

# Extracellular Vesicle-Based Therapeutic Cargo Delivery for Cancer Therapy

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**Abstract:** Extracellular vesicles (EVs) have become attractive nanoscale delivery systems in oncology because of their inherent biological advantages and distinct physicochemical properties. These cell-derived nanoparticles exhibit low immunogenicity, inherent targeting capacities, and the natural ability to facilitate precise intercellular transfer of bioactive molecules, including proteins and nucleic acids. Recently, remarkable progress has been made in EV engineering. These advancements have improved the accuracy and efficiency of delivering therapeutic agents to tumor sites. In this review, we outline the biogenesis, composition, and unique advantages of EVs compared with conventional nanocarriers, with an emphasis on their therapeutic potential in cancer treatment. Furthermore, we discuss EV cargo-loading methods and advanced engineering approaches designed to enhance tumor-specific targeting. In addition, we systematically summarize diverse therapeutic applications, including chemotherapeutic drug delivery, nucleic acid-based therapies, cancer vaccines, and multifunctional theranostic platforms. The review also discusses current clinical trials and highlights the critical challenges in clinical translation, such as limited clinical-scale manufacturing and unclear regulatory pathways. Future efforts to overcome these challenges could transform EVs into precise and personalized tools for cancer treatment. This concise overview provides valuable insights into the current developments and future perspectives of EV-mediated therapeutic delivery systems in oncology.

**Keywords:** nanocarriers, drug loading, targeted delivery, immunotherapy, clinical translation

## Introduction

Despite significant advancements in treatment, cancer remains a major health issue worldwide.<sup>1</sup> Although current cancer treatments (eg, chemotherapy, radiotherapy, targeted therapy, and immunotherapy) have substantially improved patient survival, they face several challenges, including severe systemic toxicity, limited tumor specificity, and therapeutic resistance, underscoring the need for safer, more precise, and effective strategies for the targeted delivery of therapeutic cargo in oncology.<sup>2</sup>

To address these limitations, nanoparticle-based drug delivery systems (eg, liposomes, polymeric nanoparticles, and other synthetic nanocarriers) have been extensively explored.<sup>3,4</sup> These systems can encapsulate drugs and preferentially accumulate in tumors via enhanced permeability or targeting ligands, reducing off-target effects.<sup>5,6</sup> Emerging porous and hollow nanomaterials, such as mesoporous silica and metal-organic frameworks, have expanded this toolkit: their high surface areas and tunable pore sizes enable exceptional drug loading and controlled release.<sup>7</sup> For instance, hollow polydopamine nanobowls encapsulate tirapazamine and tether MoS<sub>2</sub> nanozymes, enabling high-capacity delivery that suppresses tumor growth and limits metastasis via coordinated multimodal mechanisms.<sup>8</sup> Similarly, advanced polymeric nanoparticles have progressed considerably. Biodegradable polymers (eg, PLGA) enable precise control over particle stability, degradation rates, and surface functionalization for active targeting.<sup>9</sup> Despite these advances, the use of

**Table 1** Key Features of EVs from Different Cellular Origins

EVs Origin	Intrinsic & Delivery Traits	Reference
Dendritic cell	Antigen presentation with peptide MHC, lymph node drainage; immune activation; vaccine potential	[19]
MI macrophages	Proinflammatory and tumoricidal, innate immune activation, antitumor microenvironment reprogramming	[20]
Natural killer cell	Innate tumor targeting, enriched in NK effector molecules, selective tumor apoptosis with limited normal-cell damage	[21]
Mesenchymal stromal cell	Low immunogenicity, stromal/tumor tropism, barrier translocation, paracrine immunomodulation	[22]
Tumor cell	Organotropism, enriched tumor antigens/neoantigens (vaccine substrates)	[23]
Red blood cell	Low immunogenicity, prolonged bloodstream residence, favorable tolerability	[24]
HEK293 cell	Improved immunological compatibility, engineering friendly, versatile for cargo loading and ligand display	[25]

**Abbreviation:** MHC, Major histocompatibility complex.

synthetic nanoparticles faces challenges, including rapid clearance by the mononuclear phagocyte system, limited barrier penetration, potential immunogenicity, and inefficient cellular uptake.<sup>10–13</sup>

In this context, extracellular vesicles (EVs) have attracted considerable attention as promising bio-derived nanocarriers.<sup>14</sup> EVs are naturally occurring lipid bilayer-bound vesicles that are secreted by virtually all cell types, and their properties differ by cellular source (Table 1 summarizes EVs from common cellular origins and their key features). EVs can be classified into the following subtypes: exosomes (30–150 nm), microvesicles (100–1000 nm), and apoptotic bodies (up to several microns).<sup>15–17</sup> Among them, exosomes and microvesicles have attracted considerable interest because of their nanoscale dimensions, excellent biocompatibility, low immunogenicity, and intrinsic ability to mediate targeted intercellular communication.<sup>18</sup> These native features position EVs as a promising alternative to conventional nanocarriers for therapeutic delivery in oncology, with the potential to overcome key limitations associated with current cancer therapies.

In this review, we outline the biogenesis and molecular composition of EVs and discuss cargo-loading strategies and surface engineering approaches to improve tumor-specific targeting. Furthermore, we evaluate the diverse therapeutic applications of engineered EVs, from the targeted delivery of chemotherapeutics and nucleic acids to cancer vaccines and multimodal theranostics. Finally, we summarize the current efforts toward clinical translation, highlight ongoing challenges, and provide insights into future opportunities for advancing EV-based precision cancer therapy.

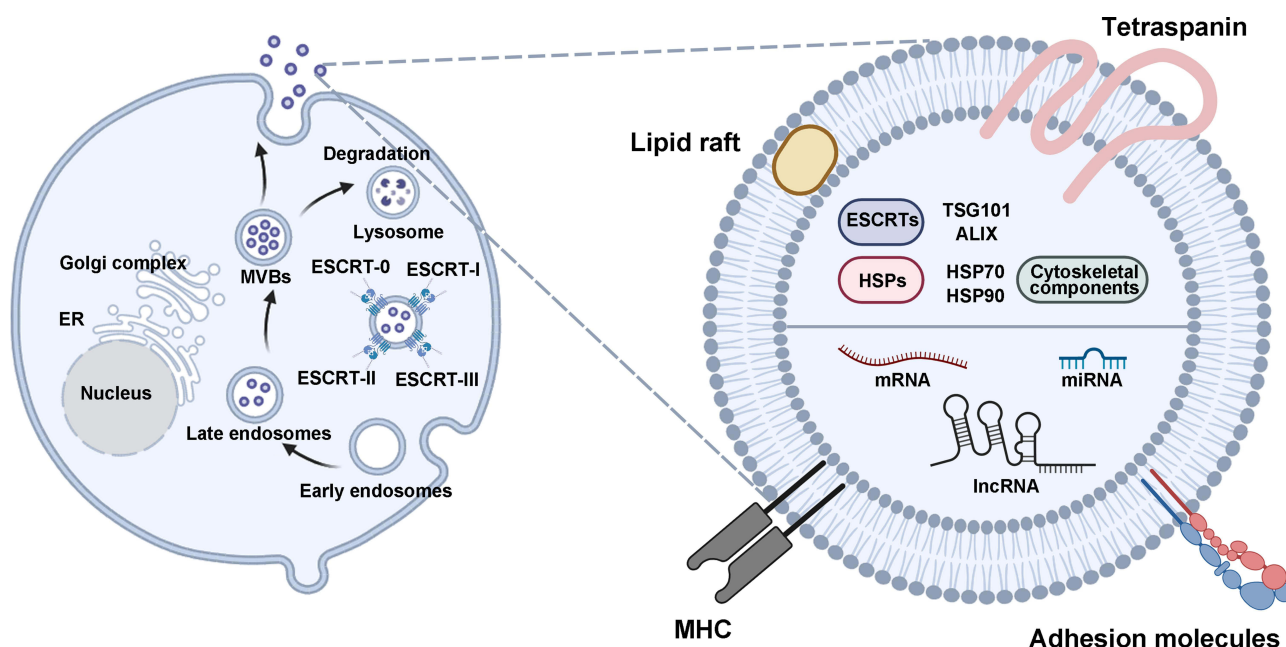
## EVs: A Concise Overview

### Biogenesis of EVs

The biogenesis of EVs involves sequential membrane remodeling events within the endosomal network (Figure 1). This process begins with the endocytosis of plasma membrane components, which generates vesicular structures that fuse to form early endosomes. As these endosomes mature into late-stage compartments, inward membrane invagination leads to the formation of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs). As exosome precursors, ILVs are either degraded lysosomally or released extracellularly upon the fusion of MVBs with the plasma membrane.<sup>26,27</sup> The formation of ILVs is predominantly controlled by the endosomal sorting complex required for transport (ESCRT) machinery. ESCRT-0, ESCRT-I, and ESCRT-II recognize and sequester ubiquitinated cargo, whereas ESCRT-III facilitates membrane remodeling and ILV scission, in coordination with accessory proteins (eg, VPS4 and ALIX).<sup>28,29</sup> Aside from the ESCRT-dependent pathway, there are alternative mechanisms, which involve ceramides, tetraspanins, and Rab GTPases that contribute to ILV formation and cargo sorting.<sup>26</sup> The interplay between these pathways determines the composition and functional properties of EVs, which vary depending on the cell type and physiological context.

### Composition of EVs

EVs have a unique molecular composition that reflects their cellular origin and biogenesis pathways. This composition enables EVs to effectively facilitate intercellular communication and serve as promising therapeutic delivery vectors.<sup>30</sup> EVs are structurally encapsulated by a lipid bilayer, which primarily comprises cholesterol, sphingomyelin, and



**Figure 1** EVs are formed through endosomal pathways, originating from early endosomes that mature into multivesicular bodies (MVBs) before release. These vesicles contain diverse array of membrane-associated proteins, lipids, and nucleic acids.

phosphatidylcholine that account for approximately 80% of the total lipids and confer membrane rigidity and stability. This bilayer preserves vesicular integrity, safeguards internal cargo, and facilitates interactions with target cells.<sup>31</sup> Membrane-associated proteins are key determinants of EV functionality. EVs are enriched in tetraspanins (eg, CD9, CD63, and CD81), which contribute to vesicle formation, membrane organization, and cellular internalization.<sup>32,33</sup> Moreover, they contain adhesion molecules, particularly integrins that facilitate target cell recognition and tissue-specific distribution. Among them,  $\alpha 6 \beta 4$  and  $\alpha 6 \beta 1$  promote lung tropism, whereas  $\alpha v \beta 5$  mediates liver-specific targeting.<sup>34,35</sup>

In addition, the intravesicular cargo contains various biomolecules, including heat shock proteins (eg, HSP70 and HSP90), ESCRT-associated proteins (TSG101 and ALIX), and cytoskeletal components. Collectively, these molecules maintain vesicular stability and regulate cargo trafficking.<sup>36</sup> EVs also contain nucleic acids, including microRNAs (miRNAs), long noncoding RNAs, and messenger RNAs (mRNAs), which modulate gene expression in recipient cells.<sup>37</sup> Given their molecular heterogeneity and natural ability to protect and transport bioactive molecules, EVs have a significant potential as therapeutic delivery vehicles.<sup>38</sup>

## EV Isolation and Characterization

EVs can be isolated through various strategies that leverage differences in size, density, or surface markers. Common isolation methods include differential ultracentrifugation, density gradient centrifugation, polymer-based precipitation, membrane ultrafiltration, size-exclusion chromatography, immunoaffinity capture, and emerging microfluidic-based techniques, each with distinct yield–purity tradeoffs.<sup>39</sup> The key attributes of these methods are summarized in Table 2. After isolation, EV preparations are characterized to confirm vesicle size, morphology, and marker profiles. Generally, nanoparticle tracking analysis is used to quantify particle concentration and size distribution.<sup>40</sup> Transmission electron microscopy is used to characterize EV morphology at the nanometer scale.<sup>40</sup> Western blotting is used to verify EV identity by detecting enriched markers (eg, CD63, CD81, and TSG101) and to assess purity by confirming the absence of cellular contaminants (eg, Golgi protein GM130).<sup>41</sup> Flow cytometry, often bead-assisted, is used to profile EV surface markers for phenotypic analysis.<sup>42</sup> Collectively, these complementary assays provide orthogonal evidence for establishing EV identity and purity.

**Table 2** Summary of EV Isolation Techniques

Isolation Method	Principle	Yield	Purity	Advantages	Disadvantages	Reference
Differential Ultracentrifugation	Sequential high-speed spins pellet EVs by size/density	Low–Moderate (some EV loss)	Moderate	Widely used, scalable	Time-consuming, co-isolation of similarly sized contaminants	[43]
Density Gradient Centrifugation	Ultracentrifugation with a density medium (eg, sucrose) separates EVs by buoyant density	Low (multi-step process)	High	High purity, resolves subtypes	Labor-intensive	[43,44]
Polymer Precipitation	Hydrophilic polymers (eg, PEG) reduce EV solubility, causing EV aggregation and low-speed co-precipitation	High (efficient particle recovery)	Low	Simple; high yield	Non-specific protein contamination	[45]
Ultrafiltration	Size-based filtration using membranes (pore size < EV diameter) to retain EVs while smaller molecules pass through	Low (EVs adsorb to filter)	Low	Fast	Filter clogging, EV loss	[46]
Size-Exclusion Chromatography (SEC)	Porous beads separate particles by size: EVs (larger than pores) elute first, while smaller molecules are delayed	Moderate–High	High	Gentle, preserves integrity	Dilution, limited volume	[47]
Immunoaffinity Capture	Antibodies or ligands bind specific EV surface markers to pull down target EV subpopulations (often on magnetic beads)	Low (only marker-positive EVs captured)	Very High	Specific	Costly, limited yield	[39]
Microfluidic Isolation	Miniaturized chip devices integrating size-based, affinity, or acoustic separation to sort EVs from small samples	Low–Moderate (small sample input)	Moderate	Rapid, small sample input	Low scale, device-dependent	[48]

## Cellular Uptake and Trafficking of EVs

Cells internalize EVs via diverse endocytic pathways, including clathrin-mediated endocytosis, caveolin/lipid raft-dependent uptake, macropinocytosis, phagocytosis, and, occasionally, direct plasma membrane fusion.<sup>49,50</sup> Multiple uptake mechanisms can operate simultaneously within a given EV population.<sup>49</sup> Following endocytosis, EVs typically traffic to endosomal compartments and often accumulate in late endosomes or lysosomes. Many internalized EVs are degraded in lysosomes or recycled to the cell surface; however, a fraction can escape the endolysosomal system to deliver functional cargo into the cytosol.<sup>51</sup> For instance, some EVs fuse with endosomal membranes in an acidification-dependent manner and release their RNA and protein cargo into the cytoplasm.<sup>51</sup> Understanding these pathways is crucial for designing therapeutic EVs. Surface modifications can considerably alter EV biodistribution. For example, precoating EVs with albumin forms a “protein corona” that shields them from phagocytes and redirects uptake toward target liver cells, thereby prolonging circulation and enhancing delivery efficacy.<sup>52</sup>

## Unique Features of EVs as Nanocarriers

### Enhanced Circulation and Immune System Compatibility

As cell-derived vesicles, EVs demonstrate extended circulation and low immunogenicity *in vivo*.<sup>53</sup> Moreover, their plasma membrane-like composition (eg, displaying the “self” marker CD47) enables them to evade phagocytic clearance by monocytes, thereby prolonging their half-life in the bloodstream.<sup>54</sup> Unlike conventional nanocarriers, which frequently trigger an immune reaction or require PEGylation to avoid rapid uptake by the mononuclear phagocyte system,<sup>55,56</sup> EVs inherently “fly under the radar” of the immune system, allowing more drugs to reach the tumor site.<sup>57</sup> This immune compatibility also translates to decreased risks of adverse immune reactions compared with synthetic nanoparticles.

### Advanced Cell-Specific Targeting

EVs have innate tissue-homing ability mediated by parent cell-derived adhesion molecules and cognate receptor–ligand pairs.<sup>33,58</sup> Notably, integrin profiles on tumor-derived EVs direct accumulation toward metastasis-prone organ sites, whereas immune cell-derived EVs preferentially target lymphoid tissues.<sup>23</sup> This endogenous targeting capacity

enables efficient delivery of therapeutic cargo to specific cell types, often surpassing that of synthetic nanocarriers, which require exogenous ligands, stimuli-responsive designs, or external fields to achieve similar specificity.<sup>59</sup> For example, a previous study reported that EVs delivered RNA interference molecules to pancreatic cancer cells (PCCs) more effectively than liposomes.<sup>54</sup> Such natural tropism enables EV-based nanocarriers to achieve high uptake in target tumor cells while sparing normal tissues, surpassing the targeting selectivity of liposomes, polymeric nanoparticles, and micelles.<sup>60</sup>

## Superior Biological Barrier Penetration

Compared with many synthetic nanocarriers, EV-based carriers can traverse physiological barriers, including the blood–brain barrier (BBB), because of their nanoscale dimensions and native membrane protein composition.<sup>61,62</sup> These properties enable EVs to deliver therapeutics to sanctuary sites (eg, brain tumors or metastases) where liposomes and polymeric micelles show limited accumulation.<sup>63</sup> Moreover, EV membranes are fluid and dynamic, facilitating fusion or uptake across cell–cell junctions. Thus, EVs frequently achieve comparable or superior tissue penetration and deep tumor infiltration than conventional nanocarriers, facilitating drug delivery to otherwise inaccessible microenvironments.

## Intrinsic Biocompatibility and Versatile Payload Capacity

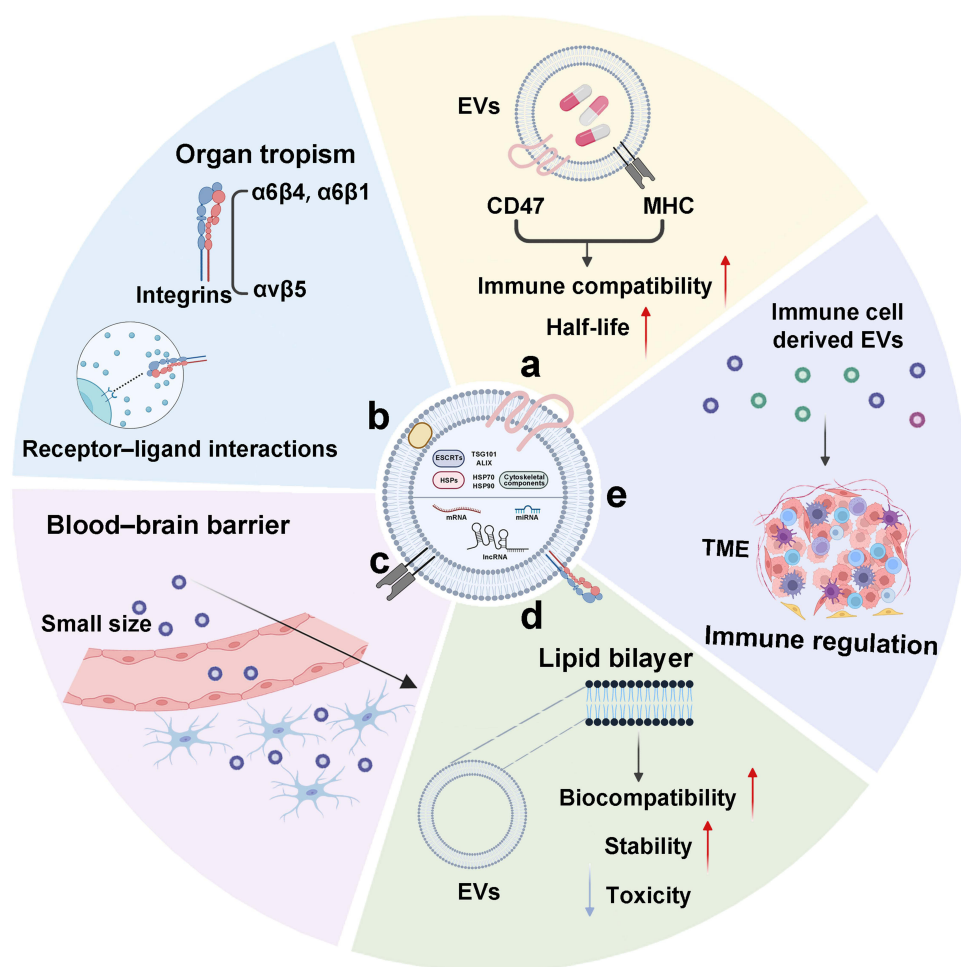
EVs exhibit excellent biocompatibility, high stability, and minimal toxicity, as they are derived from natural cell membranes.<sup>64</sup> Moreover, they are well tolerated by the body and can protect therapeutic cargos from degradation. Compared with conventional nanocarriers, EVs can accommodate various payloads, from small-molecule drugs to large macromolecules (eg, small interfering RNA [siRNA], miRNA, proteins, and even CRISPR complexes), without compromising the cargo's bioactivity.<sup>65–67</sup> The lipid bilayer of EVs shields therapeutics from enzymatic degradation and premature clearance, enabling stable delivery of diverse treatments.<sup>55</sup> This versatility and favorable safety profile provide EVs a distinct advantage in delivering complex combination therapies that challenge synthetic nanoparticle systems. [Figure 2](#) summarizes these features, highlighting the key properties of EVs that enhance their effectiveness as therapeutic nanocarriers.

## Integrated Immunomodulation and Immune Activation

In addition to passive drug delivery, EVs are unique bioactive carriers that can actively regulate immune responses. Different EV subtypes elicit distinct immunological effects. M1 macrophage–derived EVs carry proinflammatory cytokines and miRNAs that activate innate immunity and enhance antitumor responses,<sup>68</sup> whereas M2 macrophage–derived EVs convey anti-inflammatory signals suited that can reprogram inflamed milieu.<sup>69</sup> For instance, macrophage–derived EVs can function as natural adjuvants, with preserved macrophage-lineage receptors on their surface engaging antigen-presenting cells (APCs) and enhancing their activation.<sup>70</sup> Dendritic cell–derived EVs serve as potent antigen-presenting vesicles, displaying peptide–MHC complexes and costimulatory ligands that strongly activate T cells and natural killer (NK) cells;<sup>71</sup> when engineered to co-display IL-12 and anti-CTLA-4, they further drive Th1 polarization while lifting checkpoint restraint.<sup>72</sup> T cell–derived EVs likewise carry immunoregulatory miRNAs and cytokines that can dampen or steer immune reactions. In aggregate, EVs' endogenous interactions with specific cell types, together with configurable surface and cargo features, enable synchronous, multidimensional immunomodulation in the tumor microenvironment (TME), which is rarely achieved by conventional nanocarriers.

## EV-Based Cargo Loading Strategies

Successful incorporation of pharmaceutical agents into these nanoscale vesicles determines the therapeutic efficacy of EV-based drug delivery systems. Contemporary loading methods can be systematically categorized into endogenous and exogenous approaches ([Figure 3](#)), with comparative details tabulated in [Table 3](#).



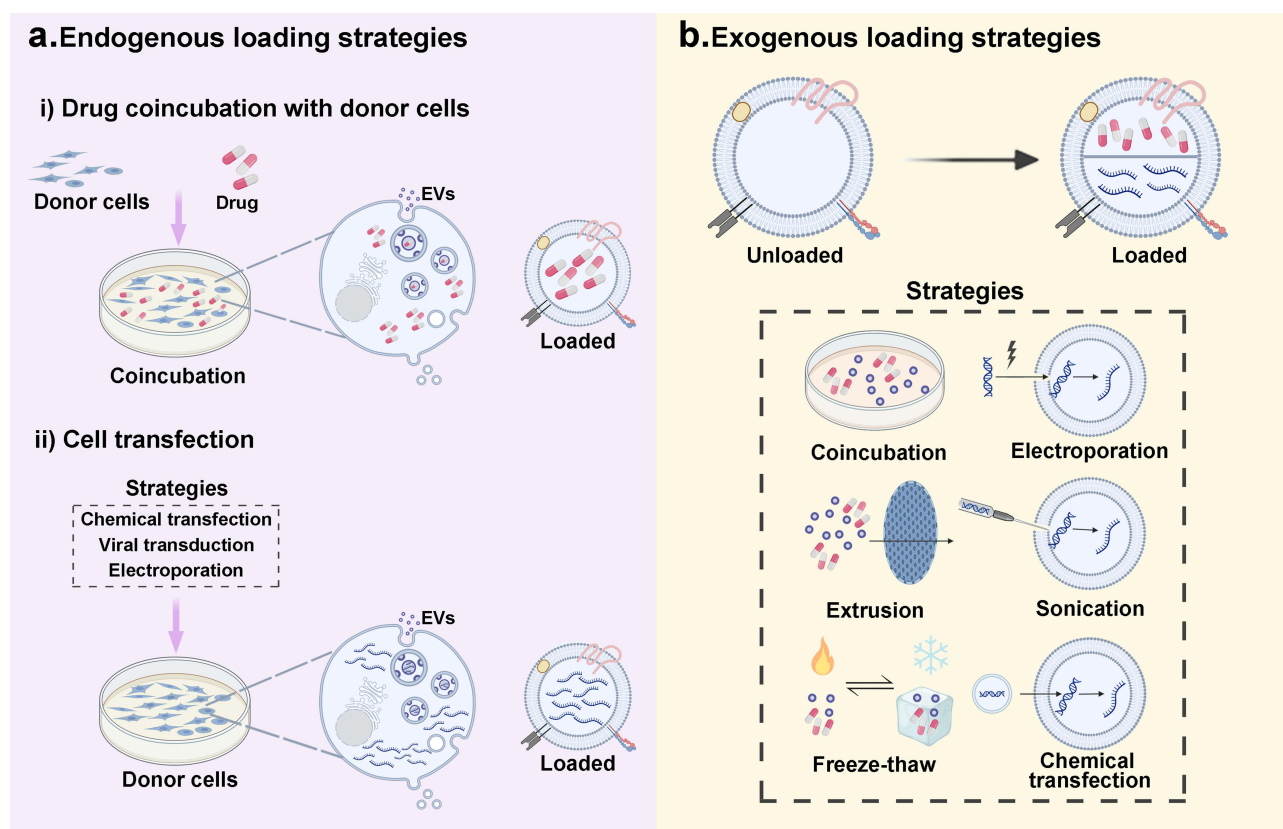
**Figure 2** Key biological advantages of EVs as carriers for therapeutic cargos. (a) Surface molecules such as CD47 and MHC enhance circulation half-life and immune compatibility, reducing immune clearance. (b) EVs achieve specific cellular targeting through integrin-mediated organ tropism and precise receptor–ligand interactions, facilitating selective therapeutic delivery. (c) Their small size and native surface proteins enable effective penetration across biological barriers, including the blood–brain barrier. (d) EVs possess a lipid bilayer membrane, providing inherent biocompatibility, low toxicity, and versatile cargo-loading capabilities. (e) Immune cell–derived EVs convey antigenic and immunostimulatory cues that modulate and remodel the tumor microenvironment (TME). Symbols: red upward arrow (↑) indicates increase; blue downward arrow (↓) indicates decrease.

## Endogenous Loading Strategies

Endogenous loading incorporates therapeutic cargo into EVs by modulating the intracellular concentrations of target molecules prior to EV biogenesis.<sup>73</sup> This strategy exploits cells' natural sorting mechanisms, whereby elevated cytoplasmic levels of specific therapeutic agents (eg, small-molecule drugs, proteins, and nucleic acids) result in their preferential enrichment during vesicle formation.<sup>74</sup> This physiological loading approach preserves the inherent structure and functional properties of EVs by avoiding post-isolation modifications that can compromise therapeutic efficacy.

## Drug Coincubation with Donor Cells

In this approach, target drug molecules are cultured with EV-producing donor cells, allowing spontaneous incorporation of therapeutics during EV biogenesis.<sup>75</sup> Pessina et al demonstrated that MSCs internalized paclitaxel (PTX) via passive uptake and secreted PTX-loaded EVs without genetic modification.<sup>76</sup> The protocol exposed MSCs to high PTX concentrations, yielding efficient drug encapsulation. The PTX-loaded EVs demonstrated significant antitumor activity, making them promising biomimetic drug delivery vehicles.<sup>76</sup> In subsequent studies, this approach was expanded to various small-molecule therapeutics, including doxorubicin (DOX) and dexamethasone, using analogous coculture protocols.<sup>77,78</sup>



**Figure 3** EV cargo-loading strategies. (a) Endogenous: donor cells are drug-exposed or genetically manipulated (chemical transfection, viral transduction, electroporation) so cargos are packaged into secreted EVs. (b) Exogenous: cargos are introduced into isolated EVs via coincubation, electroporation, sonication, extrusion, freeze–thaw, or chemical transfection.

However, despite its simplicity, this approach exhibits limited loading efficiency and low process control. Loading outcomes are mainly determined by the physicochemical properties of the drug and donor cell attributes.<sup>79</sup> A systematic comparison of DOX loading across PCCs, macrophages (MØs), and pancreatic stellate cells showed distinct capacities.<sup>80</sup> Intriguingly, although PCC-derived EVs demonstrated superior drug loading efficiency, MØ-derived EVs exhibited enhanced antitumor efficacy despite having a lower drug content, highlighting the importance of donor cell selection in endogenous loading strategies.<sup>80</sup> In addition, cytotoxicity profiles should be carefully considered when selecting therapeutic agents to maintain donor cell viability and ensure sustainable EV production.<sup>81</sup>

**Table 3** EVs-Based Cargo Loading Strategies

Loading Strategy		Factors Affecting Loading Efficiency	Advantages	Disadvantages
Endogenous loading	Drug Coincubation with Donor Cells	Cell viability	Simple, preserves EV integrity	Lower loading efficiency, limited control
	Cell Transfection	Transfection efficiency, cell viability	Preserves EV integrity	Limited control, residual reagent cytotoxicity

(Continued)

**Table 3** (Continued).

Loading Strategy		Factors Affecting Loading Efficiency	Advantages	Disadvantages
Exogenous loading	Coincubation	Temperature, pH, incubating time	Simple, cost-effective	Lower loading efficiency
	Electroporation	Voltage intensity, pulse duration, buffer composition	High controllability, high loading efficiency	Potential membrane damage, risk of aggregation
	Sonication	Sonication intensity, duration, temperature	High loading efficiency	Risk of membrane damage, potential thermal degradation
	Extrusion	Extrusion times	High loading efficiency, homogeneous particle size	Risk of damaging EVs structure
	Freeze-Thaw	Number of cycles, temperature control	Simple	Lower loading efficiency, potential drug inactivation, risk of aggregation
	Chemical Transfection	Transfection reagent choice	High loading efficiency	Residual reagent cytotoxicity

### Cell Transfection

Cell transfection is an effective endogenous loading method for introducing therapeutic cargo into EVs. This strategy encompasses chemical transfection, electroporation, and viral transduction to deliver plasmid DNA or functional nucleic acids into donor cells, inducing the expression of specific therapeutic biomolecules.<sup>82</sup> Subsequently, the transfected cells package the synthesized molecules into EVs through inherent biogenesis pathways. Zhang et al used lentiviral transduction to generate induced neural stem cells that express tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), which produced TRAIL-functionalized EVs.<sup>83</sup> These synthesized EVs exhibited preferential membrane localization of TRAIL and enhanced loading efficiency compared with fibroblast-derived EVs (351 vs 187 pg TRAIL/ $\mu$ g EVs), demonstrating superior antineoplastic efficacy against cerebral malignancies.<sup>83</sup> Similarly, Kimura et al engineered bone marrow-derived mesenchymal stem cells to express microRNA-29b (miR-29b) using lentiviral vectors. The resultant miR-29b-enriched small EVs (sEVs), which were isolated through ultracentrifugation, demonstrated selective targeting to peritoneal mesothelial cells and effectively suppressed gastric cancer metastasis.<sup>84</sup>

Although cell transfection demonstrates considerable loading efficiency, the method has several technical challenges, including variable transfection rates, potential genotoxicity, and difficulties in accurately controlling the cargo-loading capacity, which depend on the cell viability and transfection outcomes.<sup>85</sup> Furthermore, transfection reagents have been reported to accumulate in late endosomes and be released into the extracellular space via exocytosis. These transfection complexes share similar physical properties with EVs and may co-sediment during purification, potentially contaminating EV preparations and altering their characteristics.<sup>86,87</sup>

### Exogenous Loading Strategies

Exogenous loading strategies, in contrast to endogenous approaches, use physicochemical methods to incorporate therapeutic cargo into pre-isolated EVs. This post-isolation modification strategy offers distinct advantages over endogenous approaches, particularly in terms of process standardization and manufacturing scalability.<sup>73</sup>

#### Coincubation

This method offers a gentle and straightforward approach for loading therapeutic agents into EVs. It relies on concentration-gradient-driven diffusion under physiologically relevant conditions to facilitate drug translocation across EV membranes.<sup>88</sup> The EV phospholipid bilayer exhibits a preferential affinity for hydrophobic compounds because of its amphipathic nature, enabling the efficient encapsulation of lipophilic therapeutic agents.<sup>89</sup> Yang et al achieved approximately 8% drug loading efficiency by simply mixing EVs with DOX and incubating the mixture at 37°C for 30 min.<sup>90</sup>

Despite its simplicity and cost-effectiveness, conventional coincubation has inherent limitations, particularly for hydrophilic drugs, proteins, and nucleic acids, because of their low membrane permeability.<sup>15</sup> To overcome these limitations, several strategic modifications have been developed. One approach involves the use of membrane-active agents, particularly saponins, to induce transient membrane permeabilization. However, surfactant exposure must be carefully optimized to prevent compromising vesicular integrity.<sup>91</sup> Another approach focuses on cargo modification to enhance membrane penetration. Zhang et al developed a novel approach using cell-penetrating peptides (CPPs) conjugated to DOX. They achieved a loading efficiency of 37.18% via the coincubation of CPP-DOX conjugates with urinary EVs under optimized conditions (pH 9.0, 37°C, 2 h), indicating a 2.5-fold enhancement compared with conventional methods. These functionalized EVs demonstrated superior antineoplastic efficacy.<sup>92</sup> The drug loading efficiency can also be improved by optimizing the incubation conditions. Several factors, including the drug concentration, incubation temperature, duration, and pH, can significantly affect the loading process.<sup>93,94</sup> Therefore, these parameters must be carefully adjusted based on the specific characteristics of the cargo and EV source.

## Electroporation

This advanced method uses high-intensity electric fields to induce the transient membrane permeabilization of EVs. This method involves suspending EVs and therapeutic cargo in an optimized electroporation buffer and then applying calibrated electric pulses to generate temporary nanopores, which facilitate cargo translocation.<sup>73</sup> This approach is particularly versatile for hydrophilic cargos, such as small molecules and nucleic acids, which typically have poor membrane permeability. Gong et al engineered uPA-sEVs-siSrc, an advanced drug delivery system that incorporates Src-siRNA into urokinase plasminogen activator (uPA)-functionalized sEVs.<sup>95</sup> This system achieved a 45% loading efficiency under optimized electroporation parameters (400 mV, 125  $\mu$ F) while preserving vesicular integrity and functionality, resulting in robust antitumor effects. Similarly, Tian et al successfully encapsulated DOX into immature dendritic cell (DC)-derived exosomes with 20% efficiency via electroporation.<sup>96</sup> The DOX-loaded exosomes effectively suppressed breast tumor growth and markedly reduced systemic toxicity and cardiotoxicity in MDA-MB-231 tumor-bearing mice.

The primary advantage of electroporation is its high controllability, enabling precise adjustment of experimental conditions for optimal loading. However, several technical challenges must be considered. In particular, excessive electric field strength can compromise membrane integrity, and RNA cargo aggregation during electroporation remains a significant challenge.<sup>97,98</sup> To mitigate these issues, key parameters, including voltage intensity and pulse duration, must be systematically optimized based on specific EV populations and cargo characteristics.<sup>99</sup> RNA aggregation can be mitigated by incorporating stabilizing agents (eg, trehalose, citric acid, and ethylenediaminetetraacetic acid) into the electroporation buffers, thereby enhancing the loading efficiency and cargo stability.<sup>31</sup>

## Sonication

This method employs acoustic energy and mechanical shear forces to transiently permeabilize EV membranes, facilitating the internalization of therapeutic molecules.<sup>82</sup> Subsequently, membrane integrity is restored through a standardized postsonication incubation protocol (37°C, 1 h).<sup>100</sup> Studies have shown that sonication-induced membrane perturbation significantly reduces the microviscosity of the EV membrane, thereby enhancing membrane fluidity and drug permeation.<sup>101</sup> Accordingly, ultrasound-assisted loading often outperforms simple incubation. For example, Kim et al achieved approximately 28.29% loading efficiency when using sonication to load PTX into RAW 264.7 M $\phi$ -derived EVs, which was higher than that achieved via incubation (4.10%).<sup>101</sup> Similarly, in another study on 5-fluorouracil encapsulation, sonication demonstrated superior loading capacity compared to incubation (6.79% vs 4.10%).<sup>102</sup>

Although sonication shows promising potential for efficient EV drug loading, sonication parameters should be optimized to maintain EV functionality. For example, the intensity and duration of acoustic energy exposure must be precisely controlled to maintain membrane integrity and biological activity. Sun et al reported higher PTX loading efficiency in sEVs via sonication than via electroporation and incubation methods.<sup>103</sup> Paradoxically, PTX loaded via incubation exhibited enhanced cytotoxicity against pancreatic ductal adenocarcinoma (PDAC) cells, indicating that

sonication-induced membrane alterations may impair cellular uptake mechanisms.<sup>103</sup> Furthermore, sonication requires rigorous temperature monitoring to prevent the thermal degradation of vesicular structures and therapeutic cargo.

### Extrusion

This technique uses calibrated nanoporous membranes (100–400 nm) and standardized liposome extruders to load drugs into EVs.<sup>94</sup> In this technique, transmembrane pressure gradients are used to induce controlled membrane destabilization, thereby facilitating drug translocation into the vesicular lumen.<sup>31</sup> Membrane elasticity and drug–membrane interactions predominantly govern the loading efficiency. Subsequently, unencapsulated drugs are eliminated through density gradient ultracentrifugation or dialysis to ensure homogeneity and purity of the preparation, yielding highly purified drug-loaded EVs. Bi et al successfully loaded PTX into H22 hepatocarcinoma-cell-derived EVs through serial extrusion across 200-nm porous membranes, achieving an encapsulation efficiency of 92.33%.<sup>104</sup> Compared with free PTX, the engineered EVPA exhibited enhanced antitumor efficacy against hepatocarcinoma *in vitro* and *in vivo*.<sup>104</sup>

Although extrusion demonstrates superior drug loading efficiency and enhances EV size homogeneity, repeated extrusion cycles can compromise the structural integrity of EVs and alter the composition of endogenous cargo through mechanical stress–induced membrane perturbation.<sup>94,105</sup>

### Freeze–Thaw

This method increases drug loading in EVs by inducing membrane microfractures through repeated freeze–thaw cycles. This technique involves rapidly freezing an EV–cargo mixture at  $-80^{\circ}\text{C}$  or in liquid nitrogen, followed by controlled thawing at room temperature.<sup>82</sup> Multiple cycles ( $\geq 3$ ) are used to maximize the drug encapsulation efficiency.<sup>82</sup> Ebrahimian et al used a freeze–thaw method combined with coincubation and surfactants to encapsulate thymoquinone into exosomes, effectively targeting MCF7 cancer cells and inducing apoptosis.<sup>106</sup>

Despite its simplicity, this method generally results in lower drug loading efficiency than sonication or extrusion methods.<sup>107</sup> Moreover, the use of this method in drug loading investigations is limited because of the potential for EV aggregation and the risk of drug inactivation from frequent freeze–thaw processes.<sup>79</sup>

### Chemical Transfection

Chemical transfection-mediated drug loading is a promising strategy for incorporating therapeutic molecules into EVs. Because various transfection reagents are commercially available, several EV-based drug delivery systems have been developed. Recent studies have demonstrated the efficacy of chemical transfection. For example, Xing Pei et al used the Exo-Fect reagent to load siFGL1 and siTGF- $\beta$ 1 into cRGD-functionalized EVs, achieving a loading efficiency of 97%.<sup>108</sup> The resulting EVs effectively downregulated FGL1 and TGF- $\beta$ 1 expression, thereby inhibiting colorectal cancer (CRC) progression. In a parallel study, Peng et al<sup>109</sup> achieved 70% loading efficiency by transfecting siSTAT3 into tumor-derived EVs (T-EVs) isolated from 4T1 triple-negative breast cancer (TNBC) cells. A dual-therapeutic delivery system with enhanced tumor targeting and improved efficacy in TNBC models was generated by coincubating DOX with siSTAT3-loaded T-EVs.<sup>109</sup>

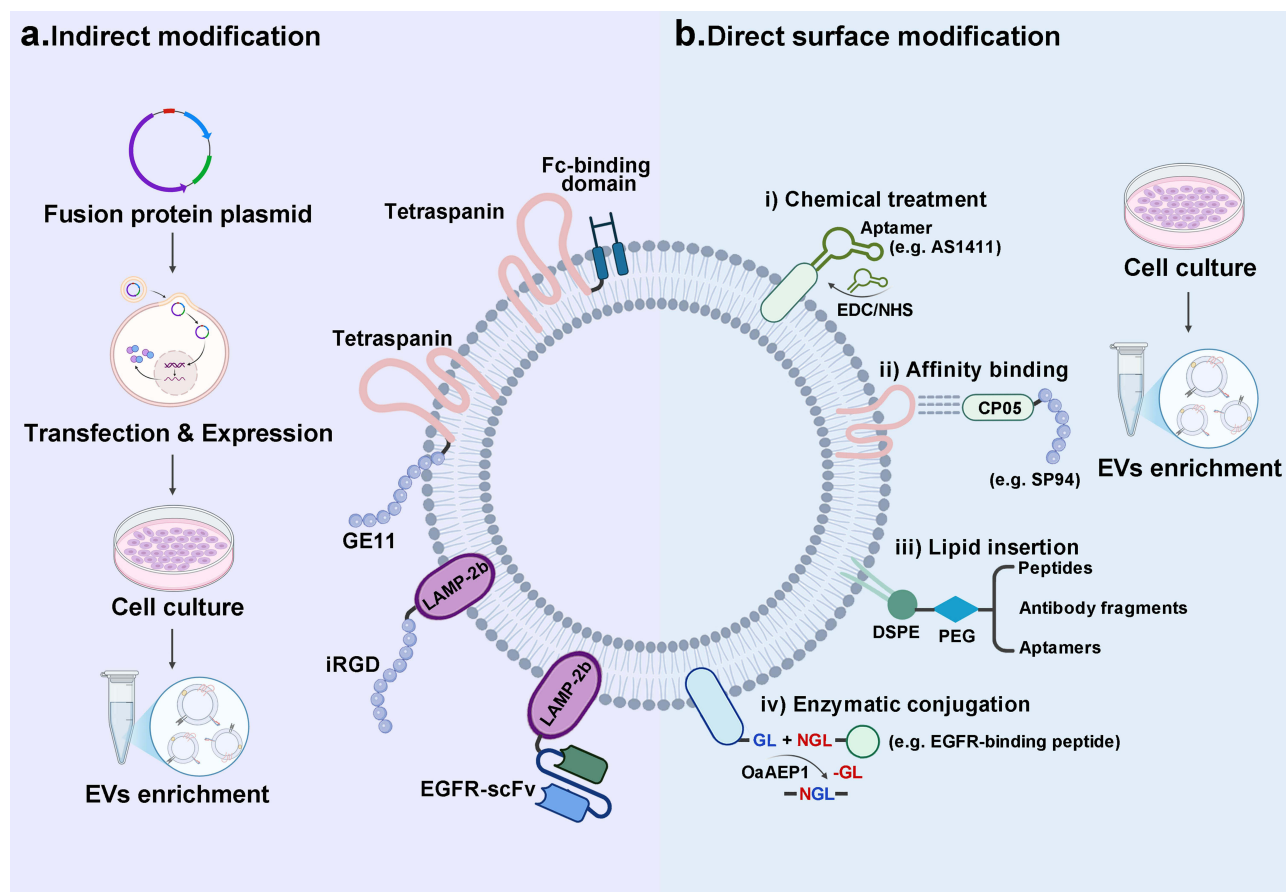
The potential cytotoxicity associated with the residual transfection reagents should be carefully considered. Moreover, comprehensive characterization studies are needed to fully elucidate the spatial distribution of the loaded therapeutic cargo (whether encapsulated within the EV lumen or surface-associated).<sup>110</sup>

## Engineered EVs for Enhanced Targeted Delivery

Although native EVs have desirable properties, they are being actively engineered to enhance their targeting ability and therapeutic efficacy in cancer treatment. In this section, several major engineering approaches are discussed and examples of how each method enhances EV-mediated drug delivery to tumors are provided. Representative EV engineering strategies are summarized in [Figure 4](#).

### Indirect Modification: Cell-Based Engineering

Targeted EV engineering can be achieved by genetically modifying donor cells via transfection by introducing engineered expression constructs. These constructs encode fusion proteins comprising targeting moieties that are genetically linked



**Figure 4** Surface modification strategies for targeted EV delivery. (a) Indirect Modification: Genetic modification of parent cells by plasmid transfection to express targeting molecules incorporated into EV membranes. (b) Direct Surface Modification: Surface functionalization of isolated EVs. Methods include i) chemical conjugation, using EDC/NHS to covalently attach ligands such as the ASI411 aptamer; ii) affinity binding, CP05 recognizes CD63 on EVs and anchors CP05–ligand conjugates for noncovalent surface display; iii) lipid insertion, incorporating DSPE-PEG–linked peptides, antibody fragments, or aptamers into the outer leaflet; and iv) enzymatic conjugation, using OaAEP1 to ligate GL/NGL-tagged peptides for site-specific display.

to EV-enriched transmembrane proteins, which function as membrane anchors.<sup>111,112</sup> Among the available membrane-anchoring candidates, lysosome-associated membrane protein 2b (LAMP-2b) and tetraspanin family members (eg, CD9, CD63, and CD81) are frequently used because of their abundant expression and stable incorporation within the EV membrane.<sup>113,114</sup>

LAMP-2b is an optimal scaffold for EV surface modification as its N-terminal domain (29-amino-acid signal peptide) can tolerate genetic fusion with diverse targeting moieties (eg, peptides and antibody fragments) while maintaining efficient protein trafficking and membrane integration, thereby ensuring the stable surface display of the targeting molecules.<sup>79</sup> LAMP-2b-based modification has been extensively validated for its versatility. For example, the LAMP-2b-fused  $\alpha$  integrin–targeting iRGD peptide has been successfully expressed on HEK 293FT cell–derived EVs, which were subsequently loaded with DOX via electroporation.<sup>115</sup> These iRGD-modified EVs demonstrate a 40% enhancement in cellular uptake by glioblastoma multiforme (GBM) cells in vitro and exhibit significantly improved antitumor efficacy in vivo compared with their unmodified counterparts.<sup>115</sup> In another study, an epidermal growth factor receptor (EGFR)–specific single-chain variable fragment (scFv), which targeted the overexpressed EGFR on cancer cells, was successfully fused with LAMP-2b and expressed on HEK 293T cell–derived EVs.<sup>116</sup> The targeting ability of these engineered EVs, loaded with LPCAT1-specific siRNA, was evaluated in lung cancer brain metastasis (BM). In vivo biodistribution studies have demonstrated that the scFv-modified exosomes have significantly greater retention than unmodified variants, showing two-fold higher residual fluorescence in the BM region at 96 h postinjection. Moreover, these functionalized

exosomes efficiently traversed the BBB, resulting in the significant downregulation of LPCAT1 and subsequent inhibition of tumor cell proliferation in the BM model.<sup>116</sup>

Tetraspanins, which are a family of abundant EV transmembrane proteins, have also demonstrated efficacy in genetic surface engineering. Yang et al engineered HEK 293T cell-derived EVs by fusing the EGFR-targeting GE11 peptide with CD63.<sup>117</sup> These GE11-modified EVs, loaded with DOX, exhibited enhanced cellular uptake in EGFR-positive CRC and GBM cells, along with improved antitumor efficacy and reduced systemic toxicity in vivo. Wiklander et al engineered EVs by fusing the Fc-binding domains to CD63, CD9, and CD81, with the CD63 C-terminal fusion displaying optimal expression and targeting efficiency.<sup>118</sup> These Fc-EVs demonstrated versatile antibody decoration capabilities, achieving broad tissue targeting. In another study, trastuzumab (a HER2-targeting antibody)-decorated Fc-EVs exhibited a 339-fold increase in cellular uptake by HER2-positive breast cancer cells (SKBR-3), and Fc-EVs functionalized with PD-L1 antibodies demonstrated comparable targeting enhancement toward PD-1-expressing cells.<sup>118</sup>

## Direct Surface Modification of Isolated EVs

In direct modification strategies, isolated EVs are functionalized by attaching targeting molecules or ligands onto the EV surface using diverse conjugation approaches.

### Chemical Treatment

Chemical modification strategies use reactive chemical groups to covalently attach targeting molecules to the EV surface.<sup>119</sup> Functional groups on EV membranes—such as amines on surface proteins or lipids (eg, phosphatidylethanolamine), carboxyl groups, and thiols—can be exploited for bioconjugation.<sup>120,121</sup> Targeting ligands can be permanently grafted onto EVs using linkers or via click chemistry. Generally, chemical ligation methods are more robust and stable than noncovalent affinity binding, although with a greater risk of altering the native structure or functions of the vesicles.<sup>122,123</sup> Current bioconjugation approaches frequently use 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC)/N-hydroxysuccinimide (NHS)-mediated amide bond formation and azide–alkyne cycloaddition reactions.<sup>124</sup> For example, Hosseini et al demonstrated the selective conjugation of the AS1411 aptamer to exosomal surface amines via EDC/NHS chemistry, enabling the specific recognition of nucleolin, which exhibits increased expression on CRC cells.<sup>125</sup> This surface engineering strategy markedly enhanced the targeting specificity and efficiency of therapeutic cargo delivery of functionalized exosomes.

### Affinity Binding

Affinity-based EV targeting relies on the selective recognition and binding of specific affinity molecules to membrane proteins, receptors, or distinct lipid molecules on the EV surface.<sup>126</sup> This noncovalent approach requires only mild incubation conditions for surface functionalization, thereby preserving the structural integrity of EVs while offering operational simplicity compared with chemical conjugation strategies. However, the stability of affinity-based modifications may be compromised under physiological conditions. Thus, environmental factors must be carefully considered.

CD63 functions as a canonical EV marker while being a highly enriched tetraspanin protein in EV membranes.<sup>127</sup> Gao et al identified CP05, an affinity peptide that specifically targets the second extracellular loop of CD63, providing a versatile platform for EV modification.<sup>128</sup> Notably, CP05 demonstrates a universal binding capability across EVs from diverse sources while preserving their structural and functional integrity. The versatility of CP05 as a modifiable targeting peptide facilitates the straightforward surface functionalization of exosomes. For example, exosome surface decoration using CP05 conjugated to SP94, a hepatocellular carcinoma (HCC)-targeting peptide, markedly enhanced tumor accumulation compared with unmodified exosomes in murine HCC models.<sup>128</sup>

In addition to peptides, other affinity pairs have been exploited. For example, reticulocyte-derived EVs inherently express transferrin receptors (TfR) on their surface, thereby enabling targeted functionalization through specific transferrin (Tf)–TfR interactions.<sup>129</sup> Qi et al functionalized reticulocyte-derived EVs were functionalized with Tf-conjugated superparamagnetic nanoparticles via selective Tf–TfR binding interactions, which conferred enhanced magnetic responsiveness to the EV carriers and facilitated targeted delivery to tumor sites.<sup>129</sup>

## Lipid Insertion

The lipid bilayer membrane structure of EVs facilitates the incorporation of hydrophobic modifying molecules. The biocompatible amphiphilic polymer 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol) (DSPE-PEG), approved by the Food and Drug Administration (FDA), serves as a prevalent surface modification agent for EVs.<sup>130</sup> DSPE-PEG conjugates feature a hydrophobic DSPE tail that inserts into the membrane and a PEG spacer that presents the ligand and enhances stealth by reducing protein adsorption.<sup>126,131</sup> The versatile PEG terminus enables the conjugation with various targeting moieties, including peptides, antibody fragments, or aptamers, facilitating diverse targeting strategies.<sup>95,132,133</sup> Recently, Zhou et al successfully incorporated integrin  $\alpha 5$ -targeting peptide (CRYRITY) into EVs through DSPE-PEG modification, leveraging the spontaneous insertion of DSPE into the lipid bilayer.<sup>134</sup> This targeted modification substantially enhanced EV specificity toward cancer-associated fibroblasts (CAFs) in pancreatic cancer, resulting in improved CAF-specific uptake and increased tumor site accumulation compared with unmodified EVs.<sup>134</sup>

## Enzymatic Conjugation

Enzymatic surface functionalization is an emerging EV modification strategy that uses specific ligases to precisely conjugate targeting molecules to the EV membrane. For example, protein ligases, such as Sortase A or OaAEP1 (an asparaginyl endopeptidase), can recognize short peptide motifs on EV surface proteins and covalently attach a ligand peptide at that site. This strategy allows precise EV functionalization with stable, high-density targeting ligands while maintaining vesicle integrity through gentle, site-specific modifications. Pham et al employed OaAEP1 ligase to display an EGFR-targeting (ET) peptide on red blood cell-derived EVs. The ligase catalyzed the covalent attachment of a biotinylated ET-NGL peptide to GL-containing EVs by recognizing the C-terminal NGL motif of the peptide and the N-terminal GL motif on the EV surface. This process facilitated the conjugation of 380 EGFR-binding peptide copies per vesicle to generate multivalent targeted delivery vehicles.<sup>135</sup> These EGFR-targeted EVs, loaded with PTX and administered to EGFR-positive lung-tumor-bearing mice, exhibited significantly enhanced tumor accumulation and superior antineoplastic efficacy compared with their nontargeted counterparts.<sup>135</sup>

# Applications of Engineered EVs in Cancer Therapy

## Chemotherapeutic Drug Delivery

Chemotherapy remains a cornerstone of cancer treatment; however, conventional chemotherapeutics are limited by broad cytotoxicity, poor solubility, and low bioavailability, which reduce their efficacy.<sup>88,136</sup> Engineered EVs offer a biocompatible delivery platform that can improve drug targeting and mitigate systemic toxicity.<sup>137</sup> Various anticancer agents have been loaded into EVs with promising preclinical results.

### Paclitaxel

PTX has been successfully delivered using EVs to overcome multidrug resistance (MDR) in tumors. The efficacy of PTX is often limited by P-glycoprotein efflux in resistant cancer cells.<sup>138,139</sup> Encapsulating PTX in macrophage-derived exosomes (termed exoPTX) can bypass these pumps, leading to higher intratumoral drug retention and potency. Kim et al demonstrated that exoPTX induced over 50-fold greater cytotoxicity in drug-resistant cancer cells *in vitro* than free PTX and markedly suppressed MDR tumor growth in mice.<sup>101</sup> Similarly, PTX-loaded exosomes from M1-polarized macrophages delivered the drug and reprogrammed the TME by activating NF- $\kappa$ B signaling in recipient cells, yielding greater tumor apoptosis and an inflammatory immune shift that synergized with chemotherapy.<sup>140</sup> Notably, recent studies have reported that EVs enhance intratumoral PTX penetration. Jafarpour et al demonstrated that mesenchymal stromal cell-derived exosomes loaded with PTX penetrated deeply into 3D breast cancer spheroids and induced significantly more cancer cell apoptosis than free PTX.<sup>141</sup> These findings indicate that EV carriers can overcome molecular resistance mechanisms and physical transport barriers in solid tumors. In addition, the surface functionalization of EVs can enhance PTX targeting. For example, PANC-1 tumor cell-derived exosomes were engineered with an RGD peptide and magnetic nanoparticles and loaded with PTX (rmExo-PTX). In a pancreatic cancer model, rmExo-PTX showed enhanced tumor accumulation and significantly reduced tumor size compared with those of free PTX.<sup>142</sup> These advances underscore the

ability of engineered EVs to enhance PTX efficacy by evading MDR, enhancing tumor specificity, and modulating the TME.

### Doxorubicin

Although DOX is a potent anthracycline, its clinical use is limited by dose-dependent cardiotoxicity.<sup>143</sup> EV-based delivery can concentrate DOX in tumors while minimizing cardiac exposure, thereby enhancing the therapeutic index. Moradi et al loaded DOX into exosomes conjugated with the SP5-52 peptide, which induced greater tumor inhibition than free DOX in vitro and in vivo, while markedly reducing cardiotoxicity in treated mice.<sup>144</sup> Xia et al developed iRGD-modified exosomes as carriers for DOX delivery, which effectively penetrated the BBB and targeted central nervous system lymphoma, achieving strong antitumor activity with minimal systemic toxicity.<sup>132</sup> These examples illustrate that EV surface engineering (eg, with tumor-homing peptides) can enhance DOX targeting to difficult-to-reach sites. Notably, the biocompatibility and innate homing ability of EVs allow repeated DOX dosing with less off-target accumulation in the heart. Overall, delivering DOX via engineered EVs has consistently achieved equal or superior antitumor efficacy compared with free DOX while markedly alleviating cardiomyopathy and other side effects.<sup>145</sup>

### Other Chemotherapeutics

Other chemotherapeutics, including gemcitabine, methotrexate, and plant-derived drugs (eg, curcumin), have also been incorporated into EVs in experimental settings, often with improved therapeutic indexes.<sup>146–148</sup> For example, methotrexate-loaded sEVs showed enhanced tumor accumulation, immune activation, and superior antitumor efficacy in a murine lung cancer model.<sup>148</sup> Cisplatin encapsulated in umbilical cord blood-derived M1 macrophage exosomes targeted ovarian tumors in vivo and reversed platinum resistance.<sup>149</sup> NK-cell-derived exosomes loaded with oxaliplatin exhibited selective tumor uptake and enhanced antitumor activity in murine CRC, consistent with FasL-mediated apoptosis and sustained release.<sup>150</sup> Autologous exosomes loaded with gemcitabine were selectively taken up by pancreatic tumors and reduced systemic toxicity, highlighting the potential of EVs to improve chemotherapy for pancreatic cancer.<sup>151</sup> Table 4 summarizes these and related studies.

## Nucleic Acid–Based Therapy

Delivering therapeutic nucleic acids to tumors remains challenging because of their instability and poor cellular uptake.<sup>162</sup> Free nucleic acids are rapidly degraded by nucleases and have difficulty crossing cell membranes.<sup>163</sup> These challenges can be addressed using EVs, which possess a protective lipid bilayer shell and innate targeting capabilities. EV encapsulation shields nucleic acids from enzymatic degradation in the bloodstream, prolonging their circulation and preserving bioactivity until delivery to target cells.<sup>164,165</sup> Moreover, EV membranes naturally fuse with recipient cells, facilitating efficient cytosolic delivery of the cargo. In the following subsections, we discuss engineered EV strategies for delivering various classes of nucleic acid therapeutics.

### siRNA

siRNA selectively silences disease-associated genes by promoting target mRNA degradation.<sup>166</sup> The primary challenge is delivering siRNA intact into tumor cells. EVs have emerged as effective siRNA carriers capable of crossing biological barriers and selectively targeting tumors. For example, Liu et al loaded anti-PD-L1 siRNA (siPD-L1) into M1 macrophage-derived EVs alongside a vesicular stomatitis virus G protein “pseudotype” to enhance EV uptake.<sup>152</sup> These engineered EVs successfully delivered siPD-L1 to colon carcinoma tumors, silencing PD-L1 expression in cancer and immune cells. The result was a repolarization of tumor-associated macrophages from an M2 (immunosuppressive) to an M1 (proinflammatory) phenotype and increased CD8+ T-cell infiltration, resulting in potent antitumor immune responses and tumor growth inhibition. In a recent study, Jiang et al targeted a metastasis-associated gene in lung cancer.<sup>116</sup> They electroporated an siRNA against LPCAT1 (an oncogenic factor promoting NSCLC progression) into HEK293T-derived exosomes decorated with an anti-EGFR nanobody for tumor targeting. Systemic administration of these exosomes in an orthotopic lung cancer model knocked down LPCAT1 in vivo, thereby suppressing tumor proliferation and substantially reducing brain metastatic lesions.<sup>116</sup> These examples showcase the precision of EV–siRNA strategies. By combining cell

**Table 4** Application of Engineered EVs in Cancer Therapy

Application	Cargo	EVs Origin	Loading Method	Loading/ Encapsulation Efficiency	Engineering	Cancer Type (Cell Lines)	Administration Route and Amount	Outcome	Reference
Chemotherapeutic Drug Delivery	PTX	Macrophages (RAW 264.7)	Sonication	LE: 28.29±1.38%	/	LLC lung metastasis (3LL-M27)	IN: 10 <sup>7</sup> particles/10 µL × 2, QOD for 7 doses	Inhibited tumor growth, reduced lung metastases, overcame MDR	[101]
	PTX	M1-polarized macrophages	Sonication	LE: 19.55±2.48%	/	Breast carcinoma (4T1)	IV: PTX-M1-Exos (5 mg/kg of PTX-equivalent), Q3D	Inhibited tumor growth, repolarized macrophages, enhanced immune response	[140]
	DOX	Serum from Balb/c mice	Sonication	LE: 30%; EE: 91%	SP5-52 peptide	Lung carcinoma (LL/2)	IV: EXO-SP-DOX (5 mg/kg DOX-equivalent), Q3D for 7 doses	Inhibited tumor growth, increased tumor accumulation, reduced systemic toxicity	[144]
	DOX	Human brain microvascular endothelial cells	Coincubation	/	iRGD peptide	Diffuse large B-cell lymphoma (U2932)	IV: iRGD-Exo-DOX*, Q3D for 5 doses	Accumulated in CNS lymphoma, inhibited tumor growth, induced apoptosis, prolonged survival	[132]
	Methotrexate	LLC or A549 cells	Coincubation	15 µg DOX/10 <sup>10</sup> microparticles	/	Lung carcinoma (LLC or A549)	IV: MTX-SMPs (10 µg MTX/ dose), QOD for 6 doses	Inhibited tumor growth, induced apoptosis, enhanced CD8+ T cell response, prolonged survival	[148]
	Cisplatin	M1-macrophage	Sonication	LE: 30%	/	Ovarian carcinoma (A2780/DDP)	IV: M1exoCIS*, QD for 7doses	Reverse cisplatin resistance, enhance tumor accumulation, reduce tumor viability.	[149]
	Oxaliplatin	NK cells	Sonication	LE: 8±2.48%	/	Colon carcinoma (HCT116)	IV: L-OHP-Exos (10 mg/kg L-OHP-equivalent), QOD	Increased apoptosis, educed proliferation, inhibited tumor growth	[150]
	Gemcitabine	Panc-1 cells	Sonication	LE: 11.68±3.68%	/	Pancreatic carcinoma (Panc-1)	IV: ExoGEM (5 or 10 mg/kg GEM-equivalent), QOD for 3 doses	Inhibited tumor growth, reduced systemic toxicity, prolonged survival	[151]

(Continued)

Table 4 (Continued).

Application	Cargo	EVs Origin	Loading Method	Loading/Encapsulation Efficiency	Engineering	Cancer Type (Cell Lines)	Administration Route and Amount	Outcome	Reference
Nucleic Acid-Based Therapy	Anti-PD-L1 siRNA	M1-macrophage	Electroporation	LE: 20%	VSV-G	Colon carcinoma (CT26)	IV: siPD-L1@V-M1 EV (1 mg/kg siRNA), QOD for 4 doses	Silenced PD-L1, enhanced CD8+ T cell infiltration, repolarized M2 to M1 macrophages, inhibited tumor growth	[152]
	LPCAT1 siRNA	HEK293 cells	Electroporation	EE: 85.19±1.21%	Anti-EGFR ScFv	Lung adenocarcinoma (PC9)	IV: siLPCAT1-loaded exosomes (5 mg/kg siRNA), Q3D for 4 doses	Reduced tumor cell proliferation, inhibited brain metastases, improved therapeutic targeting	[116]
	miR-13896	hucMSCs	Electroporation	/	/	Gastric carcinoma (HGC-27)	IV: 1×10 <sup>10</sup> particles/dose, Q3D	Reduced ATG2A expression, inhibited autophagy, suppressed tumor growth	[153]
	miR-588	MSCs	Electroporation	/	cRGD peptide	Breast carcinoma (MDA-MB-231)	IV: 1×10 <sup>10</sup> particles/mouse, Q3D for 5 doses	Inhibited tumor growth, remodeled immunosuppressive TME, enhanced NK cell cytotoxicity	[154]
	IFN-γ mRNA	MEFs	Microfluidic electroporation	/	Anti-CD71 and anti-PD-L1	Glioblastoma (GL261 or SB28)	IV: 5×10 <sup>11</sup> sEVs/mouse, Q3D	Enhanced tumor targeting, increased MHC-I expression, inhibited tumor growth, prolonged survival	[155]
	IL-12 mRNA	HEK 293T cells	Electroporation	EE: 27.6%	/	Lung carcinoma (LL/2)	INH: 2×10 <sup>9</sup> EVs/dose, Q3D for 3 doses	Activated anti-tumor immunity, increased IFN-γ secretion, reduced tumor burden, prolonged survival	[156]

Cancer Vaccines	Neoantigens (M27 & M30 or Adpgk)	DC2.4 cells	Electroporation	/	/	Colon carcinoma (MC-38), Melanoma (BI6F10)	SC: 50 µg neoantigen-equivalent, 4 doses	Enhanced antigen uptake, robust immune activation, tumor inhibition, prolonged survival	[157]
	ELANE and Hiltonol (TLR3 agonist)	MDA-MB-231 cells	Electroporation	/	/	Breast carcinoma (MDA-MB-231)	IV: HELA-Exos (containing 12 µg ELANE + 60 µg Hiltonol/dose), 8 doses	Enhanced cDC1 activation, increased CD8+ T cell response, tumor inhibition, prolonged survival	[158]
	CpG (TLR9 agonist)	BI6F10 or E.G7-OVA cells	Incubation	/	/	Melanoma (BI6F10), Lymphoma (E.G7-OVA)	SC: 7×10 <sup>8</sup> EVs, 2 doses	Improved antigen presentation, robust CTL response, tumor inhibition, prolonged survival	[159]
Theranostics and Multifunctional EV Platforms	SPION and Cur	Raw264.7 cells	Electroporation	/	RGE peptide	Glioblastoma (U251)	IV: RGE-Exo-SPION/Cur*, QOD for 7 doses	Enabled imaging, inhibited tumor growth, prolonged survival, reduced systemic toxicity	[160]
	V <sub>2</sub> C-TAT	MCF-7 cells	Electroporation	/	RDG peptide	Breast carcinoma (MCF-7)	IV: V <sub>2</sub> C-TAT@Ex-RGD (with a V <sub>2</sub> C concentration of 10 mg/kg)	Enabled multimodal imaging, induced apoptosis and necrosis, inhibited tumor growth	[161]
	STAT3 siRNA and DOX	4T1 cells	Transfection and incubation	STAT3 siRNA (EE): 70%; DOX (EE): 77.68~93.36%	/	Breast carcinoma (4T1)	IV: siSTAT3-DOX@TEV*, QOD for 6 doses	Downregulated STAT3, induced immunogenic cell death, remodeled immunosuppressive TME, inhibited tumor growth	[109]

**Note:** \*The exact dosage was not specified in the original study.

**Abbreviations:** IN, Intranasal; IV, Intravenous; INH, Inhalation; SC, Subcutaneous; MDR, Multiple drug resistance; CNS, Central nervous system; LE, Loading efficiency; EE, Encapsulation efficiency; QD, Every day; QOD, Every other day; Q3D, Every 3 days.

type-specific EVs with surface ligands and therapeutic siRNAs, gene silencing has been achieved in tumors that could not be reached with free siRNA.

### miRNA

miRNAs are short noncoding RNA molecules that participate in posttranscriptional gene regulation through mRNA cleavage or translational repression.<sup>167</sup> Recently, EV-delivered miRNAs have been found to exhibit significant antitumor effects by modulating the TME or directly inhibiting neoplastic cell proliferation. In one study, human umbilical cord MSC-derived EVs were loaded with miR-138-5p (a tumor suppressor) via electroporation.<sup>153</sup> When injected into gastric cancer models, these EVs transferred miR-138 to the tumor cells, resulting in the downregulation of ATG2A (a gene involved in autophagy) and inhibition of tumor growth and metastasis. Moreover, the treated tumors exhibited increased apoptosis and reduced prosurvival autophagy signaling, indicating that the miRNA successfully reprogrammed tumor cell fate.<sup>153</sup> In another example, Zhang et al identified miR-588 as an miRNA that can overcome immune evasion in TNBC.<sup>154</sup> They loaded miR-588 into MSC-derived exosomes and decorated the exosome surface with cRGD peptides. Systemic administration of these exosomes resulted in preferential uptake by tumors and significant remodeling of the tumor immune microenvironment. The miR-588 cargo downregulated the immunosuppressive chemokine CCL5 in tumors, leading to enhanced recruitment and activation of NK cells and cytotoxic T lymphocytes (CTL). Tumor growth and metastasis were reduced in treated mice, indicating that EV-mediated miRNA delivery can facilitate direct tumor cell attack and boost antitumor immunity.<sup>154</sup> Collectively, these findings demonstrate the versatility of EVs in delivering therapeutic miRNAs. EVs shield miRNAs from degradation, improve their pharmacokinetics, and enable targeted tumor accumulation, achieving functional gene modulation in vivo that is challenging with naked miRNA.

### mRNA

mRNA therapy has gained momentum, and EVs are being explored as natural mRNA delivery vehicles. The large size and fragility of mRNA limit its therapeutic application. However, EVs can protect mRNA and facilitate cytoplasmic uptake in target cells.<sup>168,169</sup> Dong et al engineered EVs to deliver mRNA encoding interferon- $\gamma$  (IFN- $\gamma$ ) for cancer immunotherapy.<sup>155</sup> They developed a recombinant protein anchor that displayed tumor-specific antibodies on the EV surface, thereby directing the mRNA-loaded EVs to glioblastoma cells overexpressing a particular antigen. Upon administration to tumor-bearing mice, these targeted EVs successfully translated IFN- $\gamma$  within the tumor tissue, reprogramming the TME toward an immune-active state and significantly sensitizing the tumors to checkpoint inhibitor therapy.<sup>155</sup> Liu et al developed an inhalable EV formulation delivering IL-12 mRNA for lung cancer treatment. The IL-12 EV therapy induced a robust local and systemic immune response characterized by elevated IFN- $\gamma$  levels and activation of cytotoxic lymphocytes, leading to tumor regression and prolonged survival in mice.<sup>156</sup> This approach minimized systemic exposure and toxicity, underscoring the advantage of tissue-targeted EV delivery. Overall, EV-based mRNA delivery is emerging as a promising alternative to synthetic lipid nanoparticles, with early successes in expressing cytokines, tumor suppressors, or suicide genes directly in tumors. The ability to target EVs to specific tissues via surface modification, along with their natural membrane fusion that avoids endosomal trapping, gives EVs an advantage in achieving functional mRNA protein expression at the tumor site.

## Cancer Vaccines

EVs have emerged as promising delivery platforms of cancer vaccines because of their intrinsic lymphotropism and immunomodulatory capabilities.<sup>170</sup> Previous studies have shown that EV-based vaccine formulations selectively accumulate in draining lymphoid tissues and undergo efficient internalization by APCs, significantly enhancing the antigen presentation efficiency.<sup>171,172</sup> Moreover, EVs inherently incorporate immunostimulatory molecular constituents derived from their parental cells, effectively promoting DC maturation and providing essential costimulatory signals required for T lymphocyte activation.<sup>173</sup> This intrinsic adjuvant functionality amplifies APC activation and subsequent proinflammatory cytokine secretion, thereby optimizing antigen presentation mechanisms and facilitating the generation of tumor-specific T-cell responses with enhanced immunological efficacy. To date, research has primarily focused on DC-derived EVs (DC-EVs) and tumor cell-derived EVs (T-EVs).<sup>174</sup>

## DC-EVs for Cancer Vaccination

Traditional DC vaccines are limited by their complex preparation and high production costs as well as challenges in long-term live-cell preservation.<sup>18</sup> In contrast, DC-EVs offer greater storage stability and manufacturing control while retaining key immunostimulatory components of their parent DCs.<sup>175</sup> Notably, DC-EVs naturally carry functional major histocompatibility complex class I and II molecules and costimulatory proteins (eg, CD80 and CD86) on their surface.<sup>175</sup> These properties enable EVs to directly present antigens and activate T cells in a manner similar to intact DCs. Li et al used DC-EVs as tumor neoantigen carriers for personalized cancer immunotherapy, efficiently delivering tumor-specific antigens to lymph nodes while inducing strong T-cell and B-cell immune responses.<sup>157</sup> Moreover, this nano-vaccine significantly inhibited tumor growth and prolonged survival in B16F10 melanoma and MC-38 CRC mouse models, exhibiting superior antitumor, antimetastatic, and antirecurrence effects with enhanced immunogenicity compared with liposomal formulations.<sup>157</sup> Early-phase clinical trials have also been conducted with DC-derived exosomes. In patients with metastatic melanoma and non-small cell lung cancer, autologous DC-EVs pulsed with tumor-associated antigens were administered as a maintenance immunotherapy.<sup>176–178</sup> These trials demonstrated that DC-EV vaccines are safe, well tolerated, and capable of inducing antigen-specific immune responses with no serious adverse events. However, they showed limited clinical tumor regression, suggesting the need for further enhancements.

## T-EVs for Cancer Vaccination

T-EVs are an attractive vaccine platform because they carry a natural cargo of tumor antigens that reflect the antigenic repertoire of the source cancer.<sup>179</sup> A single T-EV can present multiple tumor-associated antigens simultaneously, offering a broader immune target and potentially addressing intratumoral heterogeneity better than a single-peptide vaccine.<sup>180</sup> Moreover, T-EVs often include endogenous adjuvant molecules (eg, Hsp70) from stressed tumor cells, enhancing their inherent immunogenicity. Early studies have revealed that unmodified T-EVs can naturally promote antitumor immunity. For instance, EVs isolated from lung cancer cells were efficiently captured by host dendritic cells *in vivo*, leading to the activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and a measurable antitumor effect.<sup>181</sup> Building on this, T-EVs are currently being engineered to further enhance their vaccine efficacy. One approach involves loading additional immunostimulatory agents into T-EVs. Huang et al engineered breast cancer–derived EVs (HELA-Exos) by genetically overexpressing  $\alpha$ -lactalbumin and incorporating the immunogenic cell death (ICD)-inducing agents ELANE and Hiltonol (a TLR3 agonist) into the vesicles.<sup>158</sup> HELA-Exos demonstrated breast cancer cell–specific targeting and induced potent ICD coupled with enhanced cDC1 activation. This engineered system strongly primed tumor-specific CD8<sup>+</sup> T cells, achieving significant tumor suppression in mouse TNBC models and patient-derived tumor organoids.<sup>158</sup> Bhatta et al loaded T-EVs with the TLR9 agonist CpG, which significantly enhanced DC activation, antigen presentation, and cytotoxic T lymphocyte responses, yielding 80% and 33% tumor-free survival against E.G7 lymphoma and B16F10 melanoma, respectively.<sup>159</sup>

## Theranostics and Multifunctional EV Platforms

Developing theranostic EVs, which combine therapeutic and diagnostic functions in a single platform, is an exciting frontier in EV research.<sup>182</sup> Because of their versatile cargo capacity and modifiable surface, engineered EVs can serve as multifunctional nanoparticles for cancer therapy while enabling treatment monitoring or providing imaging contrast. In cancer therapy, this indicates that an EV can deliver a drug while simultaneously carrying a reporter (eg, a fluorescent tag or radionuclide) for imaging or it can transport therapeutic and diagnostic probes to the tumor.<sup>182–184</sup>

Several proof-of-concept studies have demonstrated the theranostic potential of EVs. For example, superparamagnetic iron oxide nanoparticles (SPIONs) and therapeutic agents have been introduced into exosomal structures to generate vesicles that can be traced via magnetic resonance imaging (MRI) or photoacoustic imaging during drug delivery.<sup>185,186</sup> In a previous study, exosomes were engineered to carry curcumin and SPIONs. In addition to curcumin-mediated chemotherapy, these constructs enabled the MRI of glioma sites and induced magnetic fluid hyperthermia under an alternating magnetic field, producing a synergistic tumor-killing effect.<sup>160</sup> Another novel approach involved loading exosomes with nucleus-targeting TAT-modified vanadium carbide quantum dots. These constructs enabled multimodal tumor imaging (eg, fluorescence imaging, photoacoustic imaging, and MRI) and provided low-temperature photothermal therapy when exposed to near-infrared laser light,

thereby achieving a synergistic tumor-killing effect through nucleus-targeted thermal ablation.<sup>161</sup> This all-in-one system highlights the potential of theranostics: enabling tumor imaging, therapy, and real-time response monitoring with a same agent.

In addition, multifunctional EVs can carry different combinations of therapeutic agents for multimodal therapy. An EV can simultaneously deliver a chemotherapeutic drug and an siRNA, targeting cancer on two fronts. Peng et al developed a bifunctional therapeutic by loading STAT3 siRNA and DOX into T-EVs. In this system, siRNA-mediated STAT3 silencing reversed immunosuppression and promoted immune activation, whereas chemotherapy via DOX synergistically amplified the antitumor efficacy.<sup>109</sup>

## Clinical Translation and Challenges

### Current Trials and Translational Status

The therapeutic potential of engineered EVs has advanced from preclinical models to early-stage clinical trials (Table 5). Several trials have explored EV-based cancer therapies, establishing their initial safety and feasibility. In one notable Phase I study, autologous tumor-derived exosomes combined with granulocyte macrophage colony-stimulating factor (GM-CSF) were used as a vaccine for CRC.<sup>187</sup> In this trial, exosomes isolated from malignant ascites of patients, which are rich in tumor antigens, were administered along with an immune adjuvant (GM-CSF). This approach was well tolerated with no serious adverse effects. Moreover, patients who received exosomes + GM-CSF exhibited tumor-specific T-cell responses, whereas those who received exosomes alone did not.<sup>187</sup> This provided a clinical proof-of-concept that exosome-based immunotherapy can safely induce antitumor immunity in humans. Similarly, early trials of DC-derived exosomes pulsed with tumor antigens in melanoma and non-small-cell lung cancer demonstrated favorable safety profiles.<sup>177,178</sup> In a phase I study of advanced lung cancer, patients were vaccinated with autologous DC-derived exosomes loaded with MAGE tumor antigens. The treatment was well tolerated (only mild grade 1–2 reactions) and resulted in detectable immune responses in some patients.<sup>177</sup> These pioneering trials confirmed that EV-based therapies are safe and provided preliminary evidence of their immune efficacy, albeit with modest clinical outcomes, laying the groundwork for interventions using next-generation engineered EVs.

Building on these early efforts, more advanced engineered EV therapeutics have recently entered the clinical pipeline. The development of iExosomes (exosomes engineered to carry siRNA against oncogenic KRAS<sup>G12D</sup>) for the treatment of

**Table 5** Clinical Trials of EV-Based Therapeutics (Data from ClinicalTrials.gov and Published References)

EVs	Target site	Status	NCT Number/ Reference
MSC-derived exosomes loaded with KrasG12D siRNA	Pancreatic cancer with metastases	Phase I	NCT03608631
Plant exosomes loaded with curcumin	Colon cancer	Phase I	NCT01294072
DC-derived exosomes loaded with antigen	Non-small cell lung cancer	Phase II	NCT01159288
Exosomes loaded with IL-12 cytokine	Cutaneous T-cell Lymphoma	Phase I	NCT05156229
Cell-derived exosomes loaded with STAT6 anti-sense oligonucleotide (ASO)	Advanced hepatocellular carcinoma/liver metastases from either primary gastric cancer or colorectal cancer	Phase I	NCT05375604
Exosomes loaded with a STING agonist	Advanced/metastatic, recurrent, injectable solid tumors with emphasis on head and neck squamous cell cancer, triple negative breast cancer, anaplastic thyroid carcinoma and cutaneous squamous cell carcinoma	Phase I/II	NCT04592484
Tumor cell-derived microparticles loaded with chemotherapeutic drugs	Malignant ascites and pleural effusion	Phase II	NCT01854866
Tumor cell-derived microparticles loaded with chemotherapeutic drugs	Malignant pleural effusion	Phase II	NCT02657460
Ascites-derived exosomes in combination with the GM-CSF	Colorectal cancer	Phase I	[187]
IFN- $\gamma$ -maturated DC-derived exosomes loaded with MHC class I- and class II-restricted cancer antigens	Non-small cell lung cancer	Phase II	[176]
DC-derived exosomes loaded with the MAGE tumor antigens	Non-small cell lung cancer	Phase I	[177]
Autologous DC-derived exosomes pulsed with MAGE 3 peptides	Metastatic melanoma	Phase I	[178]

metastatic pancreatic cancer is a prominent example.<sup>54</sup> Preclinical studies in PDAC mouse models have shown that iExosomes can specifically target KRAS<sup>G12D</sup>-mutant cancer cells and significantly suppress tumor growth while prolonging survival.<sup>54</sup> These promising results led to a phase I clinical trial (NCT03608631) evaluating iExosomes derived from MSCs in individuals with pancreatic cancer harboring KRAS<sup>G12D</sup> mutations. Interim reports from this trial indicate that iExosome therapy is feasible, safe, and well tolerated in humans, with some treated patients showing reduced circulating KRAS<sup>G12D</sup> DNA and stable disease.<sup>188</sup> Although detailed efficacy results are pending, this first-in-human trial targeting a mutant oncoprotein with exosomal siRNA is a milestone for EV-based gene therapy in cancer.

In addition to academic efforts, biotech companies have been actively translating engineered EVs to clinical applications. For example, Codiak BioSciences has developed a surface-engineered exosome platform for cancer immunotherapy. Their product exoIL-12, which displays the IL-12 cytokine on the exosome surface, was evaluated in a phase I trial for cutaneous T-cell lymphoma, aiming to concentrate immunostimulatory IL-12 within tumors while minimizing systemic exposure.<sup>189</sup> Another candidate, exoSTING—which comprises exosomes loaded with a STING agonist to activate immune cells in the TME—has completed initial clinical testing in patients with solid tumors.<sup>190</sup> Moreover, a first-in-human trial of exoASO-STAT6—exosomes that deliver an antisense oligonucleotide to inhibit STAT6 in tumors—was conducted on patients with advanced HCC.<sup>191</sup> These industry-driven trials underscore the growing clinical interest in EV-based therapeutics. Although no EV product has yet received regulatory approval, the initiation and completion of multiple phase I studies indicate rapid progress in translating EV research into potential cancer treatments. The insights gained from these trials, particularly regarding safety, dosing, and manufacturing, will be invaluable for guiding subsequent larger efficacy studies.

Overall, EV-based cancer therapeutics remain in early-stage development; however, their progress is accelerating. First-in-human studies have demonstrated their feasibility, acceptable safety, and early biological activity. Emerging regulatory engagement was demonstrated by the FDA's clearance of the first investigational new drug application for an unmodified neural EV therapy (AB126) in early 2024.<sup>192</sup> These developments indicate the growing acceptance of EVs as a viable therapeutic class. However, translating this momentum into reliable patient benefit will require overcoming persistent challenges to achieve consistent efficacy and support broad clinical use.

## Challenges and Potential Solutions

Despite encouraging early findings, translating engineered EVs into the clinic still requires addressing a series of interconnected challenges along the bench-to-bedside path. Transforming research prototypes into patient-ready products requires stricter standards, reproducible workflows, and robust evidence reflecting real-world use. The following subsections provide this context and prospective solutions.

### Delivery Efficiency and Targeting

A fundamental challenge is ensuring that EVs efficiently deliver their therapeutic payload to tumor cells *in vivo*. Natural EVs do not inherently target tumors. After systemic administration, a significant fraction of EVs can be sequestered by the mononuclear phagocyte system or taken up by nontarget cells.<sup>82,193–196</sup> This can limit the fraction of the injected EV dose that reaches the tumor. To overcome this issue, targeting moieties can be engineered onto EV surfaces. Several approaches have been shown to enhance uptake by cancer cells while reducing off-target clearance, including displaying tumor-homing peptides or antibody fragments on EV membranes.<sup>197</sup> Similarly, decorating EVs with CD47—the “don't eat me” signal—can help them evade macrophage-mediated phagocytic clearance, prolonging their circulation time. These modifications, along with optimized dosing routes (eg, intratumoral or regional administration when feasible), are being explored to improve delivery efficiency.<sup>31,75</sup>

### *In vivo* Biodistribution and Safety

Controlling EV biodistribution and assessing safety are challenges closely associated with effective targeting. Studies tracking administered EVs in animal models have shown that EVs tend to accumulate in the liver, spleen, lungs, and kidneys, irrespective of their cellular origin.<sup>195,196</sup> Although some sequestration is inevitable, excessive deposition in healthy tissues increases off-target risk, highlighting the need for comprehensive biodistribution and toxicology

assessments. Practical mitigation strategies include minimizing vesicle load via high payload-per-EV formulations, selecting administration routes that limit systemic exposure, “humanizing” membranes to reduce innate clearance, and employing stimuli-responsive architectures that release cargo within the TME (eg, pH- or enzyme-triggered), thereby sparing normal tissues.

For universal (allogeneic) EVs, immunogenicity is a critical factor. Clinical experience shows that autologous EVs are generally well tolerated, and early allogeneic platforms (eg, MSC-derived products, iExosomes) demonstrate acceptable short-term safety; however, rigorous repeated-dose immunogenicity assessment remains essential for regulatory approval.<sup>177,187,188</sup> Allogeneic vesicles present donor antigens that can trigger host recognition, accelerate clearance, and elicit anti-EV antibodies upon repeated dosing.<sup>198</sup> To mitigate immunosurveillance, developers have combined source selection (eg, low-immunogenic or immunomodulatory MSCs), genome engineering (downregulating HLA or other epitopes to yield hypoimmunogenic producers), and surface engineering (displaying CD47 “don’t eat me” cues).<sup>198</sup> When integrated judiciously, these approaches are expected to enable off-the-shelf allogeneic EV therapeutics with safety profiles approaching those of autologous products while maintaining on-target efficacy.

### Manufacturing Processes and Scalable Production

One of the most significant translational challenges in the use of EVs is the production of therapeutic EVs at scale and with consistent quality. As cells naturally release EVs in relatively small quantities, it may be difficult to isolate sufficient EVs for human dosing. Moreover, conventional ultracentrifugation methods are labor-intensive and difficult to standardize, potentially resulting in batch-to-batch variability.<sup>31,199</sup> To address this challenge, new biomanufacturing techniques (eg, culturing cells in bioreactors with high yield outputs, using tangential flow filtration or size-exclusion chromatography for scalable purification, and even inducing cells to secrete more EVs) are under development.<sup>200–203</sup> Ensuring scalability also involves developing stringent quality control measures (ie, quantifying the EV particle concentration, size distribution, cargo load, and functional potency for each batch). Collaborations among academia, industry, and regulatory bodies are beginning to establish standardized manufacturing protocols for EVs, which will be crucial for successful commercialization.

In addition to scale-up, long-term preservation is critical to maintain EV stability and potency during storage and distribution. Ultra-low temperatures ( $-80^{\circ}\text{C}$  or liquid nitrogen) better preserve particle number, size, and bioactivity than  $-20$  or  $4^{\circ}\text{C}$ ; however, repeated freeze–thaw cycles induce aggregation/fusion and functional loss.<sup>204</sup> To address these limitations, lyophilization converts EVs into a dry powder and, with appropriate lyoprotectants (eg, trehalose), allows reconstitution while preserving vesicle structure and function.<sup>205</sup> In parallel, optimized storage buffers are essential. Geng et al demonstrated that supplementing phosphate-buffered saline with human serum albumin and trehalose substantially improved the short- and long-term stability of EVs at  $-80^{\circ}\text{C}$ , facilitating much higher particle recovery after storage and fewer changes in vesicle size or uptake capacity.<sup>206</sup> Incorporating optimized buffers, careful cryopreservation, and lyophilization into manufacturing process will extend shelf life and ensure consistent performance.

### Regulatory and Standardization Hurdles

The regulatory landscape for EV-based therapeutics continues to evolve, posing a challenge for clinical translation. EV products do not clearly fall into the conventional categories of drugs, biologics, or cell therapies. Moreover, their complex composition makes them difficult to standardize.<sup>207</sup> The variations in EV isolation and engineering techniques across research groups result in heterogeneous vesicle preparations, which differ in purity, subtype (eg, exosomes vs microvesicles), and molecular cargo. This fragmentation complicates the development of clear regulatory guidelines. Compared with small-molecule drugs, demonstrating how an EV delivers its cargo and is cleared by the body is more challenging. To advance the field, consensus standards—such as those from the International Society for Extracellular Vesicles—are being developed for characterizing EVs, including defining identity, purity, potency, and safety assays.<sup>208,209</sup> In addition, robust assays to measure the critical quality attributes of EVs—such as size, charge, membrane protein markers, and nucleic acid content—are being developed. Moreover, regulatory agencies will likely require consistency in manufacturing processes to maintain reproducibility between batches. An ongoing dialogue between researchers and regulators is essential to address these issues.

## Conclusions and Prospects

### The Promise and Challenge of EV-Based Therapeutics

Engineered EVs have rapidly become one of the most innovative and versatile nanocarrier systems for cancer therapy. Based on the literature reviewed herein, EV-based delivery systems exhibit innate biocompatibility and intercellular communication while integrating the design flexibility and functional tunability of advanced nanomedicine platforms. The key findings show that EVs can outperform conventional synthetic nanoparticles in targeting tumors, penetrating biological barriers, and delivering complex therapeutic cargos (eg, RNAs and proteins) with minimal toxicity when appropriately engineered. Major applications are already demonstrating success in preclinical models. EVs loaded with chemotherapeutics achieve higher tumor cell eradication with reduced side effects. EV shuttles can deliver gene silencers deep into tumor tissue, and EV-based vaccines can effectively mobilize the immune system against cancer. These achievements underscore the distinct advantages of engineered EVs: they are inherently stealthy in the bloodstream, can be equipped with targeting ligands for precise homing, and can carry multimodal payloads that conventional vectors have difficulty accommodating. Therefore, establishing an EV-specific, screening-focused discovery pipeline is both feasible and timely. Using standardized, scalable EV workflows and identity/potency assays, a minimal, reproducible process can directly compare encapsulated versus free-form performance while capturing essential pharmacokinetic, pharmacodynamic, and safety readouts. This shared framework converts heterogeneous preclinical results into comparable, regulator-relevant evidence and supports scale-up decisions and regulatory interactions.

The clinical translation of engineered EVs remains incomplete, with several challenges necessitating continued innovation and standardization. These include ensuring batch-to-batch consistency, scaling production processes, and meeting regulatory standards. The forthcoming clinical trial data will be pivotal in determining the pharmacological behavior of engineered EVs in humans and assessing whether the antitumor efficacy observed in animal models is reproducible in patients. These uncertainties highlight the need for disciplined standardization and generation of comparative evidence to support reliable clinical development.

### Future Outlook

Looking forward, continued advances in EV engineering are anticipated to expand the therapeutic potential of these vesicles in cancer therapy. Bioengineering strategies are advancing on multiple fronts. Surface functionalization enables EV to display tumor-targeting peptides, antibodies, or aptamers, enhancing targeting specificity. In parallel, hybrid delivery systems are gaining momentum. EV–liposome constructs, formed by fusing native EV membranes with synthetic liposomal cores, combine biological interface of EVs with tunable physicochemical properties and stability of liposomes.<sup>210,211</sup> For example, one study engineered cancer cell-derived EVs fused with liposomes, achieving markedly higher drug loading and tumor-targeting efficiency than either EVs or liposomes alone.<sup>212</sup> There is also increasing interest in EV mimetics (cell-derived nanovesicles produced using extrusion or other methods), which could provide higher yields while retaining their key biofunctional properties.<sup>213</sup> In addition, alternative sources of EVs (eg, plant-derived vesicles) are being explored to overcome scalability limitations.<sup>214,215</sup> Multifunctional EV platforms capable of codelivering drugs, genes, and immunostimulators are expected to expand, enabling synergistic targeting of tumors through multiple mechanisms.

Artificial intelligence (AI) and automated manufacturing are transforming EV engineering. Machine learning algorithms can analyze large EV datasets to predict biodistribution and guide the rational design of EV surface and cargo properties.<sup>216</sup> Future workflows may employ AI-driven models to optimize EV design parameters and tailor therapies to individual patients. On the manufacturing side, scalable, GMP-compliant systems are advancing. For example, perfusion bioreactors and closed microfluidic reactors now allow high-yield EV production under controlled conditions.<sup>207</sup> Concurrently, regulatory frameworks are evolving to accommodate EV therapeutics, with global regulatory agencies aligning exosome regulations with established biologics standards, including requirements for rigorous characterization, quality control, and safety testing.<sup>207</sup> Collectively, these developments are poised to shape the next era of EV-based oncology therapeutics.

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