



Engineering the Future: Strategic Advances in Extracellular Vesicle-Mediated Drug Delivery Systems

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Abstract: Extracellular vesicles (EVs) are lipid bilayer-enclosed nanoparticles naturally secreted by cells that inherently lack replicative capacity and function as endogenous carriers of biological cargo including proteins, nucleic acids, and metabolites for intercellular communication. Leveraging their intrinsic biocompatibility and biomimetic transport properties, EVs have emerged as versatile drug delivery platforms with distinct therapeutic advantages. Recent advancements have developed two precision-engineered derivatives: structurally and cargo-modified engineered EVs, and EV mimetics integrating synthetic nanomaterials. Both types are designed to enhance targeting specificity and therapeutic efficacy, yet their strong intercorrelations frequently cause confusion. This review systematically examines the evolving landscape of EV-based delivery systems by establishing conceptual distinctions between native EVs, engineered EVs, and EV mimetics, while comparatively analyzing their preparation methodologies, clinical translation progress, and performance characteristics as drug carriers. Through systematic discussion of clinical challenges, including safety, clinical feasibility, and cross-laboratory reproducibility, we propose optimization directions integrating artificial intelligence with drug delivery systems, thereby providing insights and methodologies for next-generation EV-inspired therapeutic delivery platforms.

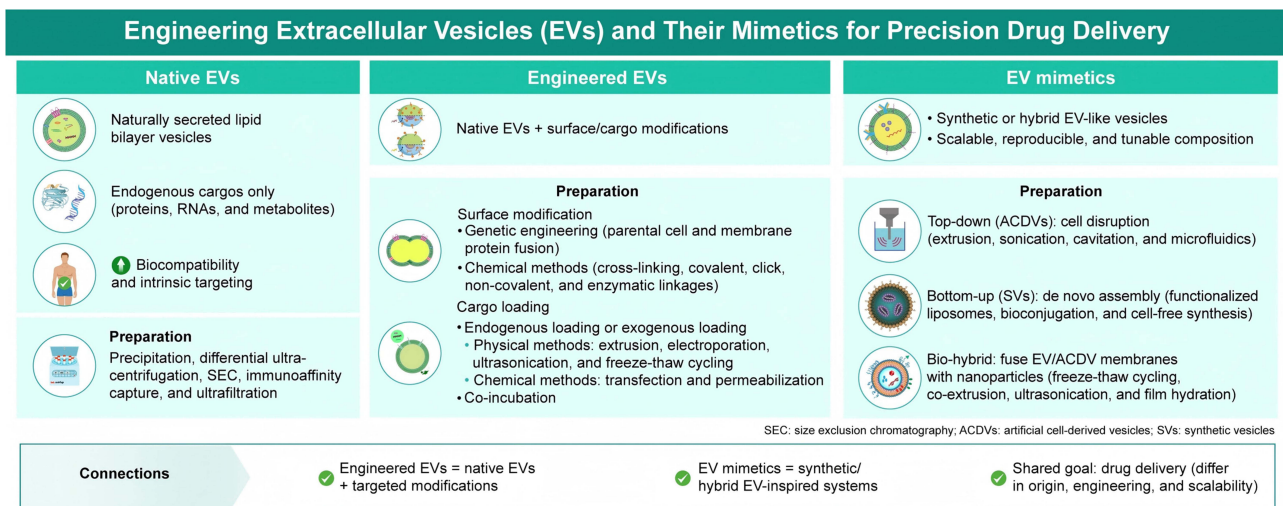
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Introduction

Extracellular vesicles (EVs) are nanoscale lipid-bilayer-enclosed particles released by eukaryotic and prokaryotic cells. They lack functional nuclei and replication capacity,¹ and mediate intercellular communication by transporting diverse biomolecules, including nucleic acids, proteins, and lipids.^{2,3} EVs were initially identified in the 1950s by Chargaff and West as “precipitates during blood coagulation”⁴ and were long considered by researchers to be mere “garbage bins” of cellular metabolites.^{5,6} It was not until the early 1980s that these vesicles, which range in diameter from 40 nanometers to 1 micrometer and are released by diverse cell types, were designated as “exosomes”.⁷ In 1996, Raposo demonstrated the antigen-presenting function of immune cell-derived EVs, a finding that fundamentally challenged the view of EVs as non-functional metabolic byproducts and marked the beginning of a new era in research focused on their role as intercellular communication vehicles.⁸ With the advent of the 21st century, the discovery that EVs could transport functional RNA ignited a surge of scientific interest. However, inconsistencies in nomenclature and classification, together with the absence of standardized isolation and detection protocols, have resulted in poor reproducibility of experimental outcomes and a fragmented progression of the field. These vesicles exhibit diverse origins, natures, and features, which results in heterogeneous nomenclature within the literature based on size (eg microvesicles, nanoparticles), cellular origin (eg oncosomes), proposed functions (eg argosomes, tolerosomes), or extracellular location (eg ectosomes, exosomes). In fact, it was not until the founding of the International Society for Extracellular Vesicles (ISEV)



Graphical Abstract



in 2011, the term “extracellular vesicles” was formally proposed. They recommended the classification of EVs considering size—small EVs (sEVs) < 200 nm vs large EVs (lEVs) > 200 nm, and biogenesis pathways—apoptotic bodies (500–1000 nm) derived from programmed cell death, microvesicles (150–500 nm) generated through plasma membrane budding, and exosomes (40–150 nm) originating from endosomal multivesicular bodies.⁹ Current isolation methods hinder precise separation of co-enriched EV subtypes, with no universal biomarkers confirming biogenesis-dependent characteristics—a consensus reflected by predominant use of “EVs” in contemporary literature, though early studies employed terms like “exosomes” (denoting sEVs isolated via 220 nm filtration or high-speed ultracentrifugation¹⁰) and “microvesicles” (lEVs obtained through differential centrifugation, ultracentrifugation, or filtration¹¹). Notably, no cited evidence confirms intracellular origins, which leads researchers to adopt “sEV” for exosome-related and “lEV” for microvesicle-related references.

EVs mediate intercellular communication and remodel the phenotype of target cells through selective cargo sorting during biogenesis, regulated release, and diverse pathways involving ligand-receptor interactions, endocytic uptake, or membrane fusion with target cells with inherent biocompatibility,¹² low immunogenicity¹³ and efficient tissue penetration,¹⁴ are promising for biomolecule delivery and non-invasive disease diagnosis.¹⁵ Despite these advantages, limitations in production scalability,¹⁶ isolation purity,¹⁷ predictability in vivo targeting precision, and storage inefficiencies¹⁸ persist. To address these issues, engineered EVs have been developed via surface functionalization and cargo engineering for precision-targeted therapy.^{19,20} Yet, their complex preparation increases cost and technical difficulty, may damage EV membrane integrity, and lacks standardized protocols leading to uncontrollable batch variation. For large-scale clinical use, EV mimetics—artificial nanovesicles mimicking EV membrane structure—including artificial cell-derived vesicles (ACDVs) fabricated via mechanical cell disruption and synthetic vesicles (SVs) constructed de novo from molecular components or hybridized with natural EVs/liposomes, have emerged as synthetic alternatives. However, they cannot replicate natural EV surface ligands or cell tropism, resulting in inferior biocompatibility, tissue penetration and higher immunogenicity.

This review systematically examines native EVs, engineered EVs, and EV mimetics to delineate their conceptual distinctions and interrelationships while evaluating their biomedical potential through comparative analysis of standardized production methodologies, therapeutic applications, and technological limitations. We categorize established production methodologies by biophysical principles and functional objectives; benchmark protocol variations for

analogous particle types; and then synthesize clinical translation outcomes across therapeutic domains including oncology, regenerative medicine, and gene therapy. Through critical analysis and discussion, we identify translational issues such as clinical safety, batch-to-batch consistency, and large-scale production capacity. Finally, we propose mechanistic optimizations and innovative strategies for artificial intelligence-integrated drug delivery systems (DDSs) to advance next-generation vesicular nanomedicines.

Production

EVs

EV Characterization

EV characterization is for confirming presence, assessing quantity, distinguishing them from non-EV components, and elucidating their biological properties, commonly conducted across morphology, particle size, and composition. Direct observation of structure and morphology via transmission electron microscope and scanning electron microscope can differentiate EVs from impurity particles. Nanoparticle tracking analysis and dynamic light scattering enable the measurement and analysis of particle size and heterogeneity.¹ Precise detection of biomarkers serves as the core basis for distinguishing EV subtypes and validating their functions. Flow cytometry and Western blotting can detect common EV surface markers such as the annexin family. Specific markers including CD9, CD63, CD81, TSG101, ALIX, and GAPDH are used to confirm EV identity.^{21,22} Additionally, microvesicle-specific markers such as integrins, selectins, and CD40L, which are associated with parental cell surface molecules, can reflect the cellular origin and targeting properties. Integrating multi-omics analyses further allows for the screening of therapeutic targets and diagnostic markers.²³ Owing to the lack of unified molecular markers, the aforementioned methods are frequently used in combination and require the exclusion of serum albumin and lipoproteins to ensure the reliability of experimental results.

Differences and Connections in EV Characterization Methods are presented in [Figure 1](#).

EV Purification

Conventional EV isolation and purification methodologies exhibit inherent trade-offs between yield, purity, and biological integrity, necessitating method selection based on experimental priorities such as reproducibility, target specificity, and operational scalability to address the persistent challenge of balancing yield with purity in EV research.

Precipitation-based EV isolation employs polymers like polyethylene glycol (PEG) or salts to reduce biomolecule solubility and aggregate vesicles from biofluids, providing a cost-effective approach for large-scale preliminary enrichment. However, its limited purity typically necessitates supplementary purification via orthogonal techniques to achieve research-grade vesicle preparations.

Differential centrifugation (DC) employs sequential separation of cellular components through incrementally increased centrifugal forces and durations, initially pelleting high-density particles like nuclei and mitochondria, while retaining lower-density materials in supernatants for subsequent processing. Ultracentrifugation (UC) achieves nanoscale particle resolution through extreme rotational speeds ($\geq 50,000$ rpm), thereby generating centrifugal force ($\sim 100,000 \times g$) sufficient to discriminate particles by size and density. Standard protocols for high-purity EV isolation typically employ three steps: (i) remove cells and large debris via low-speed centrifugation ($\sim 200\text{--}1000 \times g$, 3–15 min); (ii) filter supernatant through 0.2–0.8 μm membranes to clear residual particles, and subsequently precipitate large cell particles by medium-speed centrifugation ($10,000\text{--}12,000 \times g$, 30 min; ≥ 2 repeats); and (iii) transfer and collect exosome-like small vesicles by ultracentrifugation at ($\sim 70,000\text{--}100,000 \times g$, >70 min). The aforementioned steps require strict attention to the k-factor and rotor specifications.^{10,24–29} Further purification can be achieved through sucrose or iodixanol density gradients to isolate lipid-containing vesicles from contaminants such as proteins and protein-RNA aggregates.^{8,30–32} Differential ultracentrifugation integrates sequential centrifugation protocols that progressively escalate from low-speed separation of large cellular components to ultracentrifugation forces ($100,000 \times g$) for membrane vesicle purification. This bridges DC and UC through calibrated, stepwise force application to isolate subcellular fractions by size-density parameters.^{33,34}

Density gradient centrifugation (DGC) employs layered media (sucrose,³⁵ cesium chloride,³⁶ iodixanol³⁷) to establish a buoyancy-based separation system where particles migrate under centrifugal force until reaching equilibrium at their

EV Characterizations

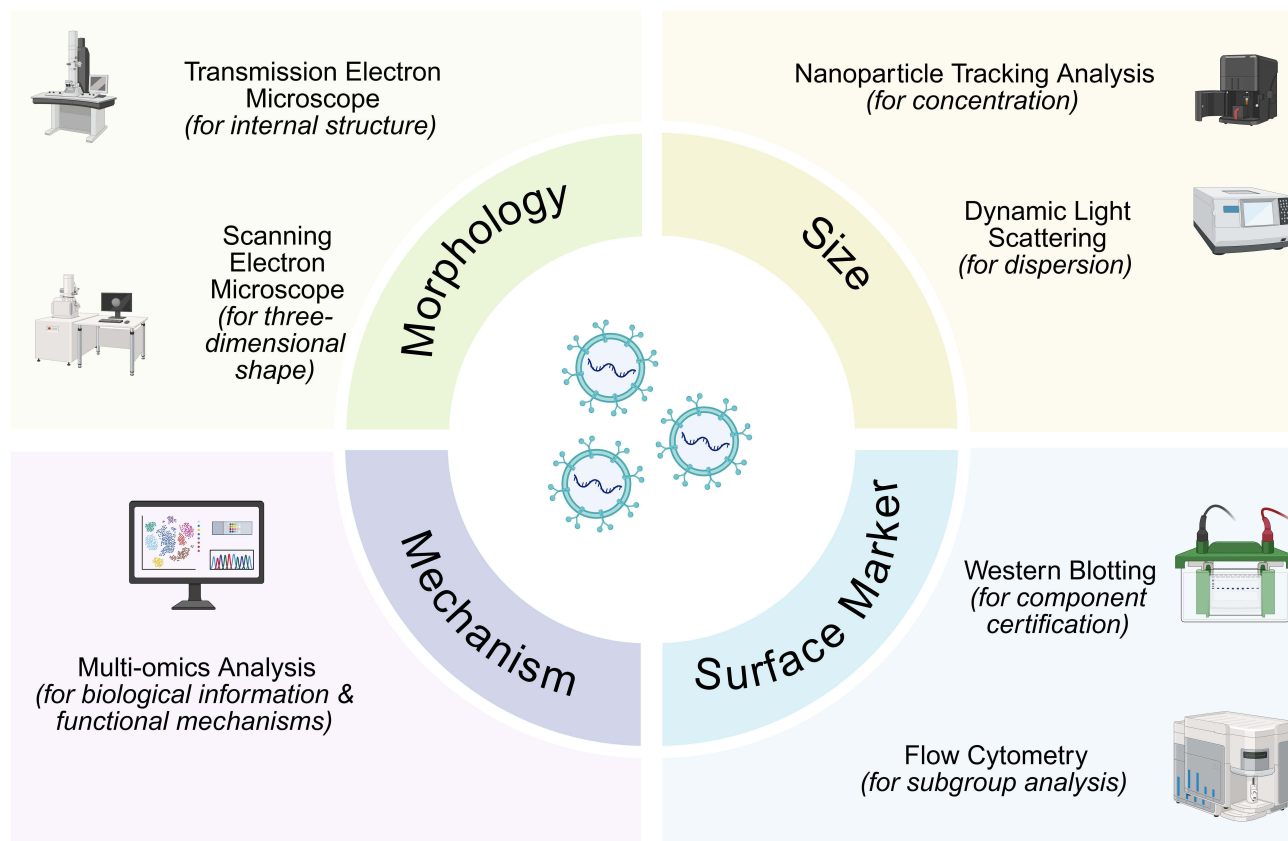


Figure 1 Differences and Connections in EV Characterization Methods. Transmission electron microscope (TEM) and scanning electron microscope (SEM) are both employed for the morphological observation of EVs. TEM provides the established morphological evidence for EVs, revealing characteristic features such as the “cup-shaped” or “double-concave disc-shaped” morphology and the lipid bilayer membrane structure. SEM enables the visualization of the three-dimensional architecture of EVs and is utilized to examine inter-particle interactions. Nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) are techniques for measuring EV size distributions; however, NTA is considered the gold standard for EV quantification, purity assessment, and the analysis of polydisperse samples, while DLS excels as a rapid tool for evaluating sample monodispersity and stability. Western blotting (WB) and flow cytometry (FCM) contribute jointly to establishing the identity and purity of EVs, with WB identifying specific EV protein markers and FCM facilitating subpopulation analysis. Multi-omics analysis, by uncovering biosynthetic pathways, elucidating sources of heterogeneity, discovering disease biomarkers, and revealing molecular mechanisms of action, serves as the core driver propelling future EV research from fundamental characterization toward functional elucidation and clinical application.

intrinsic density zones, thereby enabling high-resolution isolation of EVs from co-isolated proteins or non-vesicular particles through density matching. This method minimizes mechanical shear stress while resolving more precise density variations among biological nanoparticles, outperforming DC and UC in both separation fidelity and particle integrity preservation, particularly for discriminating EV subpopulations from protein aggregates or lipoprotein contaminants based on characteristic buoyant density profiles. Studies have shown that the buoyant density of exosomes in sucrose gradients is 1.07–1.18 g/mL, while that of apoptotic bodies is 1.24–1.28 g/mL.^{29,38–40}

Immunoaffinity separation leverages antigen-antibody specificity to isolate EVs through surface marker targeting, while employing immunoprecipitation for positive EV selection or immunodepletion for contaminant removal.^{41,42} This technique achieves superior purity relative to DC/UC/DGC by resolving EV subtypes via specific surface epitopes, though yield limitations persist due to the absence of pan-EV biomarkers for comprehensive isolation and inevitable non-specific binding during antibody-based capture.

Size exclusion chromatography (SEC) resolves EVs through hydrodynamic size partitioning using porous stationary phases, where large particles elute first via interstitial flow while smaller molecules experience delayed migration through gel matrix pores. Comparative analyses by Chandrasekera et al demonstrate that precipitation favors high EV yields but introduces copious contaminants, whereas SEC maintains superior purity, though constrained by limited throughput at

elevated sample volumes. They suggest that protocols integrating precipitation-based preconcentration with subsequent SEC purification enable high-purity EV recovery from low-abundance biofluids through synergistic yield-purity optimization.⁴³

Ultrafiltration employs semi-permeable membranes under pressure-driven flow to retain EVs while permeating smaller solutes, which achieves EV enrichment through size-based exclusion. However, this method's high recovery efficiency is countered by copious co-concentrated contaminants that compromise EV integrity and experimental reproducibility through nonspecific protein aggregation and vesicle deformation.⁴⁴

Current empirical evidence confirms that integrated isolation protocols synergizing complementary separation principles, such as UC, precipitation, and SEC, demonstrate superior efficacy in EV purification by balancing yield-purity tradeoffs inherent to single-method approaches.⁴⁵ Comparative analyses by Otani et al demonstrated that PEG isolation yields more contaminated products, UC produces lower yields, whereas PEG precipitation coupled with UC outperformed individual PEG or UC methods in both yield and purity for EV isolation from rat plasma.⁴⁶ Stam et al further reported that 60% of current EV isolation protocols employ combinatorial approaches. They systematically validated SEC initiation followed by low-speed centrifugation as an optimal strategy for achieving maximal purity while maintaining functional EV yields in sequential purification workflows.⁴⁷

Summaries of the EV separation and purification methods have been showed in [Table 1](#).

Table 1 Summary of EV Separation and Purification Methods

Classification	Methods	Core Principle	Advantages	Disadvantages
High-yield/low-purity	Polymer precipitation ⁴⁸	Solubility reduction	Low cost; Easy to handle	Precipitation of contaminating reagents
	Ultrafiltration ⁴⁹	Membrane pore size sieving	Simple operation	Filtration rate may be affected by pore size and sample concentration; Membranes are prone to clogging; Low purity
Medium-purity/medium-yield	Differential ultracentrifugation ⁴⁵	Density and sedimentation coefficient differences	Suitable for large samples and high yields	Multiple steps; Time consuming; Low reproducibility
	Field-flow separations ⁵⁰	Membrane pore size sieving	High integrity	High experience & skill required
	Size exclusion chromatography ⁵¹	Particle size differences	Simultaneous separation of different volumes of EVs, protein aggregates, and cellular debris; High integrity	High equipment costs; Complex processes
High-purity/low-yield	Density gradient ultracentrifugation ⁵²⁻⁵⁴	Density-dependent differential sedimentation	Low impurities and polymers	High experience & skill requirements; Time consuming; Low reproducibility
	Affinity separations ⁵⁵	EV surface markers targeting	Can be selectively isolated; High specificity	High cost; Altered biological function
	Flow cytometry ^{56,57}	Optical signal sorting	Allows high throughput; Multiparameter analysis	Submicron size; Low refractive index
	Microfluidics ^{58,59}	Size screening, immunoaffinity, or acoustic separation integration	High purity; High separation efficiency	Low sample size

Notes: Considering the combination of yield and purity of the sample obtained at the end of the process, EV separation procedures can be arbitrarily classified into three groups: i) methods with high yields but low purity (polymer precipitation, ultrafiltration), ii) methods that achieve a compromise between purity and yield (differential ultracentrifugation, field-flow separations, size exclusion chromatography), and iii) methods with higher purity but lower yields (density gradient ultracentrifugation, affinity separations, flow cytometry, microfluidics).

Engineered EVs

The use of EVs as drug carriers faces challenges including suboptimal cellular targeting specificity, inefficient encapsulation of therapeutic cargoes during donor cell culture, and limited bioavailability of delivered payloads in recipient cells.⁶⁰ These limitations can be addressed through engineered EV modifications that combine surface functionalization with optimization of cargo composition.

Classification of EV engineering approaches is shown in Figure 2.

Surface Modification—Improvement of Targeting

Surface modification involves adding targeting ligands to the surface of EVs through genetic engineering and chemical modification.

Genetic Engineering Methods

Genetic engineering methods for EV surface modification, including parental cell modification⁶¹ and membrane protein fusion,⁶² involve attaching gene sequences that encode guiding proteins or peptides to specific EV surface membrane proteins. Parental cell modification employs genetic engineering to overexpress fusion genes combining transmembrane proteins (eg Lamp2b, PDGFR)⁶³ with targeting peptides or proteins including neuron-targeting RVG

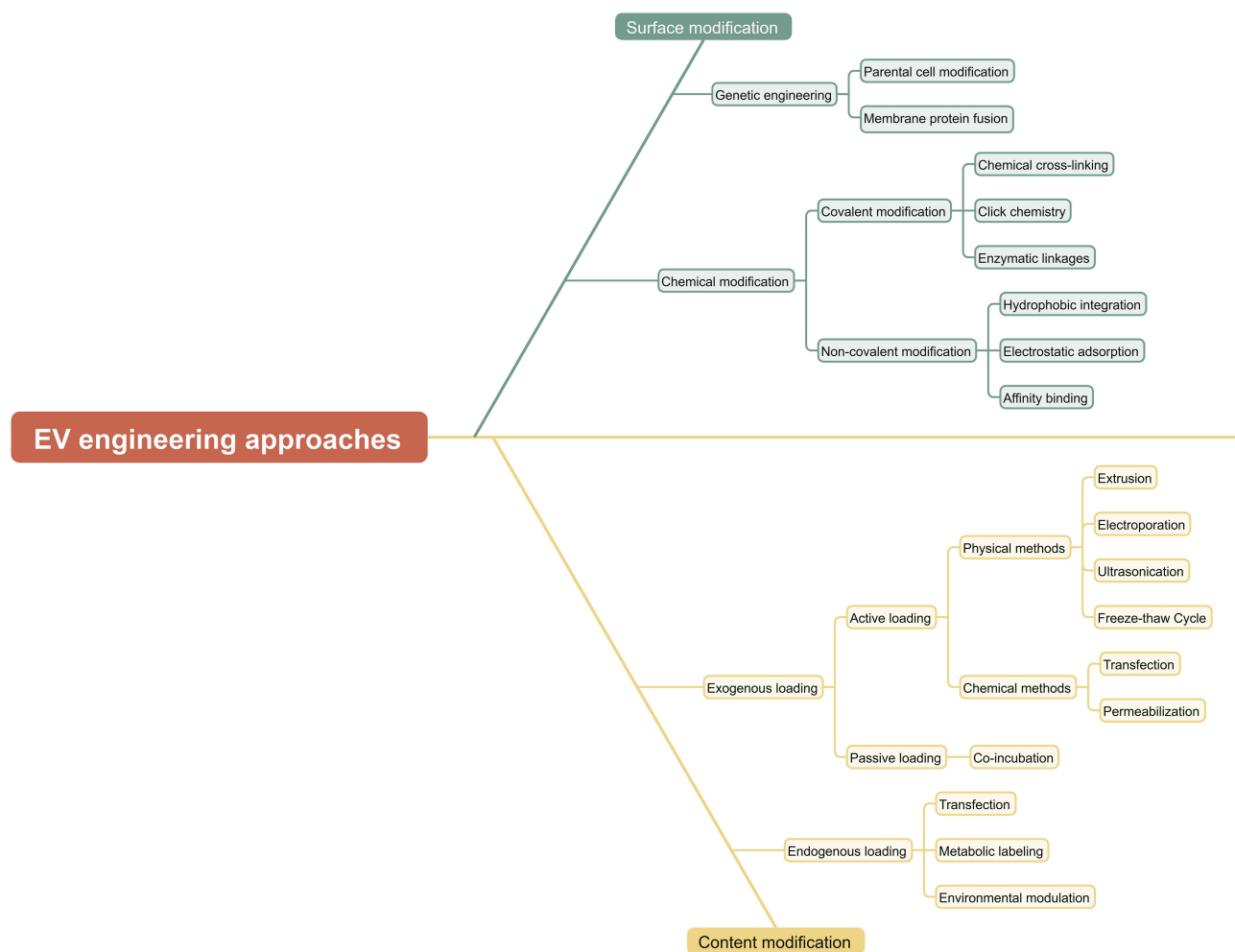


Figure 2 Classification of EV engineering approaches. EV modification can be classified into surface modification and cargo modification: surface modification can be further divided into genetic engineering modification and chemical modification based on the modification principle. Among them, chemical modification can be classified into covalent modification and non-covalent modification according to the method of chemical bond formation. Cargo modification can be divided into endogenous loading and exogenous loading based on the modification subject. Exogenous loading consists of active loading and passive loading. Different types of cargoes are suitable for different loading methods.

peptides, tumor-targeting iRGD peptides,⁶⁴ and chondrocyte-targeting CAP peptides.⁶⁵ Membrane protein fusion entails the genetic fusion of natural EV membrane proteins with functional proteins such as luciferase or antibody fragments, which are frequently applied for imaging purposes (eg Gaussia luciferase)⁶⁶ or immunomodulatory functions (eg PD-1 fusion proteins).⁶⁷ A key limitation lies in its restriction to genetically encodable targeting motifs present on peptide or protein surfaces and may be degraded by endosomal proteases inside the producing cell. To solve this problem, Hung et al engineered an RVG variant by adding a glycosylation motif. This modification both prevented peptide degradation and enhanced EV delivery to neuroblastoma cells, contributing to the development of novel sEV-based therapeutics.⁶⁸

Chemical Modification

Covalent Modification

Covalent modification involves the permanent chemical bonding of functional molecules (proteins,⁶⁹ amines,⁷⁰ fluorescent dyes^{71,72}) to reactive surface groups on EVs through three primary approaches: (i) Chemical cross-linking (eg 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS)-mediated amination reaction⁷³ and thiol–maleimide coupling for cysteine residues on the EV surface), (ii) Click chemistry (eg copper-catalyzed azidoalkyne cycloaddition; strain-promoted azide-alkyne cycloaddition;⁷³ bioorthogonal click chemistry reactions, etc) and (iii) Enzymatic linkages (eg protein ligating enzymes).⁷⁴

Non-Covalent Modification

Non-covalent modification involves transient molecular interactions (hydrophobic integration, electrostatic adsorption, and affinity binding)⁷⁵ with EV membranes. Hydrophobic integration utilizes lipid-conjugated molecules like PEG for membrane insertion to enhance stability, prolong circulation half-life, and reduce immunogenicity,^{76,77} electrostatic adsorption leverages cationic polymer-anionic EV membrane charge interactions;⁷⁸ and affinity binding includes streptavidin-biotin pairing and antibody-epitope recognition.⁷⁹ While offering rapid implementation, minimal membrane disruption, and reversible dynamics responsive to environmental stimuli, these approaches exhibit limited stability, making them suitable for only short-term applications or stimuli-responsive drug release. Sherif E. et al demonstrated that sEV PEGylation enhances *in vivo* circulation duration and plasma concentration. However, subsequent immune monitoring revealed that intravenous administration induced T cell-dependent anti-PEG IgM production can accelerate the clearance of subsequently administered PEGylated sEVs and liposomes, but selectively spare ovalbumin from immune-mediated elimination.⁸⁰

Modifying EV Content—Improving Treatment Efficiency

Engineering methods for EV content modification can be classified into two categories: endogenous loading and exogenous loading.

Endogenous Loading

Endogenous loading employs genetic engineering of parent cells to facilitate natural encapsulation of therapeutic cargo into EVs during biogenesis. This endogenous approach primarily utilizes three strategic mechanisms: (i) genetic engineering via transfection with gene-editing vectors (plasmids,⁸¹ viral vectors⁸²) to drive active packaging of target biomolecules; (ii) metabolic labeling through culture medium supplementation with bio-orthogonal precursor molecules (eg dextran sulfate)⁸³ for subsequent incorporation into EV cargos; and (iii) environmental modulation using hypoxia, inflammatory cytokines, or pharmaceutical agents to stimulate EV production with enhanced cargo-loading capacity.⁸⁴ Mizrak et al first demonstrated cancer treatment using genetically engineered IEVs that delivered therapeutic mRNA/protein by engineering donor cells to overexpress the suicide gene mRNA and cytosine deaminase-uracil phosphoribosyltransferase fusion protein. Consequently, this achieved significant growth suppression in malignant peripheral nerve sheath tumors.¹¹ This method preserves EVs' natural structure and biocompatibility but exhibits suboptimal cargo-loading efficiency, limited control over payload quantification, and potential donor cell physiological perturbations that may elevate EV heterogeneity.

Exogenous Loading

Exogenous loading is the process of introducing therapeutic agents into purified EVs. This methodology comprises two distinct strategies: active loading employs physical/chemical methods to induce cargo internalization through energy-dependent mechanisms; passive loading relies on molecules' intrinsic physicochemical properties for spontaneous transmembrane diffusion without external energy input.

Active Loading

Physical Methods

Extrusion

Extrusion-based engineering involves pressurized passage of drug^{19,85}-EV mixtures through nanoporous membranes,⁸⁶ which induces transient membrane destabilization followed by vesicle reformation. These synthetic constructs closely mimic natural EVs in key characteristics: size distribution, morphological features, and biomolecular composition,⁸⁷ while also supporting industrial-grade EV therapeutic production. However, it also causes changes in particle size distribution and membrane potentials.⁸⁸

Electroporation

Electroporation utilizes brief high-voltage electrical pulses to generate transient nanopores in EV membranes, thereby enabling efficient encapsulation of electrically charged molecules like hereditary materials.⁸⁹ Wahlgren et al compared chemical transfection versus electroporation for siRNA loading into sEVs. Chemical transfection generated contaminating MAPK-1 siRNA-containing micelles inseparable from sEVs, demonstrating its genetic material inapplicability. Thereafter, they used immunoblotting and flow cytometry to examine whether electroporation was the only means of introducing genetic material into sEV, and found that the exogenous siRNA could only be detected in the electroporated exosome samples.⁹⁰ Electroporation significantly improves loading efficiency up to 30%^{20,91} compared to passive diffusion and ultrasonication, extrusion, or saponin permeabilization.^{88,92} However, Sander et al identified key technical limitations: during electroporation, multivalent ions bind to EV membranes while buffer conductivity shifts occur, thereby collectively promoting siRNA aggregation. This phenomenon artificially inflates measured loading efficiency through false-positive signal amplification.⁹³

Ultrasonication

Ultrasonication disrupts EV membrane integrity through cavitation effects, where collapsing gas bubbles generate shear forces and microjets to create transient nanopores. These pores facilitate efficient transmembrane entry of lipophilic small molecules^{94,95} and proteins, while achieving incorporation rates of 25%–35%.⁹⁶ Notably, nucleic acid loading efficiency via ultrasonication remains inferior to electroporation.^{92,97} Studies have shown that ultrasound treatment with 20% amplitude, six cycles of 30 s on/off, and a 2-minute cooling interval between cycles enables a paclitaxel loading efficiency of $28.29 \pm 1.38\%$ in macrophage-derived exosomes. This drug-loaded system exhibits a kinetic profile characterized by an initial burst release within the first 3 hours, followed by sustained release thereafter. After one week, approximately 30% of the drug remains associated with the exosomes, and the system can be stably stored in aqueous solution for up to one month.⁸⁸

Freeze-Thaw Cycle

Freeze-thaw cycling facilitates drug encapsulation by mixing therapeutic cargo with EVs at room temperature, followed by 3 repeated cycles of flash freezing (-80°C or liquid nitrogen) and thawing (room temperature⁹⁸), exploiting ice crystal formation to disrupt plasma membranes.⁹⁹ This process preserves the bioactivity of protein, RNA,¹⁰⁰ and small-molecules,¹⁰¹ but the associated structural alterations risk EV aggregation and polydisperse size distributions, and its loading efficiency remains consistently lower than sonication or extrusion approaches. Regrettably, there are currently no direct quantitative data on the loading efficiency for biomolecules. Efficiency evaluation predominantly relies on comparative analysis with other methods and inference from membrane fusion efficiency.⁷⁷

Chemical Methods

Transfection (Calcium Chloride)

Calcium chloride (CaCl₂)-mediated transfection leverages CaCl₂-DNA precipitation complexes to enhance DNA-cell surface interactions by facilitating cellular endocytosis. For sEV-based miRNA delivery, Zhang et al adapted this technique by incorporating heat shock treatment to increase membrane fluidity, thereby enabling sEVs' uptake of CaCl₂-miRNA complexes at ambient temperatures. Comparative analysis revealed this modified approach achieves miRNA loading efficiency comparable to electroporation while eliminating dependence on specialized instrumentation.¹⁰²

Permeabilization

Chemical-mediated EV membrane permeabilization utilizes cholesterol-selective binding reagents to create transient pores for hydrophobic compounds loading. This approach employs two primary reagent categories: organic solvents (eg methanol,¹⁰³ acetone¹⁰⁴) to dissolve membrane components and detergent agents (eg saponins) to complex with membrane cholesterol for inducing controlled porosity. Saponin-based permeabilization is clinically preferred for loading therapeutic proteins into EVs^{19,105} and significantly enhances cargo-loading capacity compared to incubation techniques.^{19,106}

Passive Loading

Co-Incubation

Passive EV drug loading via room temperature co-incubation utilizes concentration gradients and molecular hydrophobicity. This approach demonstrates cargo incorporation efficiency proportional to either exogenous substance concentration or lipophilicity. It shows optimized performance for lipophilic chemotherapeutics^{99,107,108} and anti-inflammatory agents,¹⁰⁹ which exhibit enhanced EV loading capacity compared to synthetic liposomes. However, systematic evaluation of five loading strategies (passive incubation, saponin permeabilization, freeze-thaw cycles, ultrasonication, and extrusion) for oxidative enzyme encapsulation revealed that passive loading exhibits the lowest efficiency compared to other active loading methods.

Comparative studies systematically evaluate EV engineering methodologies to balance critical parameters (yield, loading efficiency), with Haney et al demonstrating superior TPP1 protein loading in macrophage-derived sEVs via ultrasonication (70 µg/10¹¹ sEVs) compared to saponin permeabilization (50 µg/10¹¹ sEVs) and transfection (10 µg/10¹¹ sEVs). This establishes that sonication is the optimal strategy for therapeutic cargo encapsulation.¹⁰⁵ Ultrasonication and extrusion induce structural reorganization of EVs, thereby enabling peroxidase diffusion through densely packed lipid bilayers. Shao et al observed that ultrasonication and extrusion enhance loading efficacy but risk vesicle aggregation and morphological alterations. In contrast, saponin permeabilization preserves native sEV morphology. Based on these findings, they hypothesized that combining extrusion with saponin permeabilization might synergize these advantages to enhance protein encapsulation efficacy. Their comparative study of SOD-loading strategies (saponin permeabilization, extrusion, saponin-extrusion hybrid) revealed via Western blotting analysis that the combined approach achieved maximal SOD incorporation in sEVs, validating the hypothesis of technique synergy.¹¹⁰ Fuhrmann et al systematically evaluated exogenous drug encapsulation strategies using hydrophobicity-varied porphyrins as model cargoes. They showed that, while EV loading efficiency primarily depends on cargo hydrophobicity, active methods like saponin permeabilization and electroporation substantially enhanced EV incorporation of moderately hydrophobic porphyrins. Particularly, saponin treatment achieved an 11-fold enhancement in hydrophilic porphyrin encapsulation while preserving vesicle delivery competence.¹¹

EV Mimetics

According to MISEV2023 guidelines, EV mimetics encompass two subtypes: ACDVs generated through artificial cellular disruption and SVs created via molecular component synthesis or hybridization. Current preparation strategies fall into three methodological categories, namely top-down, bottom-up, and bio-hybridization approaches.¹¹¹

Top-down strategies involve disintegrating complex macroscopic units like cells into nanoscale components, and these tiny membrane fragments then self-assemble to produce nanovesicle structures.¹¹² Apart from intact cells, lysed cell membranes¹¹³ and EV membranes¹¹⁴ can also be used as raw materials for coating nanoparticles. As a result, top-down-derived vesicles preserve native cellular components during membrane-to-nanovesicle transition, thereby replicating natural EV biocomplexity. Top-down techniques include chemical-induced cell blebbing, sonication nitrogen

cavitation, extrusion, filtration, and microfluidic devices. Among them, extrusion is the most widely used strategy, and the yields of EV mimetics can be increased by about 100-fold compared to natural sEVs through extrusion.⁸⁵ Additionally, nanoparticles can acquire functionalized membrane coatings through metabolic engineering, lipid insertion, membrane hybridization, or genetic modification.¹¹⁵ As their membranes originate directly from cells, these top-down vesicles are classified as ACDVs.

In contrast to top-down methods, the bottom-up approach constructs EV mimetics *de novo* using cell-derived or synthetic biomolecules, while engineering them into structurally and functionally defined architectures without employing whole cells. These EV-mimetic lipids (eg functionalized liposomes¹¹⁶) are engineered via surface modification techniques such as incubation, bioconjugation, and cell-free protein synthesis to generate diverse structures: peptide/antibody-conjugated liposomes, membrane protein-modified vesicles, protein-embedded nanoparticles, or synthesized nanovesicles.^{117–119} Though superior for large-scale vesicle production over top-down strategies, the bottom-up approach faces challenges in achieving stable EV mimetics due to significant compositional disparities from natural EVs.

Biohybrid methodologies have emerged to integrate EVs or ACDVs with synthetic nanoparticles via membrane fusion techniques, including freeze-thaw cycling, co-extrusion, incubation, ultrasonication, and film hydration, bridging the technical divide between bottom-up and top-down strategies while enhancing EV mimetic drug-loading capacity and delivery efficiency. This approach strategically merges synthetic nanocarriers' operational strengths (ease of manufacturing, scalable production, precise process control, and high drug encapsulation rates)^{120,121} with native membrane components' biological advantages (low immunogenicity, enhanced biocompatibility and safety, sustained circulatory stability, tissue-targeting specificity, and biological barrier penetration capabilities)^{122–124} to ultimately generate optimized SVs that combine engineered practicality with innate biological performance.

Current research on EV mimetics faces underdeveloped evaluation standards with inconsistent nomenclature, classification, production, and quality control. In alignment with MISEV2023 guidelines, this study categorically terms all ACDVs and SVs as EV mimetics to ensure terminological precision.

Clinical Applications

With the ongoing exploration and optimization of EV preparation technologies, their inherent advantages—such as biocompatibility, targeted delivery capability, and the capacity to transport bioactive molecules—are being progressively harnessed. This has provided crucial technical support for translating EVs into clinical diagnostic and therapeutic applications and has further propelled in-depth research related to their clinical utilization.

Natural EVs

Natural EVs originate from diverse cellular sources exhibiting unique functional properties. This prioritizes the strategic selection of cell-derived vesicles for targeted therapeutic applications and optimization of scalable production processes with enhanced drug delivery efficiency in EV-based biomedical development.

Clinically and experimentally utilized EVs predominantly originate from mesenchymal stem cells (MSCs) derived from adipose, hepatic, bone marrow, umbilical cord blood, and so on.^{125–128} These MSC-EVs inherit functional attributes from parental cells through their protein or RNA cargo, while retaining core MSC functionalities: (i) tissue protection and regeneration: MSC-EVs display universal EV markers (CD9, CD63, CD81), MSC-specific identifiers (CD44, CD73, CD90),¹²⁹ and adhesion molecules (CD29, CD59, CD166)¹³⁰ enabling targeted homing to injury sites.¹³¹ They mitigate tissue damage by suppressing apoptosis (caspase-3/8/9 inhibition)^{132,133} and pyroptosis (caspase-1 modulation),¹³⁴ while secreting cytokines, inflammatory mediators, extracellular matrix components, and antimicrobial proteins via paracrine signaling to establish pro-regenerative microenvironments, which are demonstrated in post-trauma repair in cardiac,¹³⁵ hepatic,¹³⁶ renal,¹³⁷ and neural¹³⁸ cells. Exosomes derived from menstrual blood mesenchymal stem cells effectively suppress endothelial inflammation and advanced atherosclerosis by inhibiting the miR-574-5p-mediated c-Rel/NF-κB pathway, offering new insights into stem cell therapy for inflammatory atherosclerosis.¹³⁹ Conversely, miRNA-15a-5p secreted by synovial fibroblast-derived extracellular vesicles has been identified as a key signaling molecule mediating chondrocyte apoptosis, playing a critical role in promoting chondrocyte death.¹⁴⁰ Gibello et al pioneered the use of autologous serum-derived extracellular vesicles, applying six repeated topical doses over a two-week period to four patients with chronic venous

ulcers. After a 30-day treatment period, follow-up evaluations revealed that, compared to standard therapy, EV treatment led to a significant reduction in ulcer area, accompanied by increased fibrosis and microvascular proliferation at the lesion sites.¹⁴¹ (ii) Immunomodulation: through transfer of regulatory miRNAs or proteins, MSC-EVs reprogram inflammatory immune cells (M1 macrophages, dendritic cells, Th1, Th17) into immunosuppressive phenotypes (M2 macrophages, tolerogenic dendritic cells, Tregs) This shift suppresses pro-inflammatory mediators (eg TNF- α , CCL-17, CCL-24) while elevating anti-inflammatory cytokines (eg IL-10) and TGF- β , collectively alleviating inflammation and accelerating tissue regeneration^{142,143} A Phase I clinical trial conducted by Dai et al combined sEVs derived from ascites fluid with granulocyte-macrophage colony-stimulating factor for colorectal cancer immunotherapy Results demonstrated that this combination therapy is both feasible and safe, providing an alternative immunotherapeutic option for advanced colorectal cancer.¹⁴⁴

The next most commonly used human embryonic kidney cell line, HEK293T-derived EVs, demonstrates mass production and cultivation advantages with enhanced genetic engineering potential through plasmid transfection or targeted nucleic acid modifications for precise molecular customization. While immune cell-derived EVs demonstrate functional duality: dendritic cell EVs preserve parental surface signaling molecules to activate antitumor immunity through dendritic cell-dependent and independent mechanisms,¹⁴⁵ T-cell EVs exert immunostimulatory effects, and NK-cell EVs deploy cytotoxic molecules (eg FasL, perforin) to induce tumor apoptosis.¹⁴⁶ Tumor-derived EVs, bearing tumor-specific antigens that provoke immune activation,¹⁴⁷ serve as key components in engineered antitumor EV therapeutics.

Engineered EVs

Surface Modification

Surface-modified EVs demonstrate enhanced targeting precision for delivering therapeutic cargo to hierarchical tissue structures in vivo. Genetic engineering facilitates precise display of tumor-targeting antibody fragments (scFv) on EV surfaces. Illustrating this approach, Chen et al engineered a scaffold protein with dual-displays of α PD-L1 and α CD3 scFv fragments on HEK293T-derived EV membranes. These bispecific α PD-L1 \times α CD3 BiTEs mediate T-cell redirection to PD-L1⁺ tumor cells through dual antigen recognition, simultaneously activating T-cell proliferation and cytotoxic effector functions while blocking PD-1/PD-L1 immunosuppressive signaling to enhance antitumor immunity.¹⁴⁸ Genetically engineered EVs demonstrate enhanced therapeutic precision, as exemplified by Gao et al's PSMAscFv-EVN-GSDMD platform. This system combines a prostate-specific membrane antigen-targeting scFv with the pyroptosis-inducing N-terminal domain of gasdermin D, which synergistically activates antitumor immunity as well as pyroptosis-mediated immunogenic cell death in prostate cancer models.¹⁴⁹ Wiklander et al developed Fc-functionalized EVs as modular delivery vehicles. By incubating these Fc-EVs with HER2 monoclonal antibody trastuzumab, they demonstrated enhanced tumor-targeting efficacy. This significantly increased cellular uptake in HER2-positive breast cancer cells while maintaining therapeutic payload integrity.¹⁵⁰

Surface-engineered EVs demonstrate organ-specific delivery capabilities through advanced bioconjugation techniques. Luo et al achieved spinal cord targeting by conjugating CAQK homing peptides to neural stem cell-derived sEVs via copper-free click chemistry, subsequently electroporating CCL2-specific siRNAs into these vesicles for precise delivery to spinal cord injury sites, thus, effectively enhancing neural repair outcomes. Simultaneously, Lu et al implemented a bio-orthogonal strategy where MSCs were metabolically labeled with N-azidoacetyl-mannosamine to generate azide-modified sEVs. These azide-modified sEVs were then covalently coupled with dibenzocyclooctyne-functionalized targeting ligands through strain-promoted click reactions, thereby creating chimeric antigen receptor-expressing sEVs for liver-directed therapies.¹⁵¹ Lv et al modified the surface of M2 macrophage-derived, virus macrophage inflammatory protein-II-rich EVs with a cardiac-targeting peptide and platelet membrane. This approach synergistically enhanced the vesicles' targeting specificity toward myocardial tissue and their endocytic efficiency, thereby reprogramming M1 pro-inflammatory macrophages into an M2 anti-inflammatory phenotype, providing a novel immunomodulatory therapeutic paradigm for addressing excessive inflammatory responses in viral myocarditis.¹⁵²

Engineered EVs demonstrate remarkable capabilities in crossing the blood-brain barrier (BBB) for targeted therapeutic delivery to specific brain regions. Alvarez et al transfected Lamp2b into the dendritic cells through plasmid transfection and created chimeric variants fused with RVG for neural targeting and muscle-specific peptide (MSP) for myocyte homing. The resultant MSP-Lamp2b and RVG-Lamp2b EVs exhibited cell-type-specific tropism in mice, with RVG-modified EVs achieving neuronal targeting and MSP-EVs showing muscle specificity. This culminated in

a significant reduction of β -amyloid 1–42 levels in murine models, thereby validating EV-based strategies for neurodegenerative disease intervention.²⁰

Cargo Loading

EVs exhibit remarkable versatility as delivery platforms capable of co-loading diverse therapeutic cargoes spanning small-molecule drugs, nucleic acids, and proteins. Small-molecule payloads enable cellular proliferation inhibition,^{153,154} signaling pathway modulation,^{155,156} and targeted cytotoxicity.¹⁵⁷ Nucleic acid cargoes include protein-coding plasmids or mRNAs for exogenous gene expression and regulatory RNAs for interference. Protein therapeutics, whether native or engineered, function intracellularly to activate or suppress specific biological pathways.¹⁵⁸ However, these biomacromolecules inherently lack cell membrane permeability,^{159,160} which necessitates EV-mediated delivery to achieve intracellular bioactivity.

Small Molecule Drugs

EVs exhibit intrinsic therapeutic advantages including low immunogenicity, minimal cytotoxicity, enhanced bioavailability, and inherent targeting specificity.^{122,123,161,162} Small molecule therapeutics, particularly chemotherapeutic agents, often demonstrate suboptimal pharmacokinetics characterized by non-specific biodistribution, rapid systemic clearance, limited aqueous solubility, and toxicity.¹⁶³ EV encapsulation of these pharmaceuticals enables engineered nanoformulations that enhance the therapeutic index through improved target tissue accumulation while mitigating adverse effects via reduced effective dosage requirements.

Therapeutics of engineered EV loaded with small-molecule have been showed in [Table 2](#).

Protein Cargoes

Protein-based therapeutics, including recombinant proteins,¹⁷⁷ monoclonal antibodies,¹⁷⁸ and protein kinase,¹⁷⁹ are prone to compromised efficacy due to immune recognition, rapid systemic clearance, enzymatic degradation, or off-target

Table 2 Engineered EV Loaded with Small-Molecule Therapeutics: Comparative Analysis of Loading Strategies, Physicochemical Properties, EV Sources, and Pharmacological Applications in Drug Delivery

PMID	Loading Strategies	Small Molecule Drugs	EV Sources	Applications
PMID: 35932288 ¹⁶⁴	Electroporation	Doxorubicin	HEK293T	Glioma
PMID: 37153730 ¹⁶⁵	Co-incubation	Bevacizumab	MSCs	Diabetic retinopathy
PMID: 36162554 ¹⁶⁶	Co-incubation	Adriamycin	HEK293T	Melanoma
PMID: 37473534 ¹⁶⁷	Co-incubation	Ultrasound sensitizer T-Ce6 & glycolysis inhibitor FX11	HEK-293T series	Sonodynamic cancer therapy
PMID: 36222853 ¹⁶⁸	Co-incubation	Photosensitizer Ce6 & tumor affinity agent 18F-FDG	Goat's milk	Tumor photodynamic therapy
PMID: 37719366 ¹⁶⁹	Co-incubation	Ce6 & TPP	Brain endothelial cells	Glioblastoma
PMID: 36682729 ¹⁷⁰	Co-incubation	Ce6 & TPP	HEK-293T	Chemo-sonodynamic therapy
PMID: 35154485 ¹⁷¹	Co-incubation	Indocyanine green & paclitaxel & NaHCO ₃	HEK-293T	Chemo-sonodynamic cancer therapy and photoacoustic imaging
PMID: 31037133 ⁹⁵	Ultrasonication	Paclitaxel	M1 macrophages	Breast cancer
PMID: 37935259 ⁹⁴	Ultrasonication	Caspase-I inhibitor VX-765	Bone marrow dendritic cells	Myasthenia gravis
PMID: 37992875 ¹⁷²	Ultrasonication	Benzoyloxy dibenzyl carbonate	OVCAR-8 cells	Multidrug-resistant cancer
PMID: 37867176 ¹⁷³	Ultrasonication	Corynoxine-B	HT22 Hippocampal neurons	Alzheimer's Disease

(Continued)

Table 2 (Continued).

PMID	Loading Strategies	Small Molecule Drugs	EV Sources	Applications
PMID: 35341901 ¹⁷⁴	Ultrasonication	Temozolomide & siSTAT3	BMSCs	Temozolomide-resistant Glioblastoma
PMID: 36521687 ¹⁷⁵	Ultrasonication	Resveratrol	RAW2647 murine macrophage-like cells	Multiple sclerosis
PMID: 40007431 ¹⁷⁶	Microfluidics	Verteporfin	BMSCs	Neuroblastoma

Abbreviations: MSCs, mesenchymal stem cells; T-Ce6, triphenylphosphonium-conjugated chlorin e6; TPP, triphenylphosphine; BMSCs, bone marrow mesenchymal stem cells.

molecular sequestration¹⁸⁰ Engineered EVs address these limitations by enhancing proteolytic stability through lipid bilayer encapsulation, prolonging circulation half-life via reduced immunogenicity, and enabling receptor-specific targeting through surface modifications.¹⁸¹ This DDS synergistically optimizes pharmacological performance by preserving structural integrity, enhancing cellular uptake efficiency, and minimizing nonspecific interactions, thereby amplifying therapeutic outcomes in protein-based interventions.¹⁸²

Therapeutics of engineered EV loaded with protein have been showed in [Table 3](#).

Nucleic Acid-Based Cargoes

siRNA, miRNA, and antisense oligonucleotides (ASOs) demonstrate particular utility in organ repair research. However, these polyanionic biomolecules face intrinsic pharmacological challenges due to their cell membrane-impermeable nature, susceptibility to nuclease degradation, and electrostatic repulsion.¹⁸⁹ This necessitates engineered delivery platforms to ensure intracellular bioavailability and therapeutic functionality.

Therapeutics of engineered EV loaded with nucleic acid have been showed in [Table 4](#).

EV Mimetics

Although EV mimetics remain under-investigated in clinical trials, they address critical limitations of natural EVs, notably batch-to-batch heterogeneity²⁰⁴ and low yield.²⁰⁵ Through adaptable manufacturing of ACDVs via extrusion,

Table 3 Engineered EV Loaded with Protein Therapeutics: Comparative Analysis of Loading Strategies, Physicochemical Properties, EV Sources, and Pharmacological Applications in Drug Delivery

References	Loading Strategies	Protein Cargoes	EV Sources	Applications
PMID: 35870568 ¹⁸³	Electroporation	TPP/P53	Breast cancer cell line (MCF-7, SK-BR-3 and 4T1)	Breast Cancer
PMID: 36804538 ¹⁷⁷	Electroporation	Yap1 protein	Platelets	Functional tendon regeneration
PMID: 36635966 ¹⁸⁴	Transfection	Club cell protein 16	BEAS-2B, THP-1, HEK293T cells	ALI/ARDS
PMID: 36063136 ¹⁸⁵	Transfection	Transmembrane 4 L six family member 5	SNU-761, NCI-H727, HEK293A, Huh7 and SNU449 cell lines	Blood glucose level regulation
PMID: 37119468 ¹⁸⁰	Transfection	IL-4 & IL-10	Primary dermal fibroblasts from BALB/c mice	ALI/ARDS
PMID: 37072558 ¹⁸⁶	Transfection	Wingless-type MMTV integration site family, member 3A	HEK293T	COPD
PMID: 35301052 ¹⁸⁷	Transfection	Allergen (ovalbumin) & CpG DNA	HEK293	Allergic rhinitis
PMID: 36423694 ¹⁷⁹	Transfection	AMP-activated protein kinase alpha 1	Immature dendritic cells	Allergic rhinitis
PMID: 40301355 ¹⁸⁸	Transfection	Cre recombinase & Cas9/sgRNA RNPs	HEK293T	Genome editing

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; Cas9/sgRNA, Cas9/single-guide-RNA; RNPs, ribonucleoproteins.

Table 4 Engineered EV Loaded with Nucleic Acid Therapeutics: Comparative Analysis of Loading Strategies, Physicochemical Properties, EV Sources, and Pharmacological Applications in Drug Delivery

References	Loading Strategies	Nucleic Acid Cargoes	EV Sources	Applications
PMID: 36474657 ⁸⁹	Electroporation	CCL2-siRNA	Neural stem cells	Spinal cord injury
PMID: 35198075 ¹²	Electroporation	Oligodeoxynucleotide	HEK293T	Liver fibrosis
PMID: 36845116 ¹⁹⁰	Transfection	siRNA	ADMSCs	Skin lesions
PMID: 36396626 ¹⁹¹	Transfection	Wilms' tumor 1 & miR-769-5p & histone deacetylase	Mesothelial cells	Organ fibrosis
PMID: 37351166 ¹⁹²	Transfection	<i>Ldlr</i> mRNA & CD9-MCP protein & RNA aptamer <i>Ldlr</i> -MS2	HEK293T, mouse liver 12 cells	Familial hypercholesterolemia
PMID: 36635419 ¹⁹³	Transfection	COL1A1 mRNA	Human dermal fibroblasts	Photodamaged skin
PMID: 35077741 ¹⁹⁴	Transfection	lncRNA MEG3	Human osteosarcoma cell lines, Normal human osteoblast cell line	Osteosarcoma
PMID: 33870645 ¹⁹⁵	Transfection	HOTAIR	BMSCs	Angiogenesis & Wound Healing
PMID: 36965082 ¹⁹⁶	Transfection	circRNA mSCAR	HEK239T	Sepsis
PMID: 38012307 ¹⁹⁷	Transfection	Cas9-sgRNA	HEK239FT	HIV
PMID: 39246205 ¹⁹⁸	Transfection	Ox40L mRNA	HEK293T	Melanoma
PMID: 40343195 ¹⁹⁹	Transfection	miR-141-3p	Adipose tissue	Type 2 diabetes mellitus
PMID: 40114173 ²⁰⁰	Transfection	miRNA let-7f-5p	Lip gland mesenchymal stem cells	Sjögren's syndrome
PMID: 40195092 ²⁰¹	Transfection	miR-146a	Human umbilical cord mesenchymal stromal cells	Ischemia-reperfusion injury in lung transplantation.
PMID: 40315630 ²⁰²	Transfection	cyclic RNA circ-CCND1	Pluripotent stem cell-derived mesenchymal stem cells	Ulcerative colitis
PMID: 36328741 ²⁰³	Co-incubation	A151 oligodeoxynucleotide	Umbilical cord tissue-derived MSCs	Immunomodulation & skin regeneration

Abbreviations: MSCs, mesenchymal stem cells; BMSCs, bone marrow mesenchymal stem cells; ADMSCs, adipose-derived mesenchymal stem cells; *ldlr* RNA, low-density lipoprotein receptor mRNA; COL1A1 mRNA, mRNA encoding for extracellular-matrix α 1 type-I collagen; lncRNA MEG3, long non-coding RNA maternally expressed gene 3; HOTAIR, long non-coding RNA HOX transcript antisense RNA; circRNA mSCAR, steatohepatitis-associated circRNA ATP5B regulator; Cas9-sgRNA, Cas9-single-guide-RNA.

these synthetic systems achieve parental cell-mimetic biological properties with enhanced uniformity in vesicle size, membrane architecture, and cargo composition, which demonstrates significant translational potential for industrial-scale EV-based DDSs. Concurrently, hybrid SVs engineered through the strategic integration of EV components with synthetic nanoparticles are emerging as modular platforms for precision therapeutic targeting as they combine endogenous biocompatibility with engineered functionalization capabilities.

Therapeutics of EV mimetics have been showed in Table 5.

Other Synthetic Nanoparticles

Conventional synthetic nanocarriers currently dominate clinical applications as drug delivery vehicles for proteins, small-molecule therapeutics, and other payloads. Thus, they share functional parallels with engineered EVs in target specificity, payload optimization, and mitigation of nonspecific tissue toxicity, which makes direct comparisons contextually redundant.²¹² This review specifically addresses the transformative therapeutic potential in gene therapy by synthetic nanoparticles, which is yet underexploited in engineered EVs.

Gene therapy employs nucleic acid-based interventions to modulate gene expression or cellular function through four principal strategies: (i) gene replacement, through functional gene delivery to compensate for defective alleles; (ii) gene

Table 5 EV Mimetics Therapeutics: Comparative Analysis of Material Sources, Production Strategies, Classifications, and Pharmacological Applications in Drug Delivery

References	Materials Sources	Production Strategies	Classifications	Applications
PMID: 38918594 ²⁰⁵	Red blood cells	Top-down (extrusion)	ACDV	RNA delivery
PMID: 39502438 ²⁰⁶	Mouse MSCs	Top-down (extrusion)	ACDV	Liver fibrosis
PMID: 39591514 ²⁰⁷	Mammalian cells	Top-down (extrusion)	ACDV	Lung tissue targeting delivery
PMID: 38659058 ²⁰⁸	Dendritic cells (α CD3 antibodies conjugated)	Top-down (extrusion)	ACDV	Inflammation diseases
PMID: 39624053 ²⁰⁹	Endothelial progenitor cell (cRGD peptide coupled)	Top-down (extrusion)	ACDV	Diabetic wound
PMID: 37812759 ¹¹⁶	LNP (supercharged green fluorescent protein & cardiac tissue fibronectin homing sequence conjugated)	Bottom-up	SV	Cardiac disease
PMID: 40156987 ²¹⁰	Chitosan nanoparticle (EV surface ligand transferred)	Bottom-up	SV	Cancer therapy
PMID: 39033517 ²¹¹	Ovarian epithelial ACDVs & iron oxide nanoparticles & β -Lapachone	Hybridization	SV	Ovary cancer

Abbreviations: ACDV, artificial cell-derived vesicle; MSCs, mesenchymal stem cells; SV, synthetic vesicle; LNP, lipid-based nanoparticle.

silencing, via RNA-based regulators^{213,214} to suppress aberrant gene expression; (iii) gene addition, utilizing DNA/RNA constructs^{215–217} to introduce ectopic gene expression; and (iv) gene editing, deploying CRISPR-Cas systems^{218,219} to induce precise genomic modifications for functional gain or loss. The macromolecular size and polyanionic nature of these therapeutic agents necessitate vectors to overcome cellular membrane impermeability and ensure intracellular delivery efficacy.

Classifications of synthetic nanoparticles have been showed in Table 6.

Gene Editing

Gene editing is a biotechnology for modifying genetic information through insertion, deletion, replacement, or modification of specific genomic sequences.²²⁴ CRISPR-based strategies now dominate clinical applications owing to their programmable DNA targeting capability, which facilitates permanent genomic edits and donor template integration. These advantages have enabled CRISPR's utilization in treating genetic disorders,²²⁵ developing cancer immunotherapies,²²⁶ and advancing antiviral treatments.²²⁷ The CRISPR-Cas9 system requires two components: (i) Cas9 endonuclease, for inducing double-strand DNA breaks, and (ii) single-guide RNAs (sgRNAs), for sequence-specific targeting. Synthetic nanoparticles are increasingly leveraged as non-viral delivery platforms for CRISPR-Cas9 payloads, thus enhancing transfection efficiency and tissue specificity while minimizing CRISPR-based off-target genomic perturbations.

Zhao et al engineered an oral CRISPR-Cas9 delivery platform using amphiphilic ion-polysaccharide polymer-coated nanocomplexes to disrupt the tumor necrosis factor receptor-associated protein 1 gene in colorectal cancer. They induced mitochondrial dysfunction in tumor cells, synergistically amplifying chemotherapy-driven necrotic cell death and antitumor immunity.²²⁸ It also achieved efficient in vivo gene editing in Huntington's disease models.²²⁹ Liyanage et al engineered a CRISPR-Cas9 delivery platform by covalently conjugating SpCas9 nuclease to hydroxyl-terminated PAMAM dendrimers via high-specificity inverse electron-demand Diels-Alder click chemistry, thus constructing a Cas9-dendrimer nanocomplex (D-Cas9) self-assembling with sgRNA into a neutrally charged D-Cas9-RNP. This system circumvented nuclear delivery challenges of large nucleic acids, including nuclease susceptibility, inefficient packaging, and suboptimal expression, while achieving high-efficiency cellular uptake and specificity-driven genome editing across diverse cell lines and genetic targets.²³⁰

Table 6 Classifications of Synthetic Nanoparticles: Sorted by Their Subclasses, Materials, and Production Strategies

Properties	Classifications	Common Subclasses	Materials	Production Strategies
Organic nanoparticles	Lipid-based nanoparticles	Liposomes	Cationic liposomes, phospholipids, cholesterol, polyethylene glycolized lipids	Film hydration, solvent injection, and reverse-phase evaporation
		LNPs	Ionizable lipids, cholesterol, auxiliary phospholipids, and polyethylene glycolated lipids	Detergent dialysis, ethanol loading techniques, T-mixing techniques, microfluidic hydrodynamic focusing, and staggered herringbone mixing
	Polysaccharide-based nanoparticles	-	Chitosan, dextran sulfate, sodium alginate, hyaluronic acid, adjuvant poly (inosine-cytidylic acid) (poly(I: C)), etc.	Ion cross-linking, self-assembly, enzymatic methods, etc.
	Peptide-derived nanoparticles	-	Natural short peptides, synthetic peptides, etc.	Self-assembly, electrostatic adsorption, etc.
	Polymer nanoparticles	Multi-chain bodies	Cationic polymers (eg PEI, chitosan, and biodegradable polyesters), nucleic acids	Polymer cations for spontaneous nucleic acid condensation
		Polymer bodies	PEG, polydimethylsiloxane, poly (2-methyl-2-oxazolidine), etc.	Probe sonication, film rehydration, direct dissolution of copolymers, solvent injection, solvent exchange, and phase-guided assembly
Dendritic nanoparticles		Dendritic polymers (eg PAMAM, polypropyleneimine, and PLL)	Diffusive growth method, convergent growth method, biexponential growth method, two-stage convergence method, and high monomer method	
DNA nanostructures	DNA tetrahedral nanostructures, ²²⁰ DNA nanoboxes, ²²¹ DNA nanohydrogels ²²² and DNA origami ²²³	DNA molecules	Buffering, annealing, and purification	
Inorganic nanoparticles	Metal-based nanoparticles	Gold particles, iron oxide nanoparticles, lanthanide upconversion particles, etc.	Gold, γ -Fe ₂ O ₃ , Fe ₃ O ₄ , lanthanides (Yb ³⁺ , Er ³⁺ , Tm ³⁺ , Ho ³⁺) PEG, SiO ₂ , etc.	Chemical reduction, sol-gel, thermal decomposition, electrochemistry, physical methods (eg laser ablation methods, sputtering methods), biosynthetic methods (green synthesis), template methods, microfluidic synthesis, etc.
	Carbon-based nanoparticles	Carbon nanotubes	Graphite, SiO ₂ , etc.	Arc discharge method, Chemical vapor deposition
	Mesoporous silica nanoparticles	-	SiO ₂ , cationic macromolecules (including PEI, dendritic polymers, and lipids), etc.	Sol-gel, microwave synthesis, hydrothermal synthesis, template synthesis, hard and soft template methods, etc.
Hybridized nanoparticles	Inorganic-inorganic hybrids, inorganic-organic hybrids, organic-organic hybrids, bio-abiotic hybrid nanoparticles	eg silica-lipid hybrid nanoparticles, lipid-polymer nanoparticles, polymer micelles	Inorganic constituents (eg SiO ₂ , metals), organic constituents (eg lipids, polymers)	In-situ synthesis, self-assembly, emulsification-solvent evaporation, biosynthesis methods (green synthesis), microfluidics, etc.

Abbreviations: LNPs, lipid-based nanoparticles; PEG, polyethylene glycol.

Vaccines for Infectious Diseases

A vaccine is primarily composed of immunogenic components, such as inactivated or attenuated pathogens, recombinant antigens, or nucleic acids, along with adjuvants to enhance non-specific immune responses and stabilizers to prevent the degradation of vaccine constituents.²³¹ Synthetic nanoparticles have gained significant attention in vaccine development. Their capability to encapsulate antigens enables surface functionalization for targeted delivery, and the nanoparticles act as immunostimulatory adjuvants that delay antigen degradation while activating APCs and lymphocytes. Their vaccine applications are categorized into three primary roles: antigen delivery vehicles, adjuvant delivery vehicles, and self-

contained nucleic acid vaccine carriers.²³² RNA vaccines offer a non-integrating, degradable, and non-infectious modality with enhanced manufacturability,^{233,234} thereby justifying their prioritization in this review.

The SARS-CoV-2 vaccines developed during the COVID-19 pandemic operate by delivering viral spike protein antigens via synthetic nanoparticle carriers to trigger adaptive immune responses. mRNA-1273 (Moderna) and BNT162b2 (Pfizer-BioNTech) employed N1-methylpseudouridine-modified mRNA encapsulated in LNPs formulated with SM-102 or ALC-0315 ionizable lipids, respectively, which encoded SARS-CoV-2 spike glycoprotein variants. Real-world efficacy data from 3,950 U.S. health care personnel, first responders, as well as other essential and frontline workers demonstrated 90% protection against SARS-CoV-2 infection following two doses of either vaccine.²³⁵ Accumulating evidence indicates that LNPs possess intrinsic immunostimulatory properties, thereby positioning them as potent adjuvants for recombinant protein vaccines. Dai et al systematically evaluated LNP adjuvant efficacy against conventional aluminum-based adjuvants in norovirus vaccine formulations and demonstrated that LNPs enhance recombinant protein immunogenicity by inducing cellular immunity and promoting balanced IgG2a/IgG1 ratios relative to alum. Furthermore, LNPs outperformed aluminum hydroxide in generating Th1-type cytokine-expressing CD4⁺ and CD8⁺ T cell immune responses, thus establishing their capacity to synergistically activate cytotoxic T lymphocytes and B cells, which are critical aspects for antiviral and antitumor immunity.²³⁶ Synthetic nanoparticles frequently serve as direct carriers for nucleic acid vaccines: Wang et al engineered HBsAg-encapsulating liposomes dually functionalized with mannose-PEG-cholesterol for C-type lectin receptor targeting and lipid A as an adjuvant via emulsification-freeze-drying, subsequently integrating them into microneedle arrays for oral mucosal immunization. They achieved simultaneous humoral and mucosal immunity against hepatitis B surface antigen.²³⁷ Separately, Wang et al developed a protein nanoparticle CBU-CpG-E2 vaccine co-encapsulating Behçet's disease-related CBU1910 antigen, CpG1826 adjuvant, and PLGA-PEG-PLGA hydrogel and demonstrated that combinatorial nanoparticle-hydrogel vaccination elicited enhanced magnitude and durability of humoral immunity compared to single-component systems.²³⁸

Cancer Therapies

Cancer Vaccines

Synthetic nanoparticles enhance cancer vaccine efficacy by delivering immunomodulators such as tumor antigens and adjuvants, thereby amplifying tumor-specific T-cell activation through active immunization.²³⁹ Cancer vaccine components comprise tumor antigens (tumor-associated or tumor-specific antigens), formulations (whole-cell or antigen-specific), immune adjuvants (eg granulocyte-macrophage colony-stimulating factor [GM-CSF], CpG oligodeoxynucleotide [CpG-ODN], and Toll-like receptor [TLR] agonists^{240,241}), and nanoparticle delivery systems. Among these, tumor-specific neoantigens—novel epitopes recognized by autologous T cells to initiate anti-tumor immunity independently of central tolerance²⁴²—uniquely elicit potent CD4⁺ and CD8⁺ T-cell responses via dendritic cells.

Chen et al successfully delivered a model tumor neoantigen using engineered porous hollow *Mycobacterium tuberculosis* (phMTb) that retained surface pathogen-associated molecular patterns for APC recognition and adhesion molecules facilitating APCs invasion, thereby enhancing cross-presentation of tumor neoantigens, dendritic cell activation, migration, and effector/memory CD8⁺ T cell function.²⁴³ Zhang et al developed phospholipid-coated mesoporous silica nanoparticles co-delivering ovarian cancer antigen OVA257-264 and the TLR agonist monophosphoryl lipid A adjuvant, thereby improving lymph node-targeted antigen delivery and achieving synergistic tumor antigen-adjuvant immune activation. Clinical trials demonstrate that neoantigenic tumor vaccines exhibit insufficient antigen uptake, poor tumor recognition, weak CD8⁺ T-cell responses, and impaired cytotoxic activity attributable to passive APC engagement. To address these limitations, Liang et al engineered a tumor-targeting vaccine by co-loading antigen-loaded ginsenoside-Rg3-based LNPs and GM-CSF into a macroporous hydrogel, where Rg3 serves as a targeting ligand for glucose transporter protein-1-overexpressing tumor cells. The Rg3-LNPs anchor antigens onto tumor cell surfaces and enhance antigen presentation and recognition through adjuvant synergy, thereby mitigating immune escape in antitumor therapy.²⁴⁴ Xu et al engineered a spleen- and tumor-dual-targeting vaccine by incorporating cholesterol-conjugated and thiol-modified CpG-ODN, tumor-specific neoantigen peptides, and the LNP^{D18} vector, which induced monocytic myeloid-derived suppressor cell differentiation into M1 macrophages, activated splenic dendritic cells, facilitated tumor eradication, reprogrammed the tumor microenvironment, and activated CD8⁺ T cells.²⁴⁵

Other Therapeutic Effects

Beyond their role in tumor vaccines, synthetic nanoparticles serve multifunctional purposes in oncology therapeutics—primarily as targeted drug delivery vehicles that recognize cancer-specific molecular markers to enable site-specific payload release and enhance bioavailability while sparing healthy tissues. Collagen networks critically regulate tumor proliferation, metastasis, and chemoresistance, prompting the development of collagen-targeting strategies: (i) enzymatic collagenolysis, which enhances intratumoral nanoparticle penetration^{246,247} and (ii) photothermal collagen denaturation, promoting deeper diffusion of therapeutic nanoparticles.²⁴⁸ Resultant collagen fragments further potentiate conventional therapies by improving chemotherapeutic agent permeation, immunotherapeutic antibody distribution, and immune cell infiltration into tumors. Zhou et al pioneered the use of spermine as a tumor-targeting ligand in polymeric micelles encapsulating the hydrophobic chemotherapeutic agent paclitaxel. By exploiting spermine's specific binding to the polyamine transport system overexpressed on tumor cells, they achieved active targeting for anticancer drug delivery.²⁴⁹

Synthetic nanoparticles additionally remodel the immunosuppressive tumor microenvironment by inhibiting immunosuppressive signals and activating co-stimulatory pathways. Li et al developed a multifunctional MPLO@HA nanomedicine for the targeted treatment of *Fusobacterium nucleatum* (Fn)-associated colorectal cancer.²⁵⁰ This platform conjugates metformin, oxaliplatin, and lauric acid to oligomethyleneimine, which is subsequently complexed with hyaluronic acid. The system effectively eradicates intra- and extracellular Fn to overcome Fn-induced chemoresistance, disrupt tumor immunosuppression, improve patient prognosis, and pioneer therapies for bacterial-driven cancers. Song et al engineered an STK11@PPCM biomimetic nanoplatform targeting anti-PD-1-resistant, STK11-mutant non-small cell lung cancer. This system utilizes a cationic PBAE polymer that binds NSCLC cells to enable precise delivery of STK11-encoded DNA. By restoring STK11 expression, the platform reactivates autophagy and induces immunogenic cell death, thereby providing novel clinical strategies for STK11-mutated lung adenocarcinoma.²⁵¹

Synthetic nanoparticles also provide an efficient alternative to chimeric antigen receptor-T cell generation and transduction by overcoming the limitations of conventional viral-based transduction and offering a safer approach for tumor-targeted T cell therapy. Lin et al combined microfluidic extrusion-mediated membrane disruption with LNP vector delivery, significantly enhancing transfection efficiency²⁵² and cargo delivery while reducing production costs. This maintained cell viability while preserving native immune gene expression.²⁵³

Discussion

In recent years, research on EV-based DDSs has grown exponentially, with a clear shift from in vitro experiments to preclinical and clinical studies. Approximately 201 studies have been reported using EVs as interventions or research subjects on www.clinicaltrials.gov, predominantly Phase I exploratory trials. These primarily focus on two major areas: therapeutics (including tumor immune stimulation, inflammatory immunosuppression, and tissue regeneration) and diagnostics (eg early screening via liquid biopsy) EVs have been demonstrated to be safe and well-tolerated, with a low overall incidence of adverse events (4.4%; 95% CI: 0.7–22.2%).²⁵⁴ However, conducting large-scale Phase III trials to demonstrate positive efficacy remains constrained.

Current bottlenecks in EV clinical translation primarily stem from two aspects: limitations in production technology and inherent heterogeneity. Deficiencies in production technology remain a persistent challenge, primarily stemming from the inefficiency of separation and purification techniques. This encompasses: (i) an imbalance between purity, recovery, activity, and cost;²⁵⁵ (ii) functional risks: where forces from centrifugation, chemical reagents, or membrane adsorption can readily compromise EV structural integrity, thereby reducing their biological activity and impacting subsequent diagnostic or therapeutic applications; and (iii) internationally unified quality control standards deficiency: while current advanced methods, magnetic bead separation,²⁵⁶ lipid-based nanoprobe separation,²⁵⁷ and solid-phase medium separation technologies²⁵⁸ can partially reduce inter-batch heterogeneity within a single laboratory, differences in separation parameters between laboratories continue to hinder the cross-laboratory reproducibility of data.²⁵⁹ (iv) Further bottlenecks exist in mass production. Existing methods are predominantly suited for small-scale laboratory samples and struggle to meet the high-throughput, rapid processing demands of large-volume clinical specimens. Constraints also arise from limitations in engineered modifications: genetic modifications suffer from low membrane protein fusion efficiency and narrow donor cell applicability, while chemical modifications often cause membrane damage and induce non-specific binding. Both exogenous and endogenous loading

strategies face challenges, including the loss of macromolecular bioactivity, inconsistent drug loading, significant batch variability for small-molecule payloads, and uncontrollable encapsulation rates. In vivo delivery is further hampered by phagocytic system clearance, off-target accumulation, and insufficient tissue penetration, resulting in actual targeting efficiency that falls far short of in vitro predictions.^{9,84,260}

EV heterogeneity represents a major bottleneck in clinical translation, directly impeding the standardized production and predictable efficacy of EV-based therapies. On one hand, this heterogeneity complicates the assurance of “batch consistency”: even when derived from an identical cell line, variations in culture conditions or different cell passages can alter the composition and function of the resulting EVs, leading to inconsistent quality in clinical-grade EV products. On the other hand, heterogeneity hampers the prediction of therapeutic outcomes: the composition of EV subpopulations varies among patients, meaning the same EV therapy can yield vastly different clinical results due to individual differences in the recipient’s endogenous EV landscape.

Furthermore, heterogeneity is a determinant of EV immunogenicity and potential toxicity. In fact, the purported “low immunogenicity” of natural EVs is often overstated. They can, on one hand, activate anti-infective and anti-tumor immunity by carrying pathogen-associated molecular patterns, damage-associated molecular patterns, or antigenic peptides, positioning them as ideal candidates for vaccine development.²⁶¹ Conversely, they can also mediate immune escape by expressing molecules like PD-L1 or releasing immunosuppressive miRNAs, thereby potentially accelerating tumor progression or exacerbating autoimmune diseases.²⁶² The underlying mechanism is that their immune-inert or immune-active properties are highly dependent on their cellular origin and physiological state.^{8,263} For instance, EVs derived from microbes or activated immune cells may trigger immune responses due to foreign MHC molecules or stimulatory surface components,^{264,265} whereas those from tumor cells or certain stem cells often exhibit immunosuppressive tendencies.^{266,267}

From a toxicity standpoint, while EVs themselves typically lack direct cytotoxicity, the pathological effects they mediate are characterized by their “indirect” and “covert” nature. For example, EV-mediated amplification of inflammation in sepsis,²⁶⁸ or EV-induced immunosuppression and promotion of metastasis within the tumor microenvironment,²⁶⁹ both represent forms of “secondary damage” orchestrated by EVs from distinct sources. The toxic thresholds and precise cellular targets of these effects remain difficult to quantify.

Furthermore, for artificially engineered nanoscale DDSs, functional modifications to engineered EVs inevitably increase immunogenic risks. The conjugation of exogenous molecules during surface modification may expose novel immunogenic epitopes, while the process of drug loading can disrupt membrane architecture, ultimately potentiating activation of the complement system. The current absence of standardized modifications renders this immunogenicity largely unpredictable.⁷⁴ While EV mimetics enable scalable production, their artificially synthesized membrane components inherently lack the “self-recognition” proteins present on natural EVs, rendering them more susceptible to clearance by the mononuclear phagocyte system. Additionally, EV mimetics prepared via methods such as cell lysis may retain residual immunostimulatory components.²⁷⁰

In comparison to EV-based DDS, traditional synthetic nanoparticles present toxicity profiles that extend beyond immunogenicity. This includes inherent material toxicity, the potential for chronic damage from accumulated degradation products, and the compounded effects of immunotoxicity and off-target toxicity. Consequently, their overall biosafety profile is generally regarded as inferior to that of EV-based carriers.^{270,271} Nevertheless, the capacity for large-scale production and the achievement of high batch consistency remain undeniable advantages of synthetic nanoparticle platforms.²⁷²

A comparison of the advantages, disadvantages, challenges, and future optimization directions regarding native EVs, engineered EVs and EV mimetics have been showed in [Table 7](#).

Conclusion and Outlook

As an emerging DDS, EVs demonstrate significant potential. Their clinical translation and application present a landscape of concurrent challenges and developmental opportunities. Researchers are continuously exploring novel approaches to address the aforementioned limitations.

Regarding production and preparation, studies indicate that integrating tangential flow filtration—a technique amenable to industrial-scale manufacturing—with established EV purification methods facilitates high-purity EV isolation with improved throughput.^{273–276} For standardized EV production, reproducible, Good Manufacturing Practice (GMP)-compliant process

Table 7 Comparison of Advantages, Disadvantages, Challenges, and Future Optimization Directions Regarding Native EVs, Engineered EVs, and EV Mimetics

Key Points	EVs	Engineered EVs	EV Mimetics
Advantages	<ul style="list-style-type: none"> i. High biocompatibility ii. Low immunogenicity iii. Low toxicity iv. Natural targeting v. Stability 	<ul style="list-style-type: none"> i. Retention of natural EV advantages ii. Enhanced targeting iii. Enhanced loading efficiency 	<ul style="list-style-type: none"> i. Combination of advantages of natural EV and synthetic materials ii. Extended half-life iii. Scalability iv. Controllable cost v. Potential for large-scale production vi. Personalization
Disadvantages	<ul style="list-style-type: none"> i. Limited cargo carrying capacity ii. Limited natural targeting iii. Limited production capacity iv. Difficult to isolate and purify v. Short half-life vi. High heterogeneity 	<ul style="list-style-type: none"> i. Weakness of some natural EVs ii. Complicated process iii. Possible introduction of immunogenicity iv. Uncertainty of long-term safety 	<ul style="list-style-type: none"> i. High toxicity ii. High immunogenicity iii. High production cost iv. High design difficulty
Challenges	<ul style="list-style-type: none"> i. Unknown metabolic mechanism in vivo ii. Large batch-to-batch variation iii. Difficulty in large-scale production iv. Lack of production and quality control standards 	<ul style="list-style-type: none"> i. Difficulty in large-scale production ii. Lack of production and quality control standards iii. Ethical controversy of gene modification iv. Targeted modifications need to be optimized 	<ul style="list-style-type: none"> i. Difficult to assess the toxicity of synthetic components ii. Long-term biological safety iii. Long clinical transformation period iv. Lack of production and quality control standards
Optimizations	<ul style="list-style-type: none"> i. Improve isolation purity ii. Standardize production iii. Establish sound production and quality control standards 	<ul style="list-style-type: none"> i. Optimize modification techniques ii. Establish sound production and quality control standards iii. Reduce immunogenicity iv. Improve safety 	<ul style="list-style-type: none"> i. Material biomimetic design ii. Functional modularization and integration iii. Optimize materials to reduce immunogenicity and toxicity. iv. Establish sound production and quality control standards

standards are being proposed and progressively refined through clinical trials. For instance, Humbert et al eliminated donor heterogeneity by utilizing identical human induced pluripotent stem cell (hiPSC) donors, enabling the generation of highly reproducible clinical-grade product batches.²⁷⁴ By controlling key process parameters (seeding density, medium composition, cell handling, incubation time, and temperature), they standardized the production workflow from hiPSC-derived cardiovascular progenitor cells (CPCs) vesiculation to product purification, concentration, and sterile filtration, and finally achieved excellent batch consistency between preclinical and clinical-scale batches.²⁷⁴ For characterization, advanced instrumentation such as ImageStream, Amnis, ZetaView, and ONI Nanoimager enables detailed multi-parametric analysis of EV concentration, particle size, zeta potential, and specific exosomal markers, offering a potential integrated alternative to traditional, often standalone methods like flow cytometry, Western blotting, dynamic light scattering, and nanoparticle tracking analysis.²⁷⁷

Furthermore, the integration of artificial intelligence (AI) with nanotechnology is propelling nanoscale diagnostic and DDSs toward more profound precision medicine. AI primarily encompasses the subsets of deep learning (DL) and machine learning (ML). DL is applied to predict interactions between nanocarriers and biological systems²⁷⁸ and to design ligands for precise targeting.²⁷⁹ Researchers have already employed such approaches to engineer iron oxide nanoparticles for targeted, intelligently controlled release in cancer therapy.²⁸⁰ ML algorithms analyze structural features of nanocarriers to predict their physicochemical properties and biological interactions, efficiently processing multi-dimensional data from EV multi-omics, imaging, and biophysics—patterns often unrecognizable by traditional statistical methods.²⁸¹ Simultaneously, these models can predict individual patient drug responses to customize dosage regimens and release mechanisms, aiming to minimize resistance and adverse effects while enhancing therapeutic efficacy. Through hierarchical modeling and clustering analysis, they can precisely distinguish EV subtypes and their functional differences, thereby helping to overcome heterogeneity bottlenecks. Bayesian parameter optimization and standardized data processing pipelines can further boost research and development and translational efficiency. The use of tools like SHAP and LIME helps decipher model decision-making principles, identifying key functional molecules to provide clear directions for rational EV engineering modifications.²⁸²

Although AI-driven EV DDSs represent a promising developmental trajectory, they remain constrained by data sparsity, insufficient model interpretability, and a lack of cross-platform standardization. Furthermore, current models often rely excessively on *in vitro* data, with insufficient validation of their predictions regarding batch consistency and efficacy correlation in clinical translation. Future efforts should focus on developing few-shot learning and self-supervised algorithms tailored to EV data characteristics, enhancing multimodal data integration and interpretability tools, while advancing federated learning frameworks combined with real-time analysis. By linking AI-driven process optimization with clinical outcome data, this integrated approach will enable the development of more efficient, safer, and personalized EV-based diagnostics and therapies.

Data Sharing Statement

The total number of 282 references can be found in PubMed.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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