loading efficiency of drug molecules and reduce additional damage to EVs during perforation.[102] Related studies have similarly indicated that Jia et al found that a target cargo to EVs concentration ratio of 3:1, with electroporation at 400 V, 150 μF, and a discharge time of 1 millisecond, can produce the optimal drug-loading effect.[103]

Ultrasound Treatment

Ultrasound treatment is another method for loading drug molecules such as siRNA, miRNA, ssDNA, paclitaxel, and Dox into EVs.[104] The basic principle is to use the mechanical shear force generated by an ultrasonic probe to induce deformation of the EVs membrane, allowing the target drug molecules to pass through the membrane structure for loading.[105] The amplitude, pulse frequency, cycle, and processing duration of ultrasound are key parameters affecting drug loading efficiency.[105] Compared to other loading strategies, such as incubation at room temperature and electroporation, the main advantage of ultrasound treatment is its ability to significantly increase drug loading capacity and release efficiency.[106] However, a major drawback of this method is that it can lead to changes in the EV structure, such as the formation of non-spherical EVs and/or membrane perforations, making it inconvenient for large-scale applications.[107] Related studies have reported that after ultrasonic treatment, incubating the solution at 37°C for 60 minutes helps to restore the integrity of the EVs membrane, and the mixture is then typically centrifuged at 10,000 rpm for 15 minutes, followed by three wash cycles to obtain drug-loaded EVs.[108]

Freeze-Thaw Method

This method utilizes the formation of ice crystals during freeze-thaw cycles to create transient gaps in EV membranes, thereby allowing the loading of target cargo.[109] The main advantage of this method is that it can effectively maintain the integrity of EVs membranes during repeated freeze-thaw cycles, without affecting their physicochemical and structural properties.[110] The main drawback is that the efficiency of cargo loading is unstable, and the lipid dilution ratio may affect fluorescence-based studies of EVs (Figure 2 and Table 2).

Screening and Characterization Techniques for Drug Loaded EVs

Before promoting the large-scale application of drug loaded EVs, a systematic and comprehensive evaluation of their biological characteristics is necessary. According to the MISEV2018 guidelines, qualified drug loaded EVs must have a typical bilayer lipid membrane structure without obvious membrane rupture, aggregation, or morphological distortion; Through transmission electron microscopy (TEM) or cryo electron microscopy (cryo-EM) observation, ≥90% of vesicles need to maintain a complete spherical or nearly spherical structure, with a uniform membrane thickness (about 5–10 nm) and no obvious heterogeneous impurity adsorption. Meanwhile, its size range must strictly conform to the classification characteristics of different types of vesicles, namely exosomes (30–150nm), microvesicles (100 nm-1μm), and apoptotic bodies (50 nm-5 μm). For drug loaded EVs batches with significant differences in morphological integrity or particle size distribution exceeding the preset threshold, they should be excluded to ensure the reliability of subsequent applications. In terms of drug loading efficiency, there are clear standards for drug loading requirements for different types of drugs: small molecule drugs such as paclitaxel and gemcitabine must meet drug loading requirements of≥1μg/mg total protein and

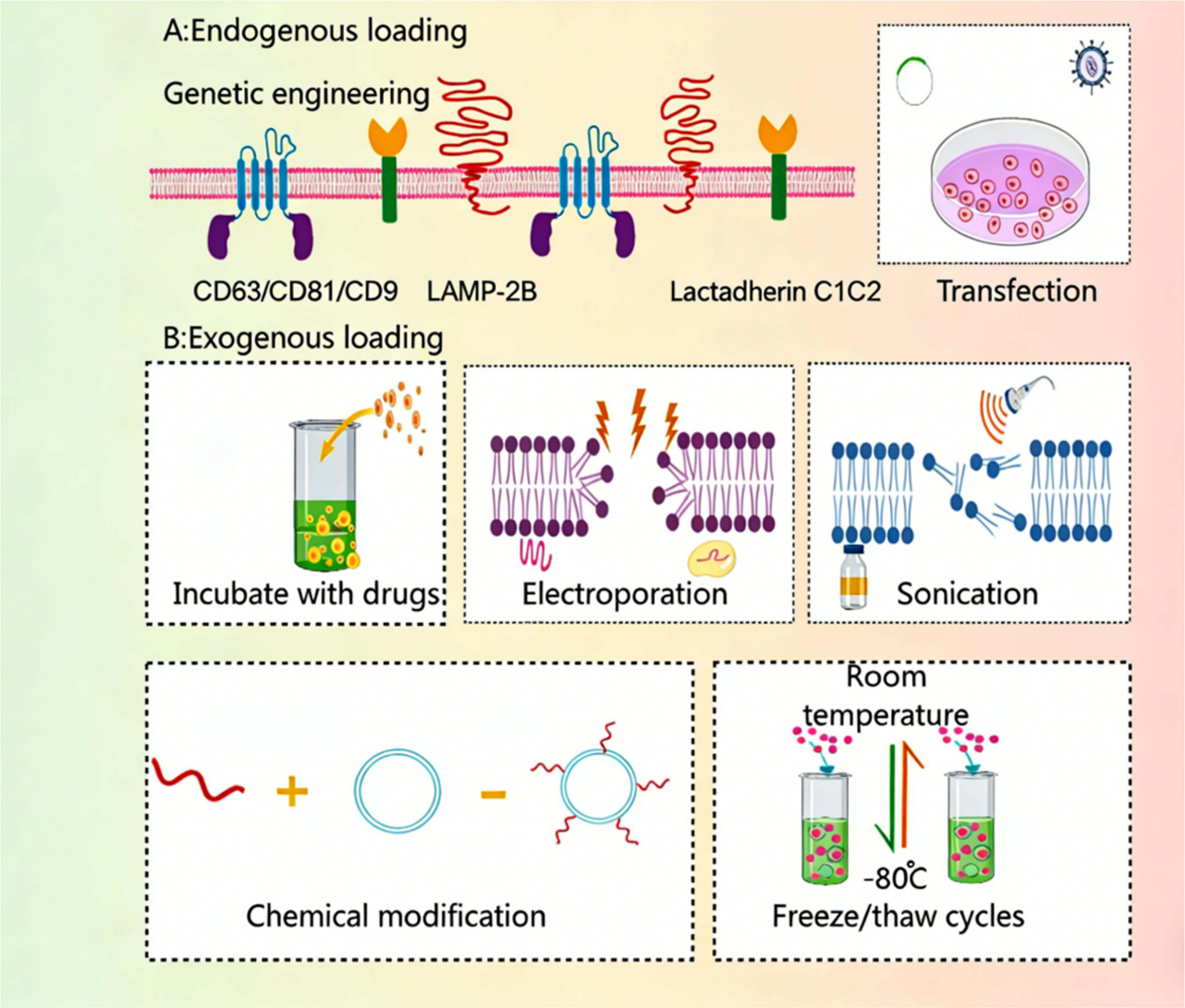


Figure 2 Loading of Therapeutic Cargo into EVs This figure depicts two primary strategies for encapsulating therapeutic agents into EVs: (**A**) Endogenous Loading Therapeutic cargo is genetically fused to EV-specific membrane components (CD63, CD81, CD9, LAMP-2B, Lactadherin C1C2) and incorporated into EVs during biogenesis, following host cell transfection. (**B**) Exogenous Loading Cargo is loaded directly into pre-formed EVs via: Physical methods: Incubation with drugs, electroporation, sonication, and freeze/thaw cycles (−80°C to room temperature). Chemical modification: A distinct method for covalent conjugation of cargo to EVs membranes or lumens.

encapsulation efficiency of≥60%; Nucleic acid drugs must meet the requirements of drug loading ≥0.1μg/mg total protein and encapsulation efficiency≥50%; Protein drugs such as enzymes and antibody fragments must meet the requirements of drug loading≥0.5μg/mg total protein and encapsulation efficiency≥40%.[15] The following text will discuss in detail the core dimensions of carrier EVs, such as morphology, particle size, and related components, based on existing related technology strategies, providing scientific references for the screening of high-quality drug loaded EVs.

Characterisation of the Key Physicochemical Properties of Drug-Loaded EVs

The characterisation of the physicochemical properties of drug-loaded EVs is a core aspect of evaluating their formulation quality and application potential. Among these, morphological integrity and particle size uniformity are two key quality control indicators. Morphological assessment relies heavily on high-resolution microscopy techniques, with commonly used observational methods including transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryo-electron microscopy (cryo-EM), and atomic force microscopy (AFM).[111] Among them, the detection processes of TEM and SEM usually require fixation and staining of EV samples.[112] By utilising electron penetration,

Table 2 Comparison of Principles, Advantages/Disadvantages, and Optimization Strategies of Commonly Used Drug Loading Strategies for Extracellular Vesicles (EVs)

Loading Strategy	Principle	Advantages and Disadvantages	Optimization Strategy	References
Electroporation	Mix EVs with target drugs in electroporation buffer, use electric field to temporarily disrupt the EVs lipid bilayer and increase permeability, allowing drugs to enter EVs, followed by incubation at 37°C to restore membrane integrity.	Advantages: High loading efficiency, suitable for various drugs. Disadvantages: Possible EVs membrane damage, potential drug inactivation by electric field.	Optimize voltage, capacitance, pulse parameters and the ratio of EVs to drugs; control the physical and chemical properties of the buffer to reduce EVs membrane damage and improve loading efficiency.	[97,103]
Co-incubation	Co-culture donor cells with target drugs in vitro, and rely on the active uptake, metabolism and vesicle sorting mechanisms of cells to allow drugs to enter the cytoplasm and integrate into newly generated EVs.	Advantages: Mild conditions, less EVs damage, simple operation. Disadvantages: Low loading efficiency, long incubation time.	Select donor cells in logarithmic growth phase and incubate in serum-free medium; establish a drug concentration gradient to balance loading efficiency and cytotoxicity.	[88,89]
Transfection	Transfect exogenous nucleic acids (drug-related) into donor cells to obtain genetically modified cells, and isolate EVs carrying therapeutic nucleic acid drugs from their culture supernatant.	Advantages: High specificity for nucleic acid drugs, stable drug loading. Disadvantages: Complex operation, potential cell toxicity.	Select efficient and low-toxic transfection methods, add cations to enhance transfection efficiency; accurately screen target mRNA to reduce co-delivery of irrelevant proteins and control transfection contamination.	[91,94]
Ultrasound Treatment	Use the mechanical shear force generated by the ultrasonic probe to induce EVs membrane deformation, allowing drug molecules such as siRNA and paclitaxel to pass through the membrane structure and enter EVs.	Advantages: Fast operation, simple equipment requirement. Disadvantages: Excessive ultrasound causes EVs fragmentation.	Optimize ultrasonic amplitude, frequency and treatment duration; incubate at 37°C for 60 minutes after loading to restore membrane integrity, and centrifuge to wash away free drugs.	[104,108]
Freeze-thaw method	Through repeated freeze-thaw cycles, use the formation of ice crystals to generate transient gaps on the EVs membrane, allowing target drugs to enter EVs through the gaps to achieve loading.	Advantages: Simple operation, no special equipment needed. Disadvantages: Repeated freeze-thaw damages EVs integrity.	Control the number of freeze-thaw cycles to maintain EVs membrane integrity; optimize the lipid dilution ratio to avoid interfering with EVs fluorescence-related detection.	[109,110]

electron scattering, or focused electron beam imaging, they enable visual analysis of EVs morphological characteristics. In contrast, cryo-EM does not require additional fixation and staining steps; by observing samples directly at low temperature, it can preserve the natural structure and original morphology of EVs to the greatest extent and has now become the mainstream method for EVs morphology identification and semi-quantitative analysis.[113,114] Additionally, AFM can exploit the interaction between the probe tip and the sample surface to provide multidimensional preliminary characterisation of EVs morphological features, mechanical properties, and biochemical characteristics.[115,116] For particle size characterisation, techniques such as nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and tunable resistive pulse sensing (TRPS) are widely used. Both NTA and DLS are based on the principle of light scattering, relying on the Brownian motion of EV particles in liquid systems to measure particle size by capturing fluctuations in scattering intensity.[117] However, DLS has lower accuracy in precisely measuring particle size and analysing the size distribution of heterogeneous EVs populations. As an optimised upgrade of DLS technology, NTA significantly improves the accuracy and resolution of EVs particle size assessment by tracking and dynamically recording individual EVs particle scattering signals in real time.[118] In addition, the detection principle of TRPS differs from the first two methods. It utilises the characteristic that EVs particles migrate towards the anode under an electric field, estimating particle volume by monitoring changes in buffer resistance during pulse excitation, thereby enabling simultaneous quantitative analysis of the absolute size and concentration of EVs particles in suspension.[119] Based on the combined application of the aforementioned morphological observation and particle size characterisation techniques, a systematic preliminary quality screening can be conducted for drug-loaded EVs formulations.[120] Batches of drug-loaded EVs with significant morphological integrity differences or particle size distributions exceeding preset thresholds should be discarded to ensure the reliability and data consistency of subsequent in vivo and in vitro functional experiments.

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Detection of Cargo Components in EVs

EVs contain a variety of key cargo molecules, including protein subunits, nucleic acids, lipids, and more, which play irreplaceable roles in communication between normal and disease-affected cells. Accurately assessing the cargo composition of large-scale produced EVs is crucial for their potential application as drug delivery vehicles for disease treatment. Mass spectrometry (MS) can comprehensively analyse the components of EVs, including proteins, lipids, and metabolites.[121,122] It should be noted that the effectiveness of EVs' mass spectrometry analysis critically depends on sample preparation; specific contaminants, such as detergents, lipids, and polymer materials, can affect MS results. Therefore, standardised EV purification steps are necessary to ensure detection sensitivity. This is closely related to the EVs separation and purification techniques mentioned earlier, as efficient separation and purification processes can provide high-purity samples for subsequent MS analysis, thereby reducing interference from contaminants.[123] Zhou et al developed a high-throughput nanobiochip liquid biopsy integration system, which uses molecular beacons (MBs) and antibody-conjugation strategies to achieve detection of multiple nucleic acids and proteins on the EV surface and inside the lumen from micro-volume samples (less than 90 μL) and with high throughput (up to 384 samples per run), offering a new approach for accurate and efficient analysis of EV cargo composition.[124]

Proteins

Traditionally, EV-specific proteins are detected based on their specific binding to target antibodies using Western blotting (WB), enzyme-linked immunosorbent assay (ELISA), and flow cytometry.[125,126] These methods are widely used in the basic detection of EVs proteins due to their strong specificity, but their inherent limitations are prominent, such as large sample dosage demand, high purchase and maintenance costs of detection equipment, and time-consuming experimental processes, which greatly limit their wide application in large-scale sample screening and trace EVs sample analysis. In recent years, in response to the limitations of traditional methods, various new EVs protein detection techniques have gradually emerged, which can be divided into two main categories: optical detection methods and non-optical detection methods.[127,128] Optical detection methods achieve precise detection based on changes in optical physical properties, including surface plasmon resonance (SPR), Raman scattering and rayleigh scattering, and enhanced flow cytometry. Their core principle is to indirectly analyse protein content and characteristics by utilising changes in refractive index, energy, and other optical parameters resulting from the interaction between proteins and the detection system as the EVs solution flows through the device. These methods offer the advantages of fast detection speed and relatively high sensitivity. Non-optical detection methods focus on the characteristic of EVs being rich in negatively charged particles on their surface, detecting them by capturing electrical signals related to protein levels (such as changes in potential or current) without the need for complex optical equipment.[129,130] These methods hold great potential for low-cost and portable detection scenarios. In addition, single-vesicle analysis (SEVA) technology has emerged as a research hotspot, enabling targeted characterisation of EVs populations and subpopulations with heterogeneous molecular markers, thereby providing in-depth insights into the molecular features of EVs proteins and their biological functional associations.[131] General protein staining techniques combined with high-sensitivity flow cytometry can also achieve comprehensive labelling of all vesicles, enabling precise comparison of protein composition differences between EVs and their parent cells and offering richer dimensions for EVs proteomics research. It is worth noting that the effectiveness of these protein detection technologies is directly related to the quality of EVs isolation and purification. The high-efficiency isolation and purification techniques mentioned earlier (such as immunoaffinity methods and combined separation strategies) can reduce interference from impurities on protein detection signals, while gentle separation techniques like microfluidics and SEC can preserve the native conformation of proteins, ensuring the accuracy of subsequent detection and forming a complete "isolation-to-detection" technical loop.[132]

Nucleic Acid

In addition to proteins, RNA is a highly functional core component in EVs cargo, and its quantitative and qualitative evaluation is crucial for the clinical application screening of EVs drug delivery vectors. A large number of literature studies have shown that the therapeutic effect and adverse reactions of EVs drug delivery vectors are closely related to the RNA content in EVs, and the presence and expression level of specific RNA molecules directly affect the suitability

of vectors.[133,134] For example, RNA molecules such as circRNA-100338, miR-378a, miR-128, miR-378d, and circRNA-002136 present in EVs have been shown to be closely related to tumour invasion, metastasis, chemotherapy resistance, and angiogenesis.[135–137] This finding highlights the core value of accurately evaluating RNA signatures in EVs, which is crucial for screening EVs as drug-delivery vectors suitable for different disease treatments, and provides a molecular basis for vector-targeting optimisation. Currently, RNA detection technologies in EVs have developed into a diversified system, with core methods including reverse transcription polymerase chain reaction (RT-PCR), molecular beacons (MBs), and RNA sequencing (RNA-seq), each with its own advantages and suitable applications.[138,139] Among them, RT-PCR is the most widely used conventional detection tool, with a detection process that includes EVs isolation and purification, RNA extraction, and subsequent quantitative analysis. The process is mature and highly specific.[140] To ensure RNA extraction efficiency and purity, various commercial kits have been widely used for EV RNA extraction, such as the Norgen Biotek Total RNA Purification Kit, Qiagen miRNeasy™ Micro Kit, and Ambion mirVana™ miRNA Isolation Kit.[141] These can accommodate the extraction needs of different types of RNA (total RNA, miRNA), laying the foundation for the accuracy of subsequent detection. RNA-seq technology, with its high-throughput advantage, can comprehensively reveal the relative abundance and origin characteristics of RNA in EVs, as well as the enrichment of therapeutic RNA in EVs, providing reliable transcriptomic data to support the quality assessment of large-scale preparation of EV-based drug delivery carriers.[142] MBs technology, on the other hand, relies on molecular conformational changes and fluorescence emission properties to achieve precise targeted detection of specific RNA or single-stranded DNA molecules in EVs.[143] It is characterised by high sensitivity and strong specificity, creating a technological complement to the previously mentioned nanobiochip system. It is worth noting that, in addition to RNA abundance, variations in post-transcriptional RNA isoforms are also closely associated with disease progression, playing a particularly key role in tumour development. Studies have shown that most transcripts exported by EVs originate from the cytoplasm and are enriched in S-phase-specific sequences. This characteristic reveals potential mechanisms by which post-transcriptional RNA isoform variation regulates disease and provides a new direction for optimising EV-based drug-delivery vectors from an RNA-modification perspective.[138] In summary, accurately assessing the RNA content, characteristics, and variation patterns in drug-loaded EVs is not only a core step in elucidating the biological functions of EVs but also essential for clarifying their therapeutic and diagnostic potential in various diseases and promoting the clinical translation of EV-based drug delivery systems. The efficiency of such assessments also relies on the aforementioned high-efficiency EVs isolation and purification techniques to ensure the integrity and purity of RNA samples (Figure 3).

Delivering Drugs for Treatment of Diseases

The EVs drug delivery system exerts its therapeutic effect through two primary mechanisms. Firstly, therapeutic cargoes are directly delivered to target cells by EVs to induce therapeutic effects. Secondly, genetic engineering or transfection of donor cells is employed to control the type of cargo carried by EVs, thereby regulating the EVs' biological effects on target cells. In the following sections, we will specifically address the EVs delivery of a variety of therapeutic cargoes (such as nucleic acids, proteins, and small molecule compounds) for application in the treatment of diseases.

Nucleic Acid Molecules

High-throughput sequencing of nucleic acid molecules and RT-qPCR experimental tools have demonstrated that nucleic acid molecules from parental cells are selectively delivered to EVs.[144] However, in in vivo therapeutic experiments, free nucleic acid molecules have been found to exhibit poor bioavailability, short half-life, and low targeting, severely reducing their therapeutic efficacy.[145] The use of EVs as a carrier delivery platform for nucleic acids appears to compensate for this shortcoming, as the closed structure of EVs protects the nucleic acid molecules from degradation by the body and subsequently enables specific binding to the target cell.

mRNA

mRNA, a class of molecules that transmit the genetic code from DNA to the ribosome for protein expression, is considered a highly promising tool for disease treatment.[146] Various types of mRNAs have been developed for vaccine

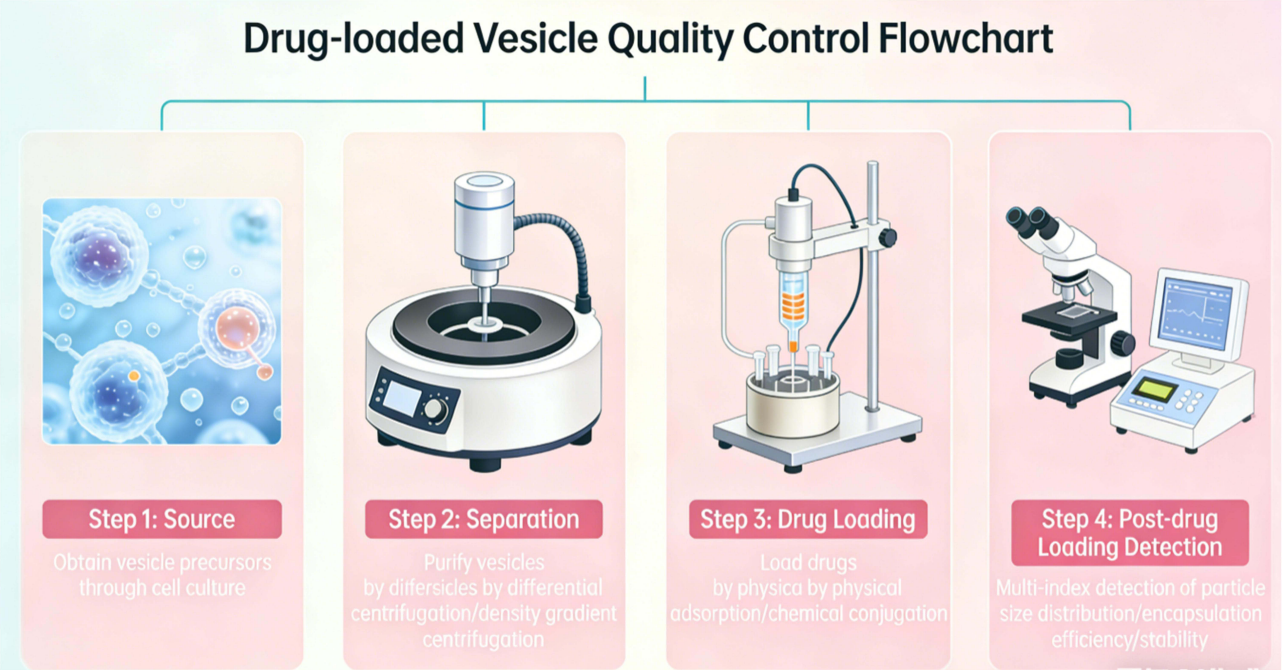

Figure 3 Drug-loaded Vesicle Quality Control Flowchart: This diagram illustrates the full quality control process for drug-loaded vesicles, which includes four key stages: First is the source preparation stage, where vesicle precursors are obtained through cell culture as the initial step in quality control of drug-loaded vesicles, providing compliant vesicle materials for subsequent processes. Next is the separation and purification stage, in which vesicles are purified using differential centrifugation or density gradient centrifugation techniques to remove cell debris and other impurities, ensuring the purity of the carrier for the subsequent drug-loading stage. The third stage is drug loading, where the drug is incorporated into the vesicles through physical adsorption or chemical conjugation. This is the core operation step for achieving the drug-loading function of the vesicles. Finally, the post-loading testing stage involves assessing multiple parameters of the drug-loaded vesicles, including particle size distribution, drug encapsulation efficiency, and system stability, which is a critical verification step to confirm the quality and functional effectiveness of the drug-loaded vesicles.

applications in different tumors and for the treatment of novel coronaviruses. However, conventional drug delivery systems for mRNA still face significant challenges in overcoming physiological barriers to achieve tissue-and cell-targeted delivery. The excellent biosafety and biocompatibility of EVs, their stability against degradation, and their ability to cross physiological barriers such as the blood-brain barrier (BBB) make them promising delivery vehicles for mRNA therapeutics.[147,148] For example, Dong et al conducted a study where they cloned an N peptide that specifically binds to the boxB sequence in RNA into the C-terminus of the EVs cytoplasmic protein CD64. They subsequently utilized the N peptide fused to the CD64 C-terminus to specifically bind to boxB, recruiting IFN-γ mRNA into EVs for targeted glioblastoma therapy. Safety assessments revealed no hemolytic toxicity from co-incubation of blood samples, and liver function indicators in the blood of healthy mice were within the normal range 24 hours after administration of imsEV. Preclinical modeling studies found that injection of imsEV significantly inhibited tumor growth in immunocompetent mice with GL261 tumors and prolonged the median survival by 24 days compared to the control group.[149]

MicroRNAs (miRNAs)

MiRs are a class of highly conserved single-stranded RNAs, typically 19–25 nucleotides in length, often located in the non-coding regions of the genome. In vitro and in vivo models have demonstrated extracellular vesicle-mediated transfer of miRNA function with a wide range of downstream effects.[150] However, miRs are highly susceptible to degradation in the circulation, and utilizing EVs can protect miRs and deliver them to target cells. EV-based miRNA drug delivery systems consist of two main forms: miRNA substitution or inhibition therapy.[151] For example, a study encapsulated a polycis-trans plasmid (pPolymiR) of three miRs into human viral protein (Gag/VSVg)-doped HEK293T cell-derived EVs. In glioma stem cells (GSCs), EV-pPolymiR significantly reduced GSC proliferation and prolonged survival in mice through high expression of miR-124a and miR-135a.[152] In another application, miR-199a was loaded into MSC-derived EVs by means of membrane protein Lamp2b fusion for the treatment of osteoarthritis. The miR-199a-loaded EVs ameliorated the pathological severity of cartilage and promoted cartilage repair through the mTOR autophagy

pathway.[153] The expression of many miRNAs has been implicated in promoting disease development, and miRNA expression can be inhibited by EV delivery to exert therapeutic effects. For example, a study demonstrated that EV-mediated delivery of a miR-501 inhibitor to gastric cancer cells improved the therapeutic efficacy of Dox by inhibiting miR-501 expression.[154]

siRNA

In the field of gene therapy, siRNAs are often applied to disrupt targeted complementary mRNAs and silence the corresponding genes.[155] However, the low stability of naked siRNAs in the somatic circulation and their short half-life limit their further development.[156] EVs with good biocompatibility and long cycling ability as carriers for siRNA delivery can compensate for these limitations to some extent. For example, Cui et al used electroporation to load siRNA of Shn3 into mesenchymal-derived EVs modified by bone-targeting diacyl lipid tail for anti-osteoporosis treatment. The results showed that BT-EV-siShn3 was able to specifically deliver Shn3 to osteoclasts, inhibiting osteoclast formation by silencing Shn3 and decreasing autologous RANKL expression.[157] Additionally, Zhan et al loaded sicPLA2 and metformin into healthy human blood-derived EVs, which were engineered to cross the BBB. This delivery system exerted a dual inhibitory effect on glioblastoma (GBM) phospholipid and mitochondrial metabolism via sicPLA2 knockdown and metformin.[158]

Circular RNA (circRNA)

circRNA is generated through the process of reverse splicing of mRNA exons.[159] It possesses a range of functions, such as acting as competing endogenous RNAs, regulating mRNA stability, and participating in gene transcription. Additionally, the deletion of the 5′ and 3′ termini confers resistance to degradation by nucleic acid exonucleases, which is essential for organismal life activities.[160] Recent studies have revealed that functional circRNAs can exert biological effects through EVs transfer to recipient cells. For example, circRNA SCAR has the ability to reduce mitochondrial reactive oxygen species (mtROS) and thus promote macrophage M2 polarization. Li et al effectively delivered therapeutic circRNA SCAR into macrophage exosomes using an EVs drug delivery system, which effectively alleviated the systemic inflammatory response triggered by sepsis and reduced mortality by driving macrophage M2 polarization.[97]

Long Non-Coding RNAs (lncRNAs)

lncRNAs are a class of RNAs that do not possess functional open reading frames exceeding 200 nucleotides.[161] They play a regulatory role in gene expression, transcription, and post-transcriptional processing.[162] Encapsulation of therapeutic lncRNAs into EVs can address the challenges of the short half-life and poor targeting of lncRNAs in the blood circulation, enhancing their efficacy. EV-mediated lncRNA delivery can be categorized as overexpression or suppression of endogenous lncRNA expression. For example, a study investigating the biological effects of exosome-derived lncRNA MEG3 on osteosarcoma (OS) revealed that loading lncRNA MEG3 into exosomes modified with cyclic RGD peptide (cRGD) for osteosarcoma treatment inhibited osteosarcoma progression by sponging miR-185[163] (Figure 4). In gastric cancer (GC), proliferative and migratory capacities are positively correlated with FRLnc1 expression. Zhang et al found that transfection of sh-FRLnc1-derived EVs significantly induced cell cycle arrest and apoptosis in GC cells and inhibited epithelial-mesenchymal transition (EMT).[164]

DNA

RNA is the main form of EVs nucleic acid molecules, being highly abundant in the circulation and exerting a wide range of biological effects. The relatively low efficiency of EVs for encapsulating large DNA molecules has resulted in most EV nucleic acid cargo studies focusing on RNA at this stage.[165] However, there are relevant articles reporting the application of EVs as a delivery vehicle for small DNA molecules, such as antisense oligonucleotides (ASOs) and plasmid DNA, for tumor cell therapy. ASOs are single-stranded DNA molecules consisting of 12–25 nucleotides and exert biotherapeutic effects through the inhibition of target RNA translation by degradation or spatial occupancy effects.[166] Loading ASOs into EVs overcomes current problems associated with vector viruses, lipid transfection reagents, and lipid nanoparticles, such as cytotoxicity, immunogenicity, and targeting bias.[167] For example, in one

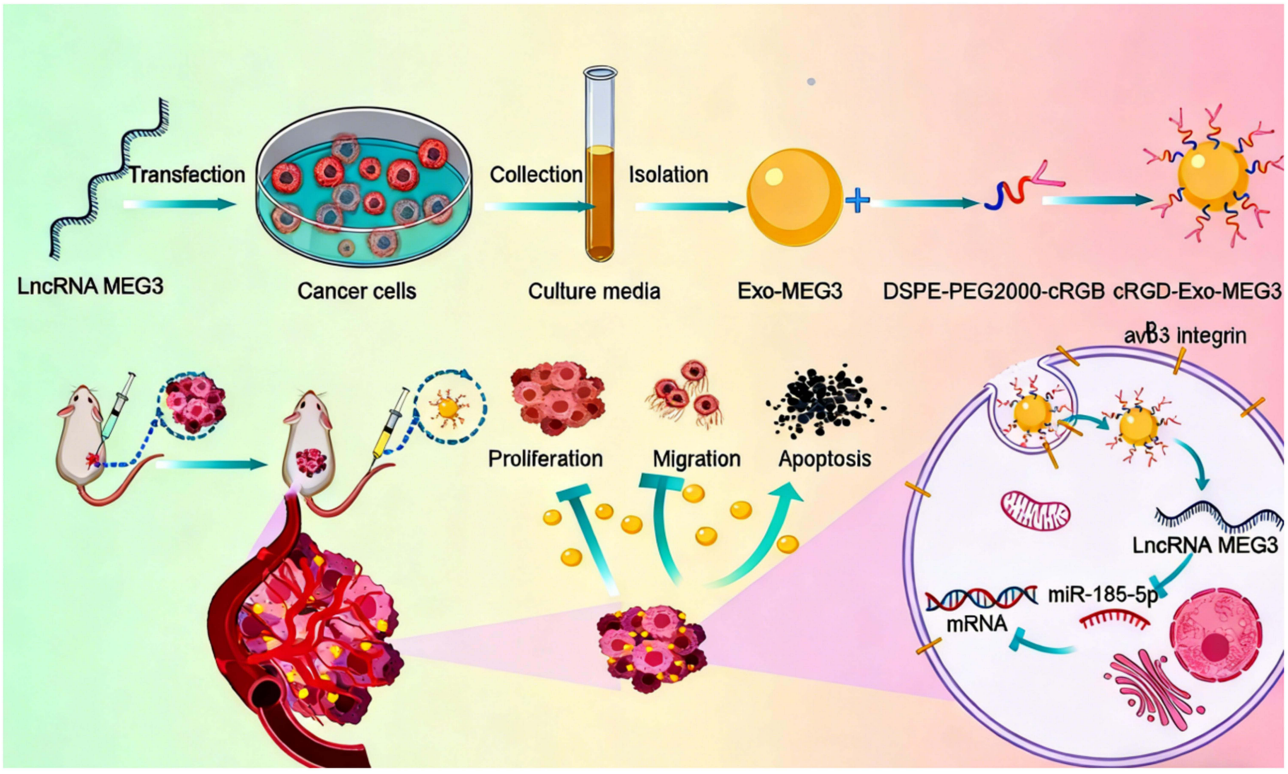

Figure 4 Therapeutic application workflow and mechanism of LncRNA MEG3-loaded exosomes: This figure illustrates the preparation, targeted modification process, and antitumor molecular mechanism of LncRNA MEG3-loaded exosomes: First, LncRNA MEG3 is transfected into cancer cells; the culture supernatant of these cells is then collected, and LncRNA MEG3-loaded exosomes (Exo-MEG3) are isolated from it. Next, Exo-MEG3 undergoes surface modification with DSPE-PEG2000-cRGD to generate cRGD-Exo-MEG3—this modification allows the exosomes to specifically target αvβ3 integrin on the surface of osteosarcoma cells, and when administered in mouse tumor models, cRGD-Exo-MEG3 exerts therapeutic effects. Additionally, LncRNA MEG3 delivered by cRGD-Exo-MEG3 acts as a molecular sponge to sequester and inhibit miR-185-5p, ultimately achieving the therapeutic outcomes of suppressing tumor cell proliferation and migration while promoting tumor cell apoptosis.

study, ASO-125b was loaded into erythrocyte-derived EVs for the treatment of refractory acute myeloid leukemia and breast cancer by inhibiting miR-125b. The results showed that EVs loaded with ASO-125b via electroporation induced 80% and 95% miR-125b degradation, respectively, thereby inhibiting tumor progression.[168] To address the issues of vector tolerance and low immunogenicity of the CRISPR/Cas9 intracellular delivery system, Jin et al encapsulated CRISPR/Cas9, consisting of a plasmid targeting PARP-1 expression, into EVs derived from the supernatant of ovarian cancer cells. This approach selectively induced apoptosis in ovarian cancer cells by inhibiting the expression of poly (ADP-ribose) polymerase-1 (PARP-1).[169] Several articles have confirmed that using EVs as a platform for delivering nucleic acid molecules is more efficient than advanced lipid formulations.[170] However, compared to liposomes or other nanoparticle realizations, loading RNA into EVs requires complex processes, demanding regulatory efforts, and significantly higher synthesis costs per agent.[171] Further understanding of the mechanisms of EVs biogenesis is crucial for developing safe, efficient engineering strategies for nucleic acid delivery, which will greatly accelerate the field of precision nanomedicine therapeutics.

Protein Molecules

Effective intracellular delivery of therapeutic proteins has become an important strategy for intervening in various diseases.[172] However, in clinical practice, this technique is limited by the cell membrane's natural barrier function, and its application is usually restricted to molecules such as monoclonal antibodies, peptide hormones, and cytokines. These molecules can bind to specific receptors on the cell membrane to trigger or block downstream intracellular signalling cascades, thereby exerting therapeutic effects.[173] In contrast, other therapeutic molecules, such as genome editing enzymes, epigenetic modification enzymes, and transcription factors, often cannot directly exert targeted therapeutic effects because they are difficult to penetrate the cell membrane and enter the cell.[174] Loading protein therapeutic cargo

into EVs can partially overcome this limitation. Currently, therapeutic proteins are loaded into EVs primarily by genetically engineering parental cells, after which the proteins are sorted into EVs in a "hitchhiking" manner. The bilayer lipid membrane of EVs can protect the protein cargo to some extent from clearance in the circulatory system and enhance the target cells' internalisation ability. For example, Han et al designed an exosome-based delivery system called MAPLEX, in which the cargo protein is fused with the photosensitive protein mMaple3, which is further fused to an EV marker protein. The fusion product is overexpressed in cells in vitro, accumulates in exosomes, is purified, and then exposed to 405 nm light to cleave mMaple3, releasing the cargo protein into the intracellular space. MAPLEXs carrying dCas9 ribonucleoprotein complexes can epigenetically edit Aβ in cortical neurons. Intranasal administration of MAPLEXs carrying dCas9 ribonucleoprotein complexes reduces Aβ, making it an important approach for treating patients with Alzheimer's disease.[174] Another study constructed a plasmid encoding a WW region-connected Cre recombinase (WW-Cre) fusion protein, which, after being ubiquitinated by Ndfip1, is secreted via exocytosis. After intranasal administration, compared with the untransfected group, the number of transfected cells in the brain exposed to purified WW-Cre-Ndfip1 exocytotic products significantly increased, demonstrating the potential of this method. Ligands can also be conjugated to transmembrane or GPI-anchored proteins on the surface of EVs using click chemistry. A promising application is the use of click chemistry to conjugate c(RGDyK) peptides to the surface of MSC-derived EVs containing curcumin. After intravenous injection, RGDyK exhibits high affinity for integrin αvβ3 in brain ischemic endothelial cells and effectively treats ischemic stroke by strongly inhibiting the expression of p-p65 and caspase-3.[175]

Small Molecule Chemical Drugs

The therapeutic effect of free small-molecule chemical drugs when used alone is unsatisfactory, with the core issue being that the drugs are difficult to reach target tissues and accumulate to effective treatment concentrations; simply increasing the dosage can cause additional damage to normal tissues and cells, and may even induce adverse effects such as hepatotoxicity and nephrotoxicity.[176–178] The main reasons are the nonspecific clearance of free drugs by monocytes/macrophages in the circulation, the selective permeability restriction of the blood-brain barrier, and the physical barrier effect of the complex tumour matrix, all of which hinder effective drug delivery to target sites. Due to their excellent biocompatibility and efficient cell-targeted internalisation, EVs are considered an ideal candidate delivery system to enhance the accessibility of free chemical drugs to targeted tissues. Recently, chemical drugs such as paclitaxel, gemcitabine, and Dox have been successfully loaded into EVs, significantly improving the therapeutic effects of these drugs. In recent years, chemotherapeutic drugs such as paclitaxel, gemcitabine, and Dox have been increasingly loaded into EVs, significantly enhancing the therapeutic effects of these drugs. For example, a study loading Dox into EVs derived from M1 macrophages found that, compared to using Dox alone, the M1 macrophage-derived EVs loaded with Dox significantly reduced cancer cell proliferation and exerted maximal cytotoxicity against cancer cells. Another comparison between free Dox, Dox-loaded EV delivery systems, and liposome-Dox delivery systems found that the Dox-EV system selectively accumulated at tumour sites 72 hours after injection, effectively inhibited tumour growth, and extended the survival of mice. Specifically, the median survival time of tumour-bearing mice was 65 days in the free Dox group, 82 days with liposome-Dox delivery, and 140 days with the Dox-EV system.[179] For pancreatic cancer, characterised by abnormal extracellular matrix proliferation and a tendency to form a dense physical barrier, studies have developed a drug-delivery system using GEM-loaded EVs (GEM-EVs) derived from Panc-1 cells. Comparative experiments confirmed that this system has a significantly better therapeutic effect on pancreatic ductal adenocarcinoma (PDAC) than free gemcitabine: the GEM-EV treatment group extended survival by 5 days compared to the free gemcitabine group. Safety evaluation showed that mice in the GEM-EVs treatment group maintained normal levels of liver and kidney function indicators, such as blood urea nitrogen (BUN) and alanine aminotransferase (ALT).[96]

In contrast, these indicators were significantly elevated in the free gemcitabine group, fully demonstrating the safety advantages of the EV-based drug delivery system.[180] When lesions occur in the central nervous system, the BBB, formed by tightly connected endothelial cells, basement membrane glycoproteins, pericytes, and astrocytic end-feet, can effectively isolate the central nervous system (CNS) from the peripheral blood circulation, thereby limiting the transport of free drugs into the brain.[181] Research reports indicate that after intravenous injection, less than 5% of the initial dose of free drug can successfully reach the lesion site in the brain. The stringent restrictions of the BBB on drug delivery,

Table 3 Multi-Pathway Sources of EVs Loaded Cargoes for Various Disease Therapeutic Applications

EVs Source	Cargoes	Loading Method	Disease Treatment	Mode of Administration	Reference
Embryonic fibroblast	IFN-γ mRNA	Electroporation	Glioblastoma	Intravenous injection	[149]
Mesenchymal stem cell	MiR-199a	Genetic engineering	Osteoarthritis	Intra-articular injection	[153]
SGC7901 cell	MiR-501 inhibitor	Plasmid transfection	Gastric cancer	Intratumoral injection	[154]
HEK293T cell	CircRNA mSCAR	Electroporation	Septicemia	Intravenous injection	[97]
Osteosarcoma cell	LncRNA MEG3	Incubate	Osteosarcoma	Intravenous/intratumoral	[164]
Red cell	ASO-125b	Electroporation	Breast cancer	Intratumoral injection	[168]
M1 Macrophages	Paclitaxel	Electroporation	Brain Diseases	Hypodermic injection	[179]

rather than a lack of candidate drugs, have become the central bottleneck in the treatment of various central nervous system diseases such as glioblastoma, Parkinson's disease, and cerebral infarction.[182] To address this challenge, Wang et al conducted an innovative study, loading Dox into neutrophil-derived extracellular vesicles (NEs-EVs), successfully achieving effective treatment of glioma. Experiments on zebrafish models to assess penetration efficiency showed that only a small amount of red fluorescence from free Dox penetrated the BBB, with no obvious fluorescence distribution in brain tissue. In contrast, in the NEs-EVs/Dox treatment group, a large amount of Dox red fluorescence was observed crossing the BBB and accumulating in brain tissue. Evaluation of therapeutic effects in tumor-bearing mice further confirmed that compared with the free Dox group, mice treated with NEs-EVs/Dox showed significantly improved quality of life, specifically manifested as slower body weight loss, reduced systemic toxicity induced by Dox, and significantly prolonged survival (median survival was 22 days in the free Dox group and 27 days in the NEs-EVs/Dox group).[182] These results confirm that loading Dox into EVs can effectively increase the accessibility of the free drug for disease treatment (Table 3).

Progress in Clinical Trial-Related Research

EVs, as natural delivery carriers, offer key advantages, including good biocompatibility, strong targeting, and low immunogenicity. Their translational application in disease treatment has become a research hotspot in the biopharmaceutical field. Currently, clinical trial research on EVs primarily focuses on EVs derived from human cells/samples and from plant sources, with applications spanning oncology, central nervous system diseases, infectious diseases, and immunological diseases.[183] Among these, the oncology field accounts for the highest proportion (54%), the central nervous system and infectious disease fields are roughly equal (both 13%), and the immunological disease field accounts for 8%.[184] In oncology, current clinical trials mostly use dendritic cells-derived EVs (DEXs) as vaccine delivery vehicles. Their core mechanism is to activate the body's specific anti-tumour immune response by loading tumour-associated antigens. As early as 2005, a Phase I clinical trial confirmed that DEXs loaded with human leukocyte antigen (HLA)-restricted melanoma-associated antigen (MAGE) peptides could be used to treat HLA-$A2^{+}$ non-small cell lung cancer (NSCLC); the study observed no severe adverse reactions after vaccination, and patients' natural killer (NK) cell activity was significantly enhanced.[185] Another Phase II clinical trial evaluated the efficacy of EVs carrying interferon-γ (IFN-γ) as a maintenance immunotherapy following first-line chemotherapy in patients with NSCLC. By enhancing the immune response of NK cells and T cells, they exerted anti-tumour effects. Among the 22 enrolled patients, only one experienced significant liver toxicity, demonstrating good safety.[186] A recent non-randomised phase I/II clinical trial further expanded the application scenarios of EVs, showing that EVs carrying squamous cell carcinoma antigen recognition sequence 1 (SART1) can be used for the treatment of advanced oesophageal squamous cell carcinoma, and SART1 peptide can induce specific cytotoxic T lymphocyte (CTL) responses after inoculation, directly killing tumour cells. None of the enrolled patients experienced significant adverse reactions.[187] The application of mesenchymal stem cell (MSC)-derived EVs in regenerative medicine is another important direction in EVs clinical research. For example, a completed phase II clinical trial confirmed that MSC-derived EVs are safe and effective for the treatment of refractory perianal fistulas in patients with Crohn's disease.[188] Clinical exploration of plant-derived EVs has also been gradually launched, and EVs derived from grape (NCT01668849), ginger, and aloe vera (NCT03493984) have been initiated. In a phase I clinical trial,

Donald Miller's team loaded the hydrophobic drug curcumin in plant-derived EVs for the treatment of intestinal diseases (NCT01294072). In addition, research on aloe vera-derived EVs is underway, and its effect on reducing insulin resistance and chronic inflammation in patients with polycystic ovary syndrome (PCOS) will be evaluated in clinical trials after enrollment is complete (NCT03493984).

Biodistribution and Pharmacokinetics of Drug-Loaded EVs

Currently, intravenous injection remains the primary administration route for drug-loaded EVs. However, the mononuclear phagocyte system (MPS) in the body's circulatory system non-specifically uptakes foreign substances, which significantly reduces the effective payload of therapeutic EVs in circulation.[189] This clearance process is mediated by multidimensional cellular and molecular interactions, with the core mechanisms mainly reflected in three aspects: ① After entering the bloodstream, surface proteins and glycans on EVs can interact with endothelial cells and initiate uptake. Overexpressed tetraspanins and fibronectin on EVs, in cooperation with endothelial cell-surface molecules such as CD106/vascular cell adhesion molecule-1 (VCAM-1), can further enhance this uptake effect.[19,190] The internalised EVs are primarily metabolised through two pathways: one is fusion with endosomes, in which their lipids and associated biomolecules are transported to lysosomes for degradation or recycling; the other is transcytosis, which returns them to the extracellular space. The intrinsic gaps between liver and spleen endothelial cells facilitate the shuttling of EVs between the stroma and the bloodstream, ultimately leading to non-specific accumulation of EVs in these organs.[191,192] ② Phagocytes can uptake EVs through a dynamin 2-dependent endocytic pathway, and the specific binding of phosphatidylserine (PS) exposed on the EV membrane to TIM-1 and TIM-4 receptors on the surface of phagocytes further promotes the uptake and degradation of EVs.[193] ③ Regulatory proteins and lipoproteins in serum can bind to EVs through Brownian motion, forming a "protein corona" under the combined action of electrostatic, ionic, and hydrophobic/hydrophilic forces. This structure can initiate an opsonisation effect, thereby significantly enhancing the recognition and clearance efficiency of phagocytes for EVs.[194] Through the synergistic action of these multiple mechanisms, relevant studies have confirmed that the half-life of circulating EVs in mice is less than 10 minutes.[195] To address this issue, the current core strategies for prolonging the circulation half-life of EVs are mainly divided into two categories: one involves modifying the surface of EVs with "do not eat me" signal molecules such as CD47, CD31, and CD24, which interact with the immunosuppressive receptor SIRPα to reduce macrophage-mediated endocytosis of EVs; the other involves coating drug-loaded EVs with inert nanomaterials like polyethylene glycol (PEG) to reduce the likelihood of recognition by the mononuclear phagocyte system (MPS).[196] Currently, in vivo tracking techniques for EVs mainly include two approaches: the first is direct surface labelling of natural EVs using lipophilic dyes such as KH26, DiD, and DiR, or radioactive isotopes like [111 In]; the second is genetic modification of donor cells to introduce fusion protein markers such as CD63, or to express proteins with fluorescent tags (eg., mCherry) or bioluminescent tags (eg., Fluc and Nluc) within the EVs lumen to enable in vivo tracking of EVs.[197]

Conclusions

EVs, with their advantages of good biocompatibility, low immunogenicity, strong targeting ability, and capacity to carry various therapeutic drugs, have emerged as highly promising drug delivery carriers. The key to their clinical translation lies in establishing a comprehensive quality control system, with donor cell selection as the foundation. EVs derived from MSCs, DCs, and other sources demonstrate outstanding performance in terms of yield and safety. In contrast, non-human EVs show potential but still require safety validation. The isolation and purification process should combine techniques such as ultracentrifugation and microfluidics to enrich EVs while maintaining their structural integrity efficiently. Drug-loading strategies need to be optimised according to the type of cargo. Pre-secretion co-incubation, transfection, and post-isolation methods such as electroporation and ultrasound treatment each have their pros and cons, requiring a balance between loading efficiency and EVs activity.

In therapeutic applications, EVs can efficiently deliver nucleic acids, proteins, and small-molecule drugs, showing significant efficacy in preclinical studies for tumours and central nervous system disorders. Related clinical trials have preliminarily confirmed their safety and effectiveness. Currently, EV applications still face challenges such as short circulation half-life, the need for improved targeting, and high costs of large-scale production. In the

future, introducing "do not eat me" signal molecules through surface modification, combining intelligent targeting strategies, and developing more efficient isolation and loading technologies will further promote the translational application of EVs in precision nanomedicine, providing new directions for the treatment of various refractory diseases.

Abbreviations

EVs, Extracellular vesicles; MVBs, Multivesicular bodies; ILVs, Intraluminal vesicles; PS, Phosphatidylserine; PI3K, phosphatidylinositol-3-kinase; MSCs, Mesenchymal stem cells; DCs, Dendritic Cells; PDEVs, Plant-derived extracellular vesicles; OMVs, Bacterial outer vesicles; UC, Ultracentrifugation; UF, Ultrafiltration; GC, Gastric cancer; AFM, Atomic force microscopy; NTA, Nanoparticle tracking analysis; DLS, Dynamic light scattering; TRPS, Tunable resistive pulse sensing; MS, Mass spectrometry; MBs, Molecular beacons; WB, Protein blotting; SPR, Surface plasmon resonance; RT-PCR, Transcription-polymerase chain reaction; RNA-seq, RNA sequencing; Dox, Doxorubicin; PDGFR, platelet-derived growth factor receptor; OVA, ovalbumin; BBB, Blood-brain barrier; GSCs, Glioma stem cells; circRNA, Circular RNA; OS, Osteosarcoma; cRGD, Cyclic RGD peptide; ASOs, Antisense oligonucleotides; SPIONs, Superparamagnetic iron oxide nanoparticles; MPS, Mononuclear phagocyte system; AD, Alzheimer's disease; MLCK, Myosin light chain kinase; KSP, Kinesin spindle protein; SEC, Size exclusion chromatography; lncRNAs, Long Non-Coding RNAs; EMT, Epithelial-mesenchymal transition; siRNAs, Small interfering RNAs; TEM, Transmission electron microscopy; SEM, Scanning electron microscopy; PTX, Paclitaxel; mtROS, Mitochondrial reactive oxygen species.

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Disclosure

The authors declare that they have no competing interests in this work.

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