International Journal of Nanomedicine

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REVIEW

Precision Engineering of Extracellular Vesicles as Programmable Carriers for mRNA Therapeutics

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Abstract: Messenger RNA (mRNA) therapeutics have rapidly evolved into a transformative modality for treating infectious diseases, cancer, and genetic disorders; however, the clinical translation of these therapeutics remains limited by the need for safe, efficient, and tissue-specific delivery vehicles. Subsequently, extracellular vesicles (EVs) have emerged as a promising next-generation platform due to the associated endogenous biogenesis, intrinsic biocompatibility, low immunogenicity, and natural ability to traverse biological barriers. Thus, this review provides a comprehensive evaluation of the major engineering strategies enabling EV-based mRNA delivery, including exogenous loading methods, endogenous genetic engineering, physical microenvironment-driven enhancement, and hybrid EV–synthetic nanoparticle systems. Moreover, this review summarizes advances in electroporation, lipid-mediated fusion, and chemical/physical loading techniques; programmable endogenous loading platforms leveraging EV-sorting proteins and RNA-binding domains; cargo release mechanisms employing self-cleaving, protease-sensitivity, and optogenetic modules; device- and substrate-based approaches that modulate EV biogenesis and cargo composition. We further highlight emerging hybrid EV systems—particularly fusogenic cubosome–EV constructs—that achieve near-quantitative mRNA encapsulation and improved biodistribution, including enhanced penetration across the blood–brain barrier. Finally, we discuss technological bottlenecks and translational considerations, including scalability, batch variability, long-term mRNA stability, and regulatory challenges associated with biologically derived carriers. Collectively, this review outlines the current landscape and future directions for precision engineering of EVs as programmable, clinically viable carriers for mRNA therapeutics.

Keywords: extracellular vesicles, drug loading, mRNA delivery, engineering EV, hybrid EVs

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Introduction

The rapid emergence of mRNA therapeutics as a transformative modality in modern medicine has enabled the treatment and prevention of a diverse array of diseases, including infectious diseases, cancer, and genetic disorders.[1,2] Meanwhile, the clinical success of mRNA vaccines, most notably during the COVID-19 pandemic, has both demonstrated the immense therapeutic potential of this technology and accelerated the development of next-generation mRNA-based therapies for a variety of indications.[3] Nonetheless, despite these advances, the clinical translation of mRNA therapeutics remains fundamentally limited by the challenge of delivering mRNA molecules safely, efficiently, and specifically to target cells and tissues. Indeed, naked mRNA is inherently unstable in biological fluids and susceptible to rapid degradation by nucleases, thereby necessitating specialized delivery vehicles to protect the cargo and facilitate cellular uptake.[1,4,5]

Presently, lipid nanoparticles (LNPs) and viral vectors have served as the primary platforms for mRNA delivery. While these systems have enabled the first wave of mRNA medicines, these platforms are not without significant drawbacks. LNPs, for example, are associated with dose-limiting immunogenicity, limited tissue targeting (often resulting in predominant liver accumulation), and potential toxicity upon repeated administration. Viral vectors, although highly efficient at gene transfer, raise concerns regarding immunogenicity, insertional mutagenesis, and manufacturing complexity. Thus, as the field advances

https://doi.org/10.2147/IJN.S595161
Received: 12 January 2026
Accepted: 30 April 2026
Published: 11 May 2026

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toward more sophisticated and personalized mRNA therapeutics, a pressing need exists for alternative delivery strategies that combine high efficiency, safety, and the ability to target tissue or cells precisely.[6–8]

EVs, such as microvesicles, have recently attracted considerable interest as natural, biocompatible carriers for mRNA delivery. EVs are nanoscale, membrane-bound vesicles secreted by virtually all cell types, and play a fundamental role in intercellular communication by transporting nucleic acids, proteins, and lipids between cells. The endogenous origin of EVs confers several unique advantages: low immunogenicity, high biocompatibility, and the intrinsic ability to cross biological barriers, such as the blood–brain barrier (BBB).[9–12] Importantly, EVs can be engineered or sourced from specific cell types to enhance tissue tropism, display targeting ligands, or carry therapeutic mRNA cargo with high efficiency and specificity. These properties make EVs particularly attractive for applications in precision medicine, where personalized and disease-specific delivery is paramount.[13,14]

Recent advances in EV engineering have further expanded the therapeutic potential of these carriers. Hybrid platforms that integrate EVs with synthetic nanocarriers, such as LNPs or liposomes, have been developed to combine the strengths of both natural and artificial systems.[15,16] A detailed comparison of the molecular principles, performance parameters, and technical bottlenecks of these platforms are summarized in Table 1. Additionally, programmable strategies for selective mRNA loading, controlled release, and surface modification are enabling the rational design of EV-based delivery vehicles tailored to specific clinical needs. Meanwhile, mechanistic studies are also shedding light on the intracellular trafficking, endosomal escape, and functional expression of mRNA delivered via EVs, providing a foundation for further optimization.[13,17]

Thus, this review presents a comprehensive and up-to-date overview of engineering EVs for mRNA delivery, with a particular focus on hybrid platforms, programmable strategies, and their translational potential in precision medicine (Figure 1). We also discuss recent technological advances, mechanistic insights, and disease-specific applications, as well as the challenges and opportunities that remain for the clinical translation of EV-based mRNA therapeutics. Indeed, by synthesizing the current state of the field, we aim to offer new perspectives and guide future research directions in the development of next-generation mRNA delivery systems.[23,24]

Table 1 Comparative Overview of Natural EVs, LNPs, Hybrid EVs Systems and Cubosome-EV Hybrids for mRNA Delivery

Platform	Loading/Fusion Principle	mRNA Encapsulation/ Delivery Performance	Advantages	Limitation	Ref.
Natural EVs	Primarily classical endocytosis and receptor-mediated endocytosis	Variable encapsulation efficiency; highly sensitive to EV biosynthesis and cargo loading techniques	Intrinsic biocompatibility, minimal immunogenicity, and allogeneic targeting (natural affinity)	Limited loading control, donor variability, scale-up challenges	[18, 19]
LNPs	Synthesis of lipid capsules during formulation; absorption primarily via endocytosis	Generally high and highly reproducible under optimized conditions	High loading capacity, scalable production, clinically validated	Dose-limiting immunogenicity, liver-centric biodistribution	[20]
Hybrid EVs	Membrane fusion or electrostatic assembly between EVs and synthetic lipids/ nanocarriers	Synergistic effect; Achieving higher payload capacity of synthetic carriers while maintaining EV-mediated targeting	Synergistic integration of electric vehicle-mediated targeting/safety with high payload capacity and structural tunability of synthetic vectors	High heterogeneity in particle formation; difficulties in large-scale purification and standardization	[21]
Cubosome-EV hybrids	Rapid membrane fusion through non-layered topological surfaces	Very high; near-quantitative mRNA encapsulation (~100%) and enhanced blood-brain barrier permeability	Synergistic effects; ~100% mRNA loading efficiency, enhanced/ modulable blood-brain barrier permeability, and maintenance of inherent EV affinity	Unresolved molecular dynamics; limitations in direct comparison with existing LNPs	[22]

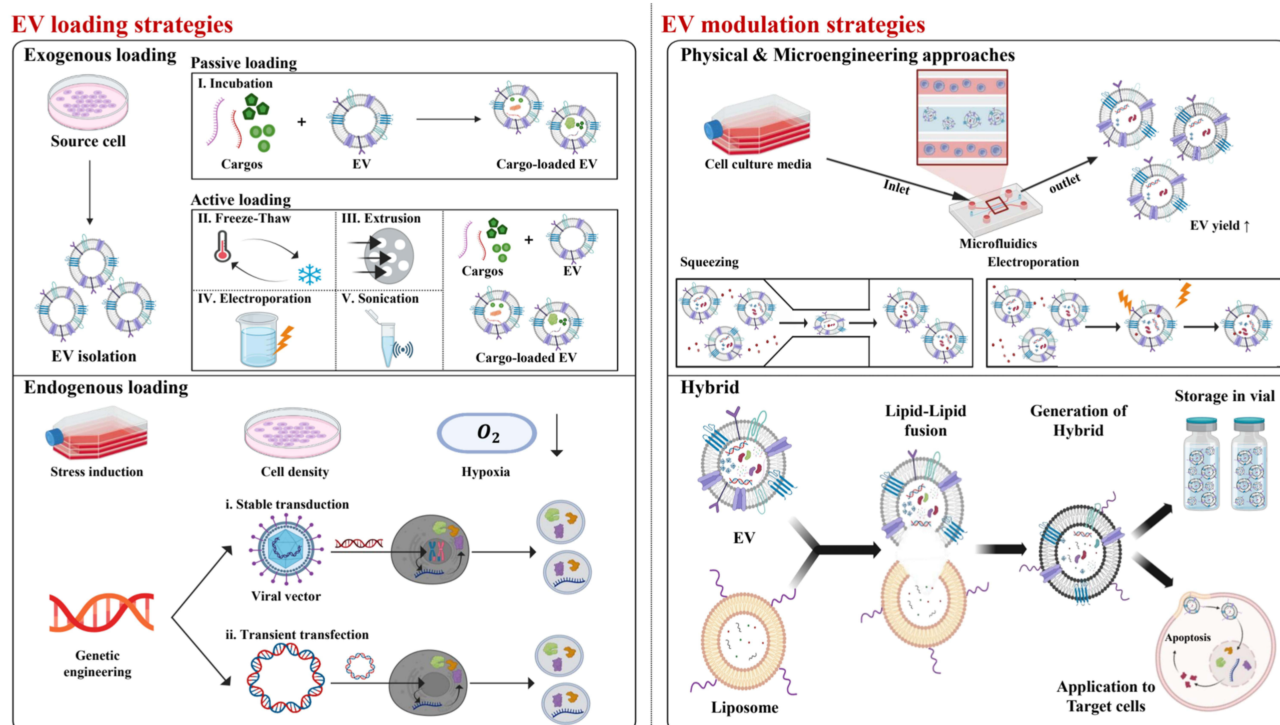

Figure 1 Overview of various strategies for therapeutics formulation using extracellular vesicles.

Extracellular Vesicles as mRNA Delivery Platforms: Key Features and Rationale

Overview and Rationale for EV-Based mRNA Delivery

EVs have emerged as highly promising platforms for mRNA delivery, owing to the associated unique biological origins and functional properties of these carriers.[25] Unlike synthetic nanoparticles, EVs are naturally secreted by cells and are inherently equipped to transport nucleic acids, proteins, and lipids between cells, reflecting the physiological role of EVs in intercellular communication.[26,27] This endogenous nature endows EVs with superior biocompatibility and low immunogenicity, which are critical for minimizing adverse immune responses and enabling repeated administration in therapeutic settings. The lipid bilayer structure of EVs provides robust protection for encapsulated mRNA, shielding these carriers from enzymatic degradation and enhancing stability and bioavailability in systemic circulation. Furthermore, EVs possess the intrinsic ability to cross biological barriers,[28] including the BBB, thereby expanding the associated potential for targeting tissues that are inaccessible to most conventional delivery systems.[17,26]

A key advantage of EVs is the associated customizability: by selecting specific cell sources or engineering parent cells, EVs can be tailored to display specific surface ligands, enhance tissue tropism, or carry therapeutic mRNA cargo with high efficiency and specificity. Meanwhile, recent advances in EV engineering have enabled the development of hybrid platforms that integrate EVs with synthetic nanocarriers, as well as programmable strategies for selective mRNA loading and controlled release. These innovations are broadening the therapeutic landscape for EV-based mRNA delivery, making these carriers especially attractive for precision medicine applications that demand targeted, safe, and efficient delivery of nucleic acid therapeutics.[29,30]

Importance of Cell Source Selection

The choice of cell source for EV production is a critical determinant of the functional and translational success of EV-based mRNA delivery systems. The parent cell dictates the molecular composition and surface protein profile of the resulting EVs, as well as the associated intrinsic targeting properties, immunogenicity, and scalability for clinical applications. For example, EVs derived from mesenchymal stem cells (MSCs) are widely favored due to their low immunogenicity, regenerative and

immunomodulatory effects, and well-established safety profile, making these cells suitable for repeated administration and use in tissue repair.[31–33] In contrast, EVs from immortalized cell lines, such as HEK293, are preferred in research and manufacturing settings for their ease of genetic manipulation and high yield. However, the use of these EVs in clinical applications requires careful safety validation.[34] Dendritic cell (DC)-derived EVs inherit antigen-presenting capabilities from their parent cells, making these cells particularly attractive for immunotherapy and cancer vaccine development.[35,36] Cardiac progenitor cell (CPC)-derived EVs exhibit natural tropism for heart tissue, which is advantageous for targeted mRNA delivery in cardiovascular disease.[37] Red blood cell (RBC)-derived EVs are notable for their minimal immunogenicity and long circulation time, supporting systemic and repeated mRNA delivery, albeit with less inherent tissue specificity.[38] Tumor cell-derived EVs, while offering the potential for tumor-specific targeting, present unique safety concerns due to the risk of transferring oncogenic material.[39] Ultimately, the rational selection and engineering of the cell source is essential for optimizing delivery efficiency, targeting specificity, safety, and scalability, and must be tailored to the intended therapeutic application and disease context.

Common Cell Sources and EV Types for mRNA Delivery

The selection of cell source for EV production is a pivotal determinant of the efficiency, specificity, and safety of mRNA delivery systems. Each cell type imparts distinct molecular signatures and functional properties to the associated EVs, influencing the suitability of these carriers for various therapeutic applications (Table 2).

MSC-Derived EVs

MSCs are widely recognized for their low immunogenicity, immunomodulatory effects, and regenerative capacity. EVs derived from MSCs (MSC-EVs) inherit these properties, making these carriers attractive for mRNA therapeutics, particularly in regenerative medicine and cardiovascular applications. Preclinical studies have demonstrated that MSC-derived EVs loaded with therapeutic mRNAs, such as vascular endothelial growth factor A (VEGF-A), can efficiently home to sites of tissue injury or inflammation, promote angiogenesis, and enhance functional recovery in models of myocardial infarction and tissue repair.[59–61] The endogenous cargo of MSC-EVs, including anti-inflammatory cytokines and growth factors, may further synergize with delivered mRNA to amplify therapeutic outcomes. Importantly, MSCs can be expanded under Good Manufacturing Practice (GMP) conditions, enabling scalable production of clinical-grade EVs with consistent quality.[62,63]

Table 2 Common Cell Sources and EV Types for mRNA Delivery

Cell Source	Key Features and Advantages	Representative Applications	Ref.
Mesenchymal stem cells (MSCs)	Low immunogenicity, regenerative/anti-inflammatory effects, scalable	Tissue regeneration, cardiovascular, immune disorders	[40, 41]
Immortalized cell lines (HEK293)	Easy genetic manipulation, scalable and standardized production	Platform development, mRNA loading studies	[17, 42–44]
Dendritic cells (DCs)	Immune modulation, strong antigen presentation	Cancer vaccines, immunotherapy	[35, 45, 46]
Cardiac progenitor cells (CPCs)	Cardiac tissue targeting, regenerative potential	Myocardial infarction, heart repair	[47–51]
Red blood cells (RBCs)	Minimal immunogenicity, long circulation time	Repeated dosing, systemic delivery	[38, 52–54]
Tumor Cell-derived EVs	Intrinsic tumor-homing, TME modulation, Molecular profiling	Tumor-targeted mRNA delivery, Cancer immunotherapy, Preclinical delivery mechanism	[55–58]

 https://doi.org/10.2147/IJN.S595161

HEK293 and Similar Immortalized Cell Line-Derived EVs

Immortalized cell lines, such as HEK293, are frequently employed for EV production due to their robust growth, ease of genetic manipulation, and reproducibility. These features facilitate the engineering of cells to overexpress specific mRNAs or display targeting ligands on EV surfaces, enabling the systematic evaluation of mRNA-loading strategies and surface modifications.[64–66] HEK293-derived EVs are commonly used as platforms for optimizing mRNA encapsulation efficiency, evaluating delivery mechanisms, and developing scalable manufacturing protocols. While these EVs are invaluable for basic research and technology development, the non-autologous and immortalized nature of these carriers necessitates careful safety and immunogenicity assessment before clinical application.[67,68]

DC-Derived EVs

DCs are professional antigen-presenting cells with a central role in initiating and regulating immune responses. EVs derived from DCs retain key immunological features, including the presentation of major histocompatibility complex (MHC)–peptide complexes and costimulatory molecules.[69] These properties make DC-EVs particularly suitable for immunotherapy and cancer vaccine development.[70] Delivery of mRNA encoding tumor-associated antigens via DC-EVs has been shown to stimulate robust antigen-specific T cell responses and modulate the tumor microenvironment, offering a natural and potent platform for cancer immunotherapy and vaccine applications. Early-phase clinical studies have explored the use of DC-EVs for personalized cancer vaccines, demonstrating feasibility and safety.[71]

CPC-Derived EVs

CPCs are specialized stem cells involved in heart development and repair. CPC-derived EVs (CPC-EVs) display a natural tropism for cardiac tissue, which is advantageous for targeted mRNA delivery in cardiovascular disease.[72,73] Preclinical models have shown that CPC-EVs loaded with therapeutic mRNAs, such as VEGFA, can selectively accumulate in injured myocardium, promote neovascularization, and improve cardiac function following myocardial infarction.[74] These findings highlight the importance of cell source selection for achieving tissue-specific delivery and maximizing therapeutic efficacy in heart regeneration.[75]

RBC-Derived EVs

RBCs are anucleate cells with a long circulation time and minimal immunogenicity.[76] EVs derived from RBCs (RBC-EVs) inherit these properties, making these carriers promising vehicles for systemic and repeated mRNA delivery. The absence of nuclear and mitochondrial DNA in RBC-EVs reduces the risk of transferring unwanted genetic material, and their high biocompatibility supports long-term administration.[38,77] However, additional engineering may be required to enhance the specificity of these carriers for particular organs or disease sites, as RBC-EVs lack inherent tissue-targeting features.[78]

Tumor Cell-Derived EVs

Tumor cell-derived EVs reflect the molecular and surface marker profiles of their parent cells, which can confer intrinsic homing to tumor tissues and the ability to modulate the tumor microenvironment. These features have prompted an investigation into their use as carriers for tumor-targeted mRNA delivery and cancer therapy. However, the potential transfer of oncogenic material and the risk of promoting tumor progression necessitate rigorous purification and safety validation before clinical translation. Despite these challenges, tumor-derived EVs remain a valuable tool for exploring tumor-specific delivery mechanisms in preclinical research.[55]

Advantages and Limitations Compared to Conventional Delivery Systems

EVs and LNPs are the two most widely studied platforms for mRNA delivery, each offering distinct advantages and presenting specific challenges. Thus, a direct, evidence-based comparison is essential to understand the potential and limitations of EV-based systems in the context of current nucleic acid therapeutics (Table 3).

Biocompatibility and Immunogenicity

EVs, which are naturally derived from cell membranes, generally exhibit lower immunogenicity and greater biocompatibility than synthetic LNPs. This endogenous origin reduces the risk of adverse immune responses, making EVs attractive

Table 3 Comparison of EVs and LNPs for mRNA Delivery

Features	EVs	LNPs	Ref.
Biocompatibility and Immunogenicity	High biocompatibility, low immunogenicity; suitable for repeated dosing and long-term use	Moderate; can induce immune activation, especially with repeated dosing; improved with new chemistries	[79–81]
Intrinsic therapeutic effects	Can exert anti-inflammatory, immunomodulatory, and regenerative effects via endogenous cargo; potential synergy with mRNA payload	Biologically inert; no intrinsic therapeutic activity	[18, 82–84]
Targeting and biodistribution	Natural tropism based on parent cell; customizable for tissue-specific delivery (eg., heart, immune system)	Predominantly accumulates in liver; extrahepatic targeting requires additional ligand engineering	[85–91]
Stability and cargo protection	Lipid bilayer protects mRNA from RNase degradation; supports functional protein expression in vivo	Lipid encapsulation protects mRNA; robust in vivo protein expression; no universal superiority in stabilization	[92–95]
Drug loading: efficiency and process	Technically challenging; variable and often lower efficiency; exogenous loading can damage EVs; endogenous loading is less scalable	Highly efficient (>90%), scalable, and reproducible encapsulation using established protocols	[96–100]
Production, scalability, and standardization	Complex, costly, and variable; involves cell culture and purification; standardization is an ongoing challenge	Established, scalable, and reproducible manufacturing; clear regulatory pathways and quality control standards	[101–105]
Endogenous cargo and safety	Contains proteins, RNAs, and biomolecules that may have unintended effects; requires rigorous safety validation	Synthetic composition; predictable and controllable; minimal risk of unintended biological effects	[18, 106–110]
Endosomal escape efficiency	Complex, Biogenic, High (~25%); 10-fold higher than LNPs; efficient functional cargo release, Immediate Function, Innate Membrane Fusion	Synthetic, Low (1–2%); major rate-limiting step; significant endosomal entrapment, Vesicle Budding-and-Collapse (VBC), Aggregated State	[111–114]

for repeated dosing and long-term therapies.[25,115] LNPs, while clinically validated and highly effective—as demonstrated by their use in COVID-19 vaccines and siRNA drugs—can trigger immune activation and inflammatory cytokine production, particularly at higher or repeated doses. Interestingly, while advances in LNP chemistry have mitigated some risks, immunogenicity remains a consideration.[82,116]

Intrinsic Therapeutic Effects of EVs

In addition to the role of EVs as delivery vehicles, EVs may exert beneficial biological effects through their endogenous protein and RNA cargo. For example, EVs derived from MSCs have demonstrated anti-inflammatory, immunomodulatory, and regenerative properties in various preclinical models. These intrinsic activities can synergize with the delivered mRNA, potentially amplifying therapeutic outcomes in tissue repair, immune modulation, and cardiovascular regeneration.[117,118] In contrast, synthetic carriers, such as LNPs, lack this associated bioactivity, highlighting a unique advantage of EV-based mRNA delivery platforms.

Targeting and Biodistribution

The surface proteins and glycans of EVs, inherited from their parent cells, can confer natural tropism toward specific tissues. For example, CPC-derived EVs show enhanced targeting to heart tissue, while DC-derived EVs interact efficiently with immune cells.[119,120] In contrast, LNPs tend to accumulate predominantly in the liver after systemic administration, limiting their utility for extrahepatic targeting unless modified with specific ligands.[121] Both platforms are being engineered for improved tissue specificity; however, EVs offer the unique advantage of leveraging cell-intrinsic targeting properties.[122]

Stability and Cargo Protection

Both EVs and LNPs protect mRNA from extracellular RNases through their lipid bilayer structures, supporting efficient delivery of intact mRNA to recipient cells. There is currently no robust evidence that one platform universally outperforms the other in mRNA stabilization under physiological conditions. Moreover, both systems have demonstrated the capacity to support functional protein expression in vivo.[123,124]

Drug Loading: Efficiency and Process

A major distinction between the two systems lies in drug loading. LNPs enable highly efficient, scalable, and reproducible mRNA encapsulation via well-established mixing protocols, often achieving >90% encapsulation efficiency.[125] In contrast, loading mRNA into EVs remains technically challenging, with lower efficiency and greater variability.[30] Furthermore, exogenous loading methods (eg., electroporation, sonication) can compromise EV integrity, while endogenous loading via donor cell engineering is less controllable and harder to scale.[96,126] Recent advances in programmable and hybrid EVs platforms are improving loading efficiency, but LNPs remain the gold standard for simple and robust mRNA encapsulation.[127]

Production, Scalability, and Standardization

LNPs benefit from established, scalable, and reproducible manufacturing processes, with well-defined regulatory pathways and quality control standards.[128] In contrast, EV production remains more complex and costly, involving cell culture, isolation, and purification steps that can introduce batch variability and limit scalability. Additionally, standardization and large-scale production of clinical-grade EVs represent ongoing challenges for the field.

Endogenous Cargo and Safety Considerations

A unique consideration for EVs is the presence of endogenous proteins, RNAs, and other biomolecules, which may have unintended biological effects on recipient cells. This complexity necessitates rigorous characterization and safety validation, especially for clinical applications. LNPs, being synthetic, have more predictable and controllable compositions.

Endosomal Escape Efficiency

A key advantage of EVs is their high endosomal escape efficiency, which is 10-fold higher than that of synthetic LNPs. While LNPs are often limited by significant endosomal entrapment and a slow “Vesicle Budding-and-Collapse” process, EVs leverage innate membrane fusion to enable immediate functional cargo release. This biological machinery allows EVs to bypass degradative pathways more effectively, resulting in superior intracellular delivery.

Strategies for mRNA Loading and Delivery via EVs

The development of efficient and precise mRNA delivery systems using EVs relies on advanced engineering strategies for mRNA loading, surface modification, and the integration of synthetic nanotechnologies.[129,130] This section reviews the main approaches, the associated technical considerations, and recent innovations in the field.

Exogenous Loading Approaches: Techniques and Efficiency

Exogenous loading refers to strategies in which EVs are first isolated from their producer cells and subsequently loaded with mRNA outside the cellular environment using various physical, chemical, or engineering-based methods.[131,132] This approach offers substantial flexibility in selecting and exchanging cargo molecules without genetically altering the donor cells, making this method attractive for rapid preclinical testing and scalable manufacturing.[133,134] Nevertheless, mRNA poses unique challenges for exogenous encapsulation due to its large molecular size (often >1000 nucleotides), strong negative charge, and complex secondary structure, all of which can hinder membrane passage and increase the risk of degradation or vesicle damage during the loading process.[135]

Electroporation remains one of the most widely employed techniques for nucleic acid incorporation into EVs. By applying controlled electrical pulses, transient pores form in the vesicle membrane, enabling the entry of macromolecules such as mRNA. However, efficient mRNA loading is particularly challenging compared to smaller oligonucleotides, and

improper parameter settings can lead to vesicle aggregation, partial RNA fragmentation, or loss of functional surface proteins.[136–138] Studies with tissue-derived vesicles, such as lung EVs, highlight that careful optimization of electroporation parameters is critical to maximize uptake while preserving vesicle integrity.[96,139]

Sonication and freeze-thaw cycling are alternative physical approaches that disrupt membranes via ultrasound or repeated temperature shifts, respectively. While effective for small molecules or short RNAs, these methods can compromise the structural integrity of EVs and reduce bioactivity when applied to large, fragile mRNA cargo, thereby limiting the applicability of these carriers without further refinement.[140,141]

Chemical transfection strategies employ cationic agents, including polymers such as polyethyleneimine (PEI) or lipids such as 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), to condense mRNA and facilitate the subsequent fusion with EV membranes.[142] For example, milk-derived EVs loaded with SARS-CoV-2 RBD mRNA via DOTAP achieved a high loading efficiency (~57%), retained vesicle morphology, and, when administered by oral gavage or intraduodenal injection in mice, elicited strong neutralizing antibody responses.[143] Similarly, plant-derived EVs subjected to osmotic stress and proprietary cationic protein treatment reached ~45% loading efficiency, maintained structural stability after lyophilization, and remained functional at room temperature for over a year,[144] enabling robust mucosal and systemic immunity upon oral administration in rats.[145]

Direct incubation, sometimes in optimized buffers with gentle agitation, permits passive diffusion of cargo into vesicles. This method is minimally disruptive but generally yields negligible encapsulation for large mRNA molecules due to poor membrane permeability; several recent studies have illustrated the versatility and limitations of these approaches (Table 4).

Popowski et al compared lung-derived EVs (lung-EVs), HEK293-derived EVs, and LNPs for inhaled delivery of green fluorescent protein (GFP) mRNA and red fluorescent protein (RFP) proteins. The physical properties of these nanoparticles were thoroughly characterized, including morphology via TEM (Figure 2a), the presence of the EV-specific marker CD63 in EV lysates compared to liposomes (Figure 2b), and size distribution analysis through Nanoparticle Tracking Analysis (NTA), which confirmed the modal diameters of the vesicles. (Figure 2c and d) Cargo was loaded into purified vesicles using optimized electroporation (Figure 2e), and all formulations were shown to retain vesicle integrity. The success of cargo loading was confirmed by RFP expression in the lysates (Figure 2f), and the structural stability of the nanoparticles was maintained for up to 72 hours, as evidenced by consistent NTA size measurements. (Figure 2g) After jet nebulization into mice, lung-EVs achieved up to 22–24-fold higher protein expression and significantly enhanced mRNA translation in the bronchioles and parenchyma compared with LNPs, and outperformed HEK-EVs. Quantitative fluorescence analysis further demonstrated that lung-EVs significantly increased cargo delivery to lung parenchymal cells compared to other groups. (Figure 2h) Biodistribution analysis showed increased lung retention and reduced off-target delivery for lung-EVs, whereas LNPs localized mainly to the trachea and underwent rapid systemic clearance. The authors attribute this advantage to the native lung-specific molecular signature of lung-EVs, which enhances pulmonary targeting and persistence, highlighting the potential of lung-EVs as a superior carrier for inhaled mRNA therapeutics targeting respiratory diseases (Figure 2). [146]

Table 4 Comparison of Exogenous mRNA Loading Methods into EVs

Type	Loading Method	Technical Description	EV Source	mRNA Type	LE (%)	Key Outcomes and Features	Ref.
Post-loading	Electroporation	Electric pulse-induced mRNA entry into purified EVs	Lung-EV, HEK293-EV	GFP/RFP mRNA	30-40%	Up to 22× greater lung expression vs. LNPs; low off-targets	[146]
	Cationic lipid fusion	mRNA-DOTAP complex (lipoplex) incubated and fused with EVs	Milk-EV	SARS-CoV-2 RBD mRNA	~57%	Induced mucosal/systemic immunity; oral vaccine route	[143]
	Hypotonic buffer and cationic protein	EVs incubated in hypotonic buffer with cationic protein (eg., protamine) and mRNA	Orange-EV	SARS-CoV-2 S1 mRNA	~45%	Stable >12 months; mucosal and systemic immunity	[145]

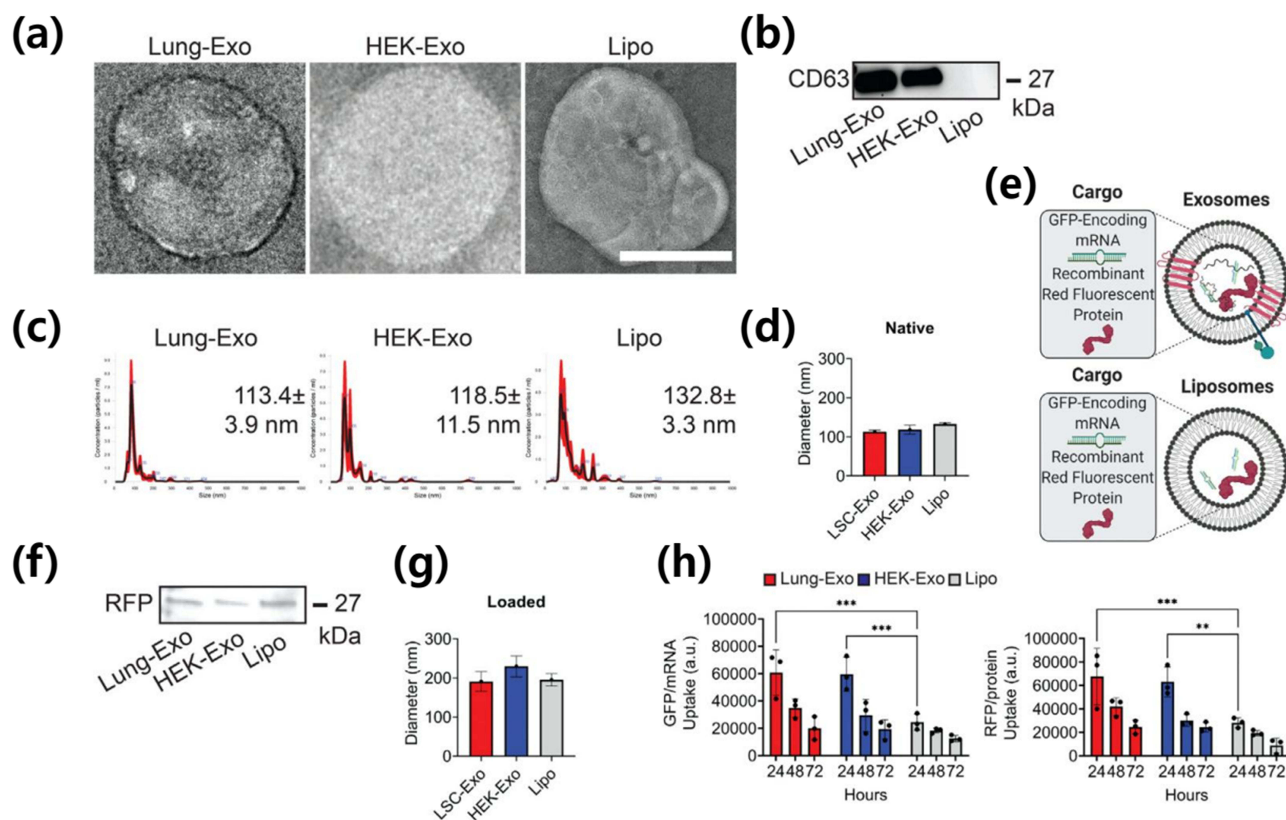

Figure 2 Comparison of lung-EVs, HEK-EVs, and liposomes for cargo loading and delivery efficiency. (**a**) Representative TEM image of native Lung-EVs, HEK-EVs, and liposomes. (**b**) CD63 expression in EV and liposome lysate. (**c**) Nanoparticle Tracking Analysis (NTA) size distribution and modal nanoparticle diameters quantified using a NanoSight NS300 (Malvern Panalytical). (**d**) Quantitative analysis of average nanoparticle size measured by NTA. Data are presented as mean ± standard error from five replicates. (**e**) Illustration of mRNA and protein loading into EVs and liposomes. (**f**) RFP expression in EV and liposome lysates. (**g**) Average nanoparticle size measured by NTA at indicated time points (24, 48, and 72 hours). Data are presented as mean ± standard error from five replicates. (**h**) Quantitative Fluorescence intensity of Lung-EVs, HEK-EVs, and liposomes normalized to nuclei in lung parenchymal cells; n = 3 per group. $^{**}p \le 0.01$, $^{***}p \le 0.001$. Reprinted from Extracellular Vesicles, Volume 1, Popowski et al, with permission from Elsevier.[146]

Zhang et al investigated the use of bovine milk-derived EVs loaded with SARS-CoV-2 RBD mRNA to induce a neutralizing antibody response in mice. The mRNA was complexed with DOTAP and fused with purified EVs, achieving a high loading efficiency (~57%) and maintaining vesicle morphology. The formulations were administered by oral gavage and intraduodenal (intestinal) injection, leading to robust neutralizing antibody production and detectable antigen expression in the intestinal tract. The study highlights the feasibility of producing non-invasive oral mRNA vaccines using food-grade EVs and demonstrates the potential for scalable, stable, and effective mucosal immunization approaches.[143]

Pomatto et al developed an oral mRNA vaccine platform using plant-derived (citrus sinensis) EVs loaded with SARS-CoV-2 S1 mRNA via osmotic stress and proprietary cationic protein treatment. This method yielded a loading efficiency of ~45%, with vesicle integrity preserved after lyophilization, supporting stability at room temperature for over 12 months. Oral capsule administration to rats induced strong mucosal and systemic immune responses, including IgG, IgA, and neutralizing antibodies, with no observed toxicity. The work demonstrates the versatility and durability of plant EVs as carriers for oral nucleic acid vaccines, offering potential for mass production and temperature-stable distribution across clinical settings (Figure 3). [145]

In summary, exogenous mRNA loading into EVs is technically demanding but continues to advance through optimization of physical, chemical, and device-based techniques. Therefore, selecting an appropriate method requires consideration of mRNA physicochemical properties, the EV source, the intended route of administration, and the therapeutic application. Moreover, critical factors influencing success include encapsulation efficiency, preservation of vesicle integrity and surface functionality, mRNA stability, and confirmed protein expression in target tissues.

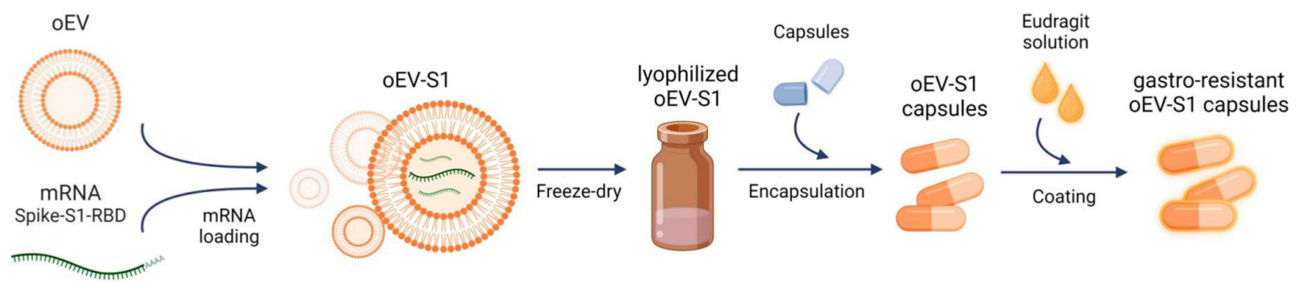

Figure 3 Workflow of plant-derived EV loading, lyophilization, and gastro-resistant capsule production for oral mRNA vaccination. Reprinted from Cells, Volume 12, Pomatto et al, with permission from MDPI.[147]

Meanwhile, ongoing innovation in buffer chemistry, loading devices, and vesicle characterization will be essential for translating these systems into safe and effective clinical platforms.[67,96,148]

Endogenous Loading and Genetic Engineering Strategies

Endogenous loading approaches for mRNA into EVs harness the intrinsic cellular machinery governing vesicle biogenesis, cargo selection, and secretion. Unlike exogenous strategies, which involve post-isolation cargo incorporation, endogenous methods rely on precise genetic manipulation of the producer cell. This enables mRNA to be selectively packaged into EVs while undergoing formation, thereby maintaining the physiological integrity and molecular complexity of the vesicles (Figure 4 and Table 5).[149,150]

Fundamentally, endogenous mRNA loading involves the integration of nucleic acid sequences into EVs via cellular processes. Producer cells are either transiently transfected or stably engineered to synthesize the desired mRNA, which then enters the endosomal sorting complex and is encapsulated within multivesicular bodies destined for EV secretion. However, passive mRNA packaging often yields suboptimal loading efficiency and lacks selectivity, presenting major hurdles for therapeutic applications.[96,106,149,154] To provide a clear decision-making framework, a comprehensive horizontal comparison between exogenous and endogenous loading techniques is summarized in Table 6. By evaluating core performance parameters, technical bottlenecks, and selection logic, this analysis clarifies how to optimize EV-based mRNA delivery according to specific research or clinical needs.

Passive Overexpression of Target mRNA

The most straightforward strategies involve overexpressing the target mRNA in donor cells via plasmid transfection, mRNA electroporation, or viral transduction. This overexpression approach leverages the natural transcriptome of producer cells,

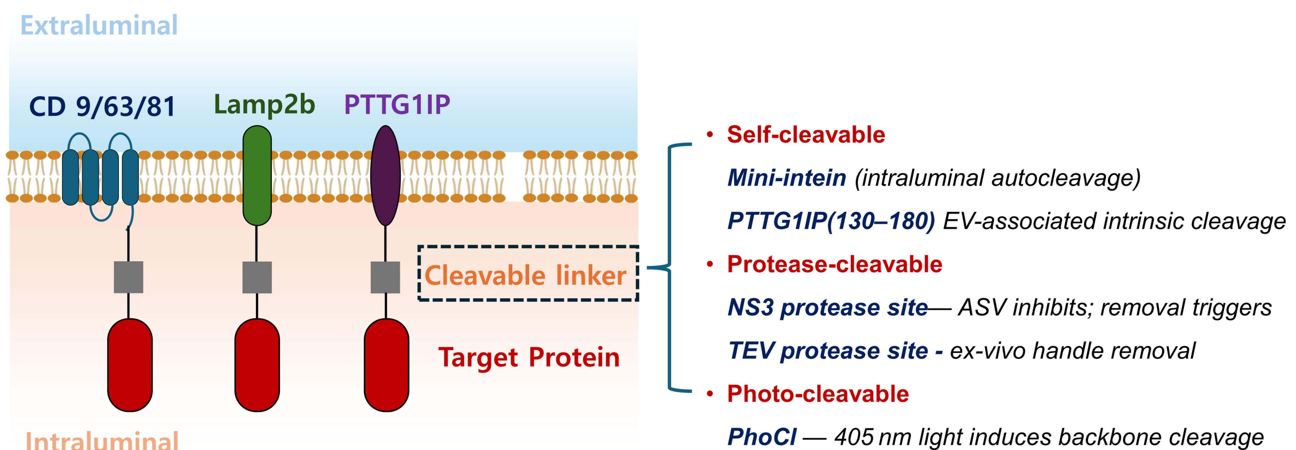

Figure 4 Strategies for selective EV cargo loading and controlled intravesicular target release using membrane scaffold proteins and engineered cleavable linkers.

Table 5 Representative Endogenous Passive mRNA Loading Strategies for EVs

Loading Strategy	Cell Type/EV Source	mRNA Cargo	Sorting/Enrichment Method	Loading Efficiency	Ref.
Passive overexpression	HEK293	HChrR6	IVT mRNA + PEI transfection	Not quantified	[151]
	Mouse liver cells/MSC/HEK293	LDLR	Plasmid transfection	10–20× enrichment	[152]
	HEK293T	IL-10	Plasmid transfection	Not quantified	[153]

Table 6 Comparative Analysis and Selection Logic for mRNA Loading Strategies

Loading Category	Strategy	Efficiency & Integrity	Selection Logic	Main Bottleneck	Ref.
Endogenous	Passive Overexpression	Low / Excellent	Best for mass production with high biocompatibility	Low loading efficiency; limited to smaller mRNA	[96, 155–157]
Exogenous	Electroporation	Medium / Moderate	Best for rapid screening of diverse mRNA sequences	RNA aggregation and membrane damage	[96, 158–161]
	Freeze-thaw	High / High	Best for maximizing cargo density per single EV	Scalability & Cargo loss	[96, 161–164]
	Sonication	High / Low	Suitable for robust EV types	Loss of EV structural integrity	[162–164]

allowing stochastic packaging of cytoplasmic transcripts into EVs. Passive overexpression is technically simple, cost-effective, and broadly applicable across cell types and target mRNAs, with minimal engineering requirements.

While encapsulation efficiency and cargo specificity tend to be relatively low and variable, passive overexpression has distinct advantages, such as preserving the physiological composition and microenvironment of EVs; moreover, passive overexpression is less likely to disrupt vesicle structure, biogenesis pathways, or critical cell signaling compared to exogenous manipulation or aggressive sorting systems.[127,165] Passive strategies also facilitate initial proof-of-concept studies and high-throughput screening of mRNA–EV interactions, guiding subsequent optimization steps.

Li et al (2021) demonstrated passive loading of mRNA into EVs by simply overexpressing low-density lipoprotein receptor (LDLR) mRNA in donor AML12 cells, without any sorting motifs or scaffold proteins. The resulting EVs (EV-LDLRs) showed a dramatic increase in encapsulated LDLR mRNA, which remained stable and could be translated into protein upon delivery to recipient cells. In a hypercholesterolemia mouse model, intravenous injection of EV-LDLRs effectively restored LDLR expression in the liver, improved lipid profiles, and reduced atherosclerotic plaque formation. Therefore, the study provides clear proof of concept that passive overexpression in donor cells is sufficient for effective mRNA encapsulation and in vivo functional delivery via EVs.[152,166]

Forterre et al (2020) reported that transfecting HEK293 cells with in vitro transcribed HChrR6 mRNA using PEI enabled efficient passive loading of functional mRNA into EVs. These EVs, further equipped with a targeting antibody, selectively delivered the mRNA to HER2+ tumors in mice, where the EVs activated a prodrug and inhibited tumor growth. Although this approach relies on passive cytosolic enrichment, this method involves direct mRNA transfection rather than solely endogenous gene expression. These models prove that passive, cell-mediated mRNA loading by transfection can enable effective and specific therapeutic mRNA delivery with EVs in vivo.[151]

Nevertheless, for clinical or manufacturing applications requiring precise dose control and high-level functional loading, passive strategies are often supplemented or replaced by active sorting modules or genetic fusion tags to enhance both efficiency and selectivity.[167]

Engineering Vesicle Sorting via Scaffold Proteins and RNA-Binding Domains

To address the inherent limitations of passive loading, contemporary research has increasingly turned to genetic engineering strategies that actively direct mRNA sorting and packaging into EVs.[168] Indeed, by expressing engineered scaffold proteins, membrane anchors, or RNA-binding domains within the producer cell, these approaches enable selective and efficient

recruitment of target mRNAs into vesicles during their biogenesis. Typically, this is achieved by fusing EV surface or membrane-associated proteins with targeting peptides, RNA-binding motifs, or functional tags, thereby facilitating specific interactions with cargo mRNA via engineered RNA sequence motifs or protein-protein associations.[131,169]

Engineered sorting scaffolds, including tetraspanins (CD63, CD9, CD81), membrane anchors (Lamp2b), glycosylation-dependent transmembrane proteins (PTTG1IP), and surface or luminal adapters (eg., MFGE8), have been genetically fused with peptide ligands or RNA-binding domains, such as MS2, PUFe, SYNCRIP, or hnRNPA2B1.[131,169–171] These modifications provide tunable control over mRNA encapsulation efficiency and cargo specificity, with additional inspiration from naturally evolved capsid proteins, such as Arc, which mediate packaging through viral-like mechanisms. Table 7 summarizes key scaffold proteins and domains used to load endogenous mRNA into EVs, along with the associated mechanistic roles and engineering principles.

The following section will provide a detailed overview of how EV-mediated mRNA delivery is achieved through EV sorting proteins and RNA-binding domains.

Hung & Leonard (2016) present the targeted and modular EV loading (TAMEL) platform for actively loading cargo RNA into EVs. This system utilizes EV-enriched membrane proteins (Lamp2b, CD63, etc.) fused to an MS2 RNA-binding domain, while the target mRNA is engineered to contain MS2 stem-loop motifs. This enables high specificity and efficiency in packaging mRNA into EVs. Using this strategy, the authors achieved up to a 6-fold increase in mRNA loading into EVs and up to 40-fold in vesicles containing the vesicular stomatitis virus (VSV-G) protein. Importantly, TAMEL-mediated active loading is far more effective for short RNAs (under 0.5kb) than for longer mRNAs (over

Table 7 Key EV Sorting/Scaffold Proteins for Endogenous mRNA Loading

Category	Protein	Core Mechanistic Function	Loading Principle/Engineering Notes	In vivo	Ref.
Membrane scaffold	Lamp2b	Transmembrane exosomal protein that anchors genetically fused targeting motifs/cargo and supports targeted EV delivery.	Genetic fusion of targeting peptides (eg., CTP, vMIP-II) to Lamp2b enables selective surface display and enhanced cargo loading/delivery.	BALB/c male	[172]
	CD9, CD81	Tetraspanins act as EV membrane scaffolds that organize membrane microdomains and support vesicle budding and membrane fusion-mediated cargo delivery.	Genetic fusion of functional cargos (eg., tTA) to CD9/CD81 enables efficient EV loading and cytosolic cargo release upon membrane fusion.	N/A	[173]
Cargo sorting module	CD63	Tetraspanin family; organizes intraluminal vesicles, nucleic acid/protein sorting.	Genetic fusion of RBDs or aptamer tags; enables efficient mRNA/protein sorting.	Melanoma mouse	[174]
Glycosylation-based	PTTG1IP	N-glycosylation-dependent transmembrane; facilitates luminal cargo sorting and release.	Genetic fusion of large cargo proteins or RNAs, self-cleaving modules added if needed.	Mouse xenograft	[175]
Surface anchor	MFGE8 (C1C2)	Binds EV surface lipids (C1/C2 domains); functions as a stable anchor for displaying fused proteins/peptides on the EV exterior.	Direct fusion of proteins to the MFGE8 sequence enables efficient EV surface display and enhances targeted delivery (eg., $\alpha v\beta 3$-directed uptake).	N/A	[176]
RNA-binding protein	SYNCRIP	Recognizes RNA EXO motif; guides high-fidelity miRNA/mRNA sorting during EV biogenesis.	Overexpression or introduction of corresponding RNA motifs; motif-engineering enhances cargo loading.	N/A	[177]
	hnRNPA2B1	Recognizes motif-tagged miRNAs; SUMOylation increases cargo recruitment specificity.	RNA motif engineering, hnRNPA2B1 overexpression modulates selective miRNA encapsulation.	N/A	[178]
Capsid particle	Arc	Forms capsid-like complexes with mRNA; mediates encapsulation and intercellular EV transfer.	Exploits natural Arc sorting or engineered complex formation for mRNA packaging in EVs.	N/A	[179]

1.5kb), demonstrating that RNA size is a key determinant of loading efficiency. In summary, TAMEL is a versatile genetic engineering technology for selectively and efficiently packaging mRNA into EVs by fusing RNA-binding domains to EV proteins and engineering cognate motifs onto cargo RNA.[30]

Zickler et al (2024) present a novel EV engineering platform that enables highly selective and efficient mRNA loading by fusing the EV marker CD63 to the PUFe RNA-binding domain and stably expressing target mRNAs in producer cells. Co-expression of the fusogenic protein VSV-G further boosts endosomal escape and functional mRNA delivery. The authors demonstrate the broad utility of this system for reporter and therapeutic mRNA delivery and show that, in a melanoma mouse model, mRNA-loaded EVs induce durable tumor remission at ultra-low doses—far surpassing conventional nanoparticle approaches. This platform overcomes major barriers to therapeutic mRNA delivery and opens new avenues for mRNA-based cancer immunotherapy and gene editing applications (Figure 5).[174]

Perez et al (2024) developed a new EV delivery platform based on the transmembrane protein PTTG1IP, leveraging the associated N-glycosylation sites for highly efficient cargo loading. Indeed, by fusing therapeutic proteins (such as Cre recombinase and Cas9) or protein-RNA complexes to PTTG1IP and employing self-cleaving sequences for cargo release, the authors achieved superior delivery and functional activity in both cultured cells and mouse tumor models compared with

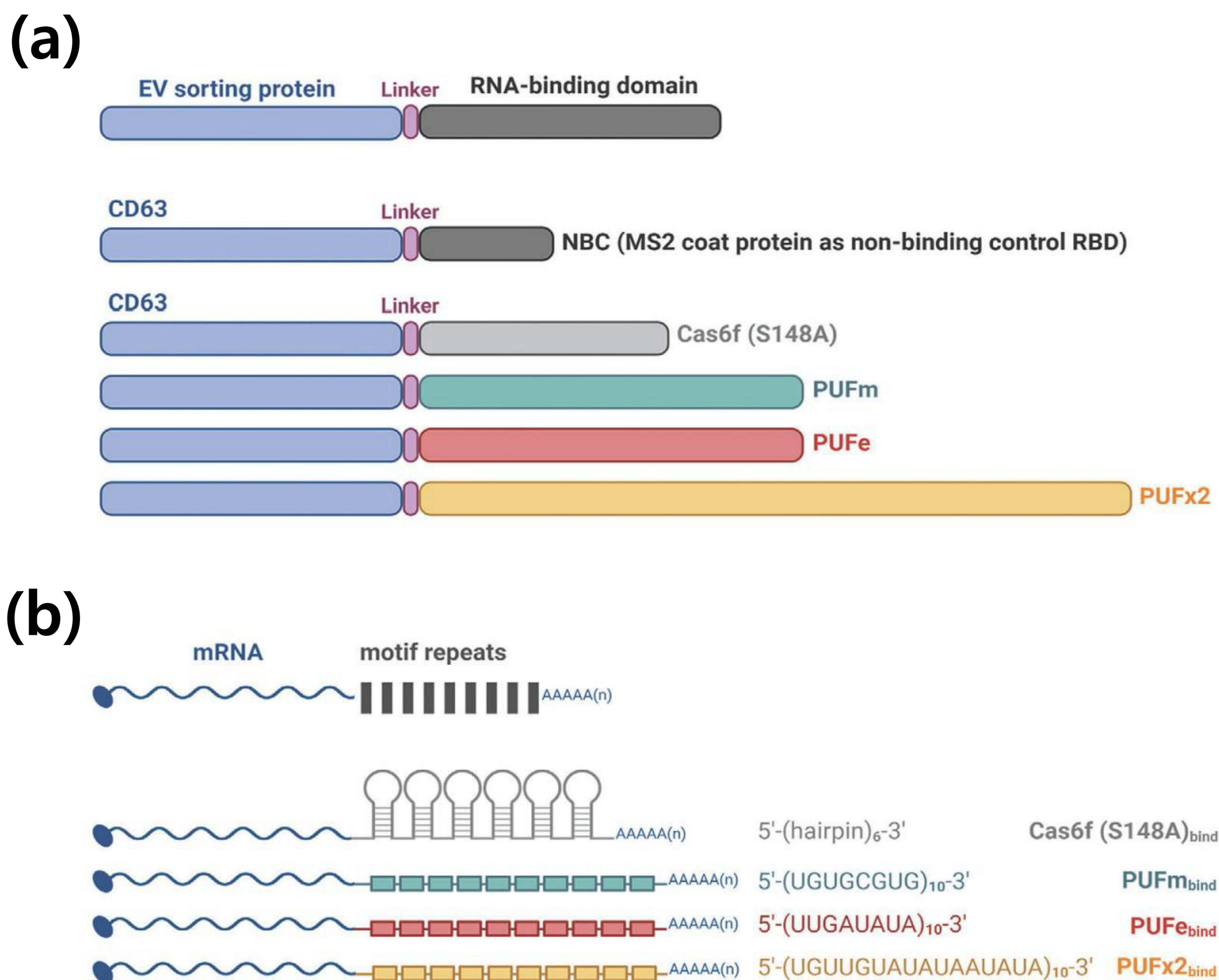

Figure 5 Engineering strategy for selective mRNA loading into extracellular vesicles via CD63–PUF fusion proteins and motif-optimized target mRNAs. (**a**) EV sorting mechanism: To enable RNA recruitment, the C-terminus of CD63 was conjugated with several RNA-binding modules—including a non-catalytic Cas6f mutant and engineered PUF variants (PUFm, PUFe, and PUFx2)—via a glycine-rich linker. (**b**) mRNA design strategy: The cargo mRNA sequences were codon-optimized and modified to incorporate multiple,[6–10] high-affinity recognition motifs within the 3'UTR, ensuring specific interaction with the corresponding sorting domains. Reprinted from Advanced Science, Volume 11, Zickler et al, with permission from Wiley.[52]

traditional EV scaffolds, such as CD63. Meanwhile, further engineering of PTTG1IP improved the associated loading and targeting capabilities of this platform, and the system demonstrated potency for gene editing via Cas9 delivery. Thus, this platform offers a versatile and effective solution for EV-mediated delivery of complex therapeutic payloads (Figure 6). [175] Table 8 introduces representative endogenous mRNA-loading and engineering strategies for EVs, highlighting key design principles and mechanistic features that enable efficient, programmable cargo incorporation.

Endogenous Cargo Release Mechanisms in Engineered EVs

Incorporating controlled cargo-release mechanisms into engineered EVs can enhance the functional performance and versatility of therapeutic protein or enzyme delivery. Techniques that allow separation of the active cargo from anchoring scaffold proteins, such as Lamp2b, CD63, or PTTG1IP, inside the recipient cell environment help maximize bioactivity at the target site.[184] A range of endogenous release strategies, including self-cleaving mini-inteins, protease-sensitive linkers, and photo-inducible/optogenetic modules, have been validated, each contributing unique advantages for specific applications and delivery contexts.[175,185] Table 9 summarizes major approaches and the associated features, reflecting the diversity and growing sophistication of controlled EV cargo release technologies.

Physical Enhancement: Device-Driven and Substrate-Based Strategies

The productivity and cargo profile of EVs are highly sensitive to the physical microenvironment experienced by producer cells. Sophisticated physical stimulation techniques have emerged as robust alternatives to chemical or hypoxic priming, offering new opportunities to engineer EVs at scale with enhanced loading of functional nucleic acids and proteins. These strategies employ device-guided physical cues, such as electrical, mechanical, acoustic, and topographical signals, to modulate cell behavior, vesicle biogenesis, and selective cargo packaging.[189–191] While direct evidence on mRNA modulation through such physical priming remains limited, several studies indicate notable shifts in small RNA and miRNA profiles under these conditions, suggesting that broader nucleic acid cargo remodeling is achievable and potentially relevant for mRNA delivery applications (Table 10). [192,193]

Biochip Stimulation and Cellular Nanoporation

Cellular nanoporation is performed on microfabricated biochips that contain arrays of conductive nanochannels (typically ~500 nm in diameter). When cells are cultured atop these chips and exposed to programmed electrical pulses, transient nanoscale pores form in the plasma membrane. This physical perturbation facilitates rapid intracellular uptake of large biomolecules (eg., plasmid DNA, mRNA).[199,200] The electrical stimulation triggers endosomal trafficking and accelerates multivesicular body maturation, leading to the robust secretion of EVs and the preferential loading of mRNA and

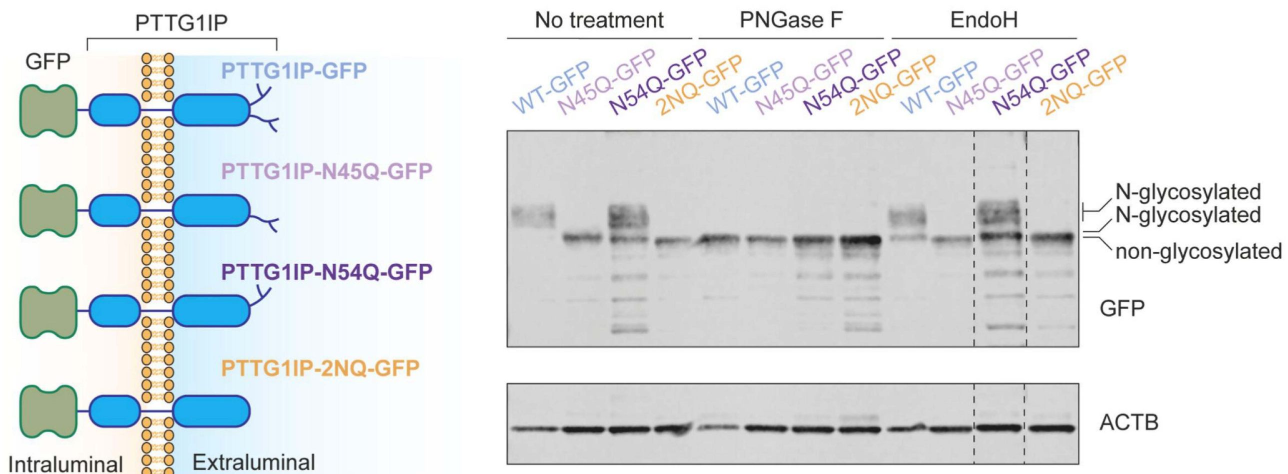

Figure 6 Engineering strategy for selective mRNA loading into extracellular vesicles via CD63–PUF fusion proteins and motif-optimized target mRNAs. [Representative immunoblotting images were acquired using a Bio-Rad Imager (Bio-Rad, Hercules, CA, USA) with standardized auto-exposure settings to ensure consistent signal detection across all samples.] Reprinted from Extracellular Vesicles, Volume 11, Perez et al, with permission from Elsevier.[53]

Table 8 Representative Endogenous mRNA Loading and Engineering Strategies for EVs

Loading Strategy/ Scaffold	Cell Type/ EV Source	mRNA Cargo and Tag	Sorting/Enrichment Method	Loading Efficiency	In vivo	Ref.
Translational repression (puromycin-mediated) + P1h3–Lamp2b fusion (HER2 targeting) + Ce6 (sonodynamic therapy)	HEK293T	GSDMD-N mRNA; P1h3-Lamp2b (HER2-specific scFv)	Puromycin suppresses mRNA translation for efficient EV encapsulation; P1h3–Lamp2b fusion for HER2+ cell targeting; Ce6 for enhanced release/ translation in recipient cells	~6 mRNA copies per 100 EVs (qPCR)	Nod scid female mice, BALB /c female mice	[180]
DNA aptamer-mediated encapsulation (translation inhibition + CD9-ZF)	HEK293T, mouse adipocytes, colon	*Pgc1α* mRNA, IL-10 mRNA; (aptamer targeting AUG, ZF motif, adipocyte-targeting ATS-Lamp2b)	DNA aptamer blocks ribosome at AUG for translation inhibition; aptamer-ZF interaction promotes active sorting into CD9-ZF EVs; ATS-Lamp2b for tissue targeting	~1.2 *Pgc1α* mRNA/EV (qPCR); IL-10 mRNA 3× vs. control EVs (qPCR)	Inflammatory bowel disease (IBD) mouse	[181]
Microfluidic nanosecond electroporation (active loading + boxB-N peptide affinity) + surface antibody docking	HEK293T, MEF, mouse glioma	IFN-γ mRNA (boxB tag); CD64 overexpression, anti-CD71/PD-L1 antibody docking	nsEP dramatically increases small EV yield; CD64 overexpression docks targeting antibodies; C-terminal N-peptide binds boxB-tagged mRNA for active recruitment into small EVs	IFN-γ mRNA: 3.5× fluorescence intensity per small EV; 20% ↑ IFN-γ mRNA-positive small EVs; small EV yield: 32× (HEK293T)	GBM model	[17]
Lamp2b-RVG fusion for neuron targeting (NGF delivery)	HEK293	NGF mRNA + protein	Lamp2b fusion with RVG peptide targets neurons, EVs loaded with mRNA and protein, systemic delivery	mRNA: ~0.87% total RNA in EV; protein: 0.03%	C56BL/6 mice	[182]
MS2 aptamer-mediated sorting + CD9-MCP fusion (controllable release via competitive “releaser” aptamer)	HEK293T	LDLR mRNA (LDLR); MS2 stem-loop aptamer; “LDLRreleaser” aptamer	Co-transfection: CD9-MCP fusion, aptamer, competitive releaser; ultracentrifugation	≥3× protein expression ↑ (1:10 releaser/EV); mRNA highly enriched, efficient translation	Ldlr-/- mouse model	[166]
CD63-based NoMi/ E-NoMi (mCherry in/ out, FLAG tag, nanobody tethering, TEV removable handle), VSV-G fusogen	HEK293T	Protein: EGFP, Cre recombinase, SaCas9, fused with anti-mCherry nanobody; mCherry inside EV, 3xFLAG tag outside	Immunocapture using external 3xFLAG; optional TEV-protease cleavage for “clean” EVs	EGFP: 55% (Nb-tethered, IC), ~3.6% (non-specific-Nb); 3.4× enrichment over SEC; Cre/ SaCas9 delivery: ~10× more efficient than SEC, protein-level activity validated	Brain tumor model	[183]
Endogenous: CD63-RBD (PUFe, etc). fusion; stable genomic integration (ϕC31)	HEK293T	Nanoluc, Cre, *mOx40L* mRNA (10x PUFe motif in 3’UTR), optional VSV-G	CD63-PUFe RBD binding for active mRNA loading, VSV-G for enhanced endosomal escape	230× more by CD63-PUFe (vs. passive control); 89% mRNA - enclosed in EV; in vivo protein at 50 ng/kg dose	C57BL/6 mice	[174]

proteins. Cellular nanoporation enables precise control over EV yield and cargo content by modulating device parameters, such as voltage, pulse duration, and channel size.[194]

Yang et al (2020) developed a cellular nanoporation approach using microfabricated biochips with conductive nanochannel arrays, in which programmed electrical stimulation induces transient nanoscale pores in the cell membrane and triggers rapid uptake of nucleic acids, such as plasmid DNA. The process was optimized using a 200 V electric field with 5 pulses at 10 ms per pulse and 0.1 s intervals, which significantly minimized cellular stress while maximizing cargo delivery. This physical perturbation markedly enhances EVs biogenesis and multivesicular body maturation, resulting in robust secretion of EVs that are highly enriched in therapeutic mRNAs and targeting peptides. Notably, this method achieves up to 50-fold greater EVs yield and more than 1000-fold increase intra-EV mRNA content compared to conventional bulk electroporation or chemical transfection, exemplifying the precise control over EV cargo loading provided by biochip stimulation and cellular nanoporation mechanisms.[194]

Table 9 Established Endogenous Cargo Release Platforms in Engineered EVs

Platform/ Category	Trigger	Module	What Actually Happens	Notes/EV-Validated Examples	In vivo	Ref.
Mini-intein-engineered EVs	Self (no exogenous trigger)	Engineered mini-intein (eg., DnaB family)	Intraluminal protein splicing/self-cleavage at the extein junction detaches the cargo from the EV anchor; fusogens (eg., VSV-G) can boost endosomal escape after uptake.	Functional delivery demonstrated for Cre, Cas9; increased loading with spontaneous release.	C57BL/6 mice	[185]
Intein + TimeSTAMP	Drug-switchable protease	TimeSTAMP (HCV NS3 protease) gated by Asunaprevir (ASV) + mini-intein	ASV present → NS3 cleavage inhibited; ASV removal → NS3 cleaves the tag, releasing the cargo; the intein arm also provides self-release.	Enables temporal control of release; functional Cre delivery shown.	C57BL/6N male mice	[186]
PTTG1IP-anchored with self-cleaving linker	Self (context-dependent)	PTTG1IP (aa130-180) anchor with EV-associated intrinsic cleavage and a designed self-cleaving linker	Cargo fused to PTTG1IP is separated via intrinsic/context-dependent cleavage (reported in EV settings), increasing the fraction of free, active protein.	Human-sequence scaffold; Cre/Cas9 delivery validated. (Avoid "exclusively within EVs"; use "EV-associated").	C57BL/6 female mice	[175]
Optogenetic control (non-cleavage)	Blue light ON/OFF	CRY2-CIBN reversible dimerization (control module; not cleavage)	Light ON promotes binding and loading; light OFF triggers dissociation from the anchor, freeing the cargo (no peptide cleavage).	Efficient soluble-protein delivery with temporal control; multiple cargos shown.	C57BL/6 male mice	[187]
Photo-cleavable EVs	Violet light (405 nm)	PhoCl (photocleavable protein)	405 nm irradiation causes backbone cleavage of PhoCl, detaching the cargo from the tether.	Demonstrated with mCherry, apoptin, catalase; non-invasive optical activation.	BALB/c mice	[188]

Table 10 Cell Culture-Based Physical Stimulation Strategies for Modulating EV Cargo Profiles

Physical Enhancement Method	Principle and Mechanism	Effect on EV Cargo Profile	Representative Cell Types	In vivo	Ref.
Cellular nanoporation (biochip)	Nanochannel arrays (~500 nm) + electric pulses (200 V, 5 pulses, 10 ms/pulse) promote membrane permeabilization and endosomal trafficking	Up to 1000× increase in EV mRNA; robust gene delivery	MEFs, DCs, HEK293	BALB/c-nu and C57BL/6 mice	[194]
Nanopriming (substrate topography)	Nanotopography modulates cell morphology and signaling, especially cytoskeleton and adhesion, leading to altered EV biogenesis and selective loading of pro-osteogenic miRNAs.	Increases osteogenesis-related miRNAs (eg., miR-210-3p, miR-497-5p) in EVs, enhancing bone regeneration function.	Human bone marrow-derived mesenchymal stem cells (hBMSCs)	Mouse femoral fracture model	[195]
Ultrasound stimulation	Low-intensity pulsed ultrasound (1 MHz, 500 mW/cm^2 for 10 min) transiently permeabilizes membranes, elevates intracellular Ca^{2+}, and stimulates EV release.	Increases EV yield and can enhance mRNA/miRNA/protein loading into EVs (eg., miR-328-5p, miR-487-3p3).	Human umbilical cord MSCs (hUC-MSCs)	N/A	[196]
Fluid shear stress/ microfluidic	Controlled fluid flow induces mild shear force and mechanosignaling changes in cell metabolism and EV biogenesis	Greatly increases EV yield; shifts miRNA and protein cargo-enriches pro-regenerative and angiogenic signatures	hMSCs/MSCs (human mesenchymal stem cells)	N/A	[197, 198]

Nanopriming via Substrate Topography and Micropatterning

Substrate-based nanopriming leverages engineered micro- and nanotopographical cues, such as ridges, grooves, pillars, or hierarchical patterns, on cell culture surfaces to physically "prime" producer cells and modulate the biogenesis and cargo profile of EVs. This approach provides robust, non-chemical control over both the quantity and quality of secreted EVs, enabling the enhancement of functional cargo loading of various nucleic acids and proteins through microenvironmental mechanotransduction.[191] When cells interact with patterned substrates, integrin-mediated adhesion and cytoskeletal

remodeling transmit mechanical signals that can alter gene expression, intracellular trafficking, and vesicle packaging pathways. This results in significant shifts in the molecular composition of EVs, often favoring enhanced loading of desired regulatory molecules, and can be tuned by altering the geometry, scale, and chemical properties of the substrate features.[191,195]

Ma et al (2022) showed that nanotopography-engineered titanium surfaces induce human MSCs to secrete small EVs with a miRNA profile that shifts dramatically over time, particularly boosting osteogenic-specific miRNAs, such as miR-210-3p and miR-497-5p, by day 21, and thereby strongly promoting bone regeneration both in vitro and in vivo. Although comprehensive mRNA analysis was not performed, this work is significant as the data exhibited that nanoscale surface cues can program the miRNA cargo of small EVs to enhance osteogenesis via key bone-related signaling pathways. The approach employed in the study underscores the therapeutic potential of using engineered nanotopographical cues to harness cell-derived vesicle contents for advanced bone tissue engineering (Figure 7).[195]

Ultrasound-Induced Cellular Stimulation

Ultrasound stimulation employs acoustic waves to apply mechanical forces to cultured cells. This physical perturbation leads to microbubble formation and transient increases in membrane permeability,[201] allowing for a surge of calcium entry and

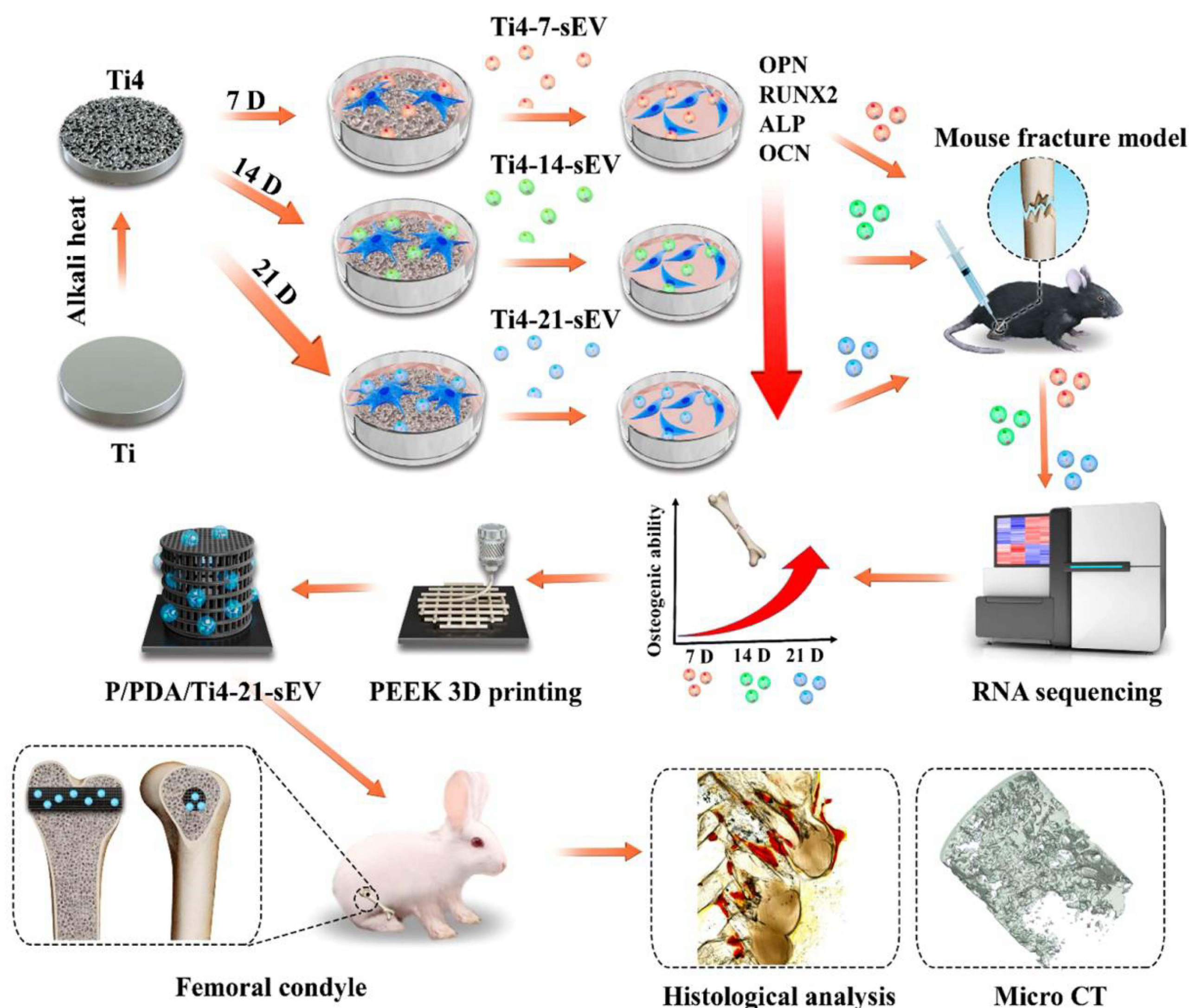

Figure 7 Schematic illustration of nanotopography-engineered titanium-induced secretion of osteogenic EVs and their application in three-dimensional-printed polyether-etherketone (PEEK) scaffolds for bone regeneration. Reprinted from ACS Nano, Volume 16, Ma et al, with permission from American Chemical Society.[95]

 https://doi.org/10.2147/IJN.S595161

activation of stress-response pathways.[202,203] The resulting intracellular signaling cascade, including calcium-triggered activation of endosomal machinery such as the endosomal sorting complex required for transport (ESCRT) complex, enhances EVs biogenesis and promotes the efficient encapsulation of regulatory miRNAs, therapeutic mRNAs, and various proteins within EVs. Thus, adjusting ultrasound application parameters enables optimized particle production while maintaining cell viability, making this method a versatile and scalable approach for therapeutic EV manufacturing.[204–206]

Yin et al (2025) systematically investigated how low-intensity pulsed ultrasound (LIPUS) preconditioning modifies the miRNA cargo of stem cell-derived EVs using both deep sequencing and qPCR. In this study, LIPUS treatment was optimized at a frequency of 1 MHz and an intensity of 500 mW/cm^2 for a duration of 10 minutes, with the ultrasound probe positioned 1 cm above the cell monolayer to ensure reproducible stimulation. Yin et al (2025) identified a set of miRNAs that were significantly up- and downregulated in EVs after LIPUS treatment. Using bioinformatics, the study predicted that these miRNA changes could affect a broad network of target mRNAs involved in major pathways, including cell cycle, MAPK, and Hippo signaling. Although the direct mRNA content within EVs was not measured, pathway network analysis identified regulatory hubs that may underpin the enhanced therapeutic effects of LIPUS-primed EVs. This work highlights that physical priming by LIPUS can deliberately reprogram EV molecular content—particularly miRNAs—suggesting new strategies to optimize stem cell EV therapies for regenerative medicine.[196]

Fluid Shear Stress and Microfluidic Stimulation

Fluid shear stress, delivered through controlled fluid flow in microfluidic systems or dynamic agitation in bioreactors, serves as a powerful physical cue to modulate EV biogenesis and cargo composition.[190,207,208] As fluid moves across the cell surface, mechanical forces activate stretch-sensitive ion channels and trigger cytoskeletal reorganization, leading to altered gene expression[209] and upregulation of vesicle biogenesis pathways.[192] These changes enhance endosomal trafficking and vesicle budding, resulting in increased EV secretion and selective enrichment of bioactive molecular cargo, including signaling peptides, miRNAs, and proteins, tailored to the intensity and duration of mechanical stimulation.[193,208,210] Hence, by precisely tuning fluid dynamics and microenvironmental parameters, researchers can customize the yield and molecular profile of EVs for specific therapeutic and diagnostic applications.[208,211]

Jeske et al (2023) investigated the effects of bioreactor-based fluid shear stress and three-dimensional (3D) microenvironment on the production and molecular characteristics of EVs derived from human mesenchymal stromal cells (hMSCs). Using a novel vertical-wheel bioreactor system with 3D microcarriers, Jeske and co-authors demonstrated that bioreactor culture significantly increased EV yield (2.5- to over 5.5-fold per cell compared to static two-dimensional (2D) culture) and robustly altered EV cargo profiles at both the miRNA and protein levels. Notably, bioreactor-derived hMSC-EVs were enriched in "mechano-miRNAs" such as miR-10, miR-19a, miR-19b, miR-21, miR-132, and miR-377, all implicated in angiogenic and neuroprotective processes. Proteomic analysis revealed upregulation of metabolic, autophagy, and reactive oxygen species (ROS)-related proteins in EVs harvested from the bioreactor cultures. The authors further showed that the observed changes correlated with upregulation of genes involved in EV biogenesis and glycolysis, and that the scalable bioreactor platform consistently produced EVs with these enhanced cargo features. This study provides direct evidence that fluid shear stress and dynamic bioreactor environments can be leveraged to upscale hMSC-EV production while modulating the therapeutic molecular payload for regenerative applications.[197]

Hybrid EVs

Hybrid EVs have recently emerged as a powerful strategy to overcome the intrinsic limitations of natural EVs, particularly the low and variable efficiency of mRNA loading.[158,212] Among the various designs, membrane fusion between EVs and synthetic lipid-based carriers such as liposomes, LNPs, or cubosomes has become the predominant approach. Moreover, the lipid bilayers of EVs and synthetic nanoparticles can merge to form hybrid vesicles through physical processes such as freeze-thaw cycling, extrusion, or electroporation. These constructs effectively combine the high loading efficiency of synthetic carriers with the biocompatibility, immune tolerance, and tissue tropism of EVs, enabling superior delivery outcomes compared with either system alone (Table 11). [213–215]

Table 11 Representative Hybrid EVs Strategies for mRNA Delivery

Hybrid Strategy	Lipids Used	EV Source	Headline Findings	In vivo	Ref.
DNA-lipid (LiNA) EV-liposome fusion with toehold-release purification	Liposomes with lipidated ssDNA (LiNA); also compared SM-102 LNPs	Mouse C2C12 myoblast EVs	~2 mRNA copies/EVL; scalable to 10^{13}; higher expression vs. LNP; low cytotoxicity	N/A	[216]
Rapid cubosome-EVs fusion ("mix-and-load"; ~10 min)	GMO/DOTAP/PEG-lipid ≈ 95/4/1 mol%	HeLa, MDA-MB-231, HBMEC EVs	~98% mRNA encapsulation; full fusion in 10 min; ~2× BBB transport; sTable 3 weeks at RT	N/A	[22]
Freeze-thaw fusion of FA-modified LNPs with EVs (ELNPs)	LNP: DOTAP/Chol/DOPE/DSPE-PEG-FA	Human HEK293T EVs	235 nm, PDI 0.24; 89% encapsulation; tumor inhibition and survival ↑ in CRC models; no toxicity	Mouse xenograft, AOM/DSS model	[217]
CELLNP (microfluidic EV-LNP mix) vs HEV (pH-driven EV-LNP fusion)	LNP with ionizable lipid, helper lipids, PEG-lipid	Human hiPSC-derived EVs	EndoEV <0.05%, CELLNP ~2.5%, HEV ~73.3% fusion; strong reporter expression in vitro	N/A	[218]

Lipid Composition and Fusion Efficiency

The success of membrane fusion depends heavily on the lipid composition of the synthetic partner. Helper lipids, such as dioleoylphosphatidylethanolamine (DOPE), induce negative curvature in the bilayer, lowering the energy barrier for stalk and hemifusion intermediates, and thereby promoting efficient EV-liposome fusion.[219] In contrast, saturated phosphatidylcholines, such as DSPC or DOPC, stabilize the lamellar phase, thereby reducing membrane fluidity and slowing fusion. Cholesterol plays a dual role: at moderate levels, particularly when combined with PE lipids, cholesterol stabilizes hemifusion structures and supports membrane remodeling; however, excessive cholesterol content increases bilayer order and rigidity, reducing both fusion efficiency and subsequent endosomal escape.[220] PEGylated lipids, while critical for prolonging circulation and reducing nonspecific uptake, can sterically hinder vesicle-vesicle contact during fusion.[221] Therefore, fusion is optimized by minimizing the PEG-lipid mol% during the fusion step, with PEGylation reintroduced post-insertion if long-circulating formulations are required.[222] Interestingly, short-chain PEG-lipid conjugates (C9-C12) have been reported to facilitate initial membrane contact and even enhance hybrid EVs formation, suggesting that PEG chemistry can be tuned rather than eliminated.[223] Collectively, these findings underscore that lipid composition is not merely a formulation parameter but a central determinant of hybrid EVs formation and function.

Cubosome–EV Hybrids: A Recent Breakthrough

In addition to classical liposomes and LNPs, cubosomes—nanoparticles with an internal bicontinuous cubic lipid phase typically composed of glycerol monooleate (GMO) or phytantriol stabilized by pluronic F127—have recently been adapted for hybridization with EVs.[224,225] Cubosomes possess a highly curved and interconnected internal structure, which offers a high loading capacity and promotes strong fusogenic interactions with biological membranes.[226] A landmark study published in *Nature Communications* (2025) demonstrated that simply mixing cubosomes loaded with in vitro-transcribed mRNA with EVs at room temperature for 10 minutes was sufficient to induce spontaneous membrane fusion, yielding hybrid EVs with nearly 100% mRNA encapsulation efficiency. Remarkably, the resulting hybrids maintained the biological identity and targeting tropism of the parental EVs, while achieving efficient mRNA expression in recipient cells. An in vivo analysis demonstrated that these cubosome-EV hybrids could cross the BBB; meanwhile, by adjusting the EV-to-cubosome ratio, the researchers could fine-tune the balance between BBB absorption and systemic transport.[22] This study highlights the potential of cubosome-EV hybrids to simplify manufacturing workflows while unlocking new therapeutic opportunities, particularly for neurological diseases where BBB penetration remains a major bottleneck (Figure 8).

Advantages, Challenges, and Future Perspectives

Hybrid EVs offer several advantages over either single, natural EVs or synthetic carriers: (i) enhanced mRNA encapsulation efficiency comparable to or exceeding LNPs, (ii) improved biocompatibility and reduced immunogenicity

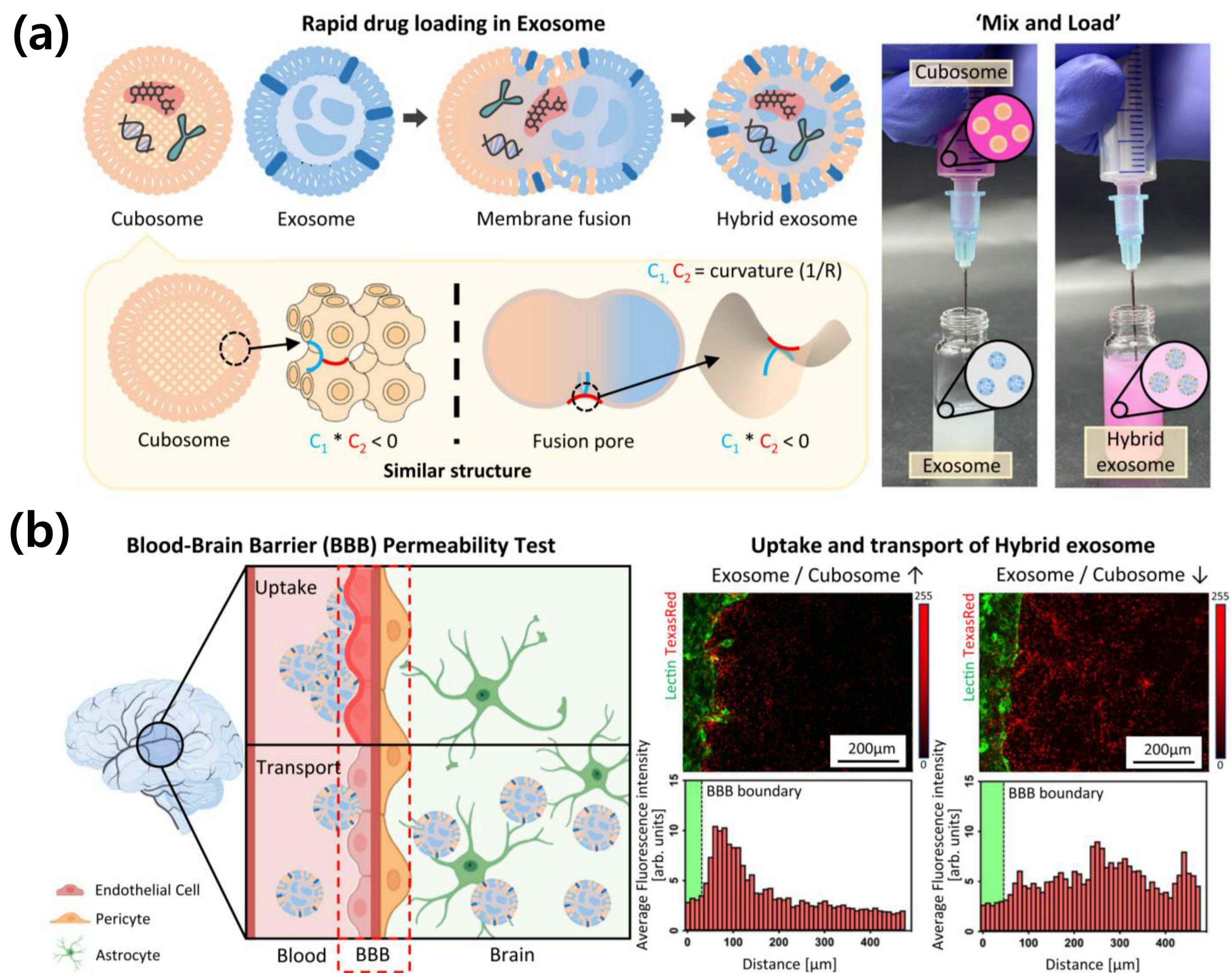

Figure 8 Fusogenic cubosome–EV hybrid system enabling rapid drug encapsulation and enhanced BBB permeability. (**a**) Schematic illustration of the membrane fusion mechanism between fusogenic lipid nanoparticles (cubosomes) and EVs, designed for rapid and highly efficient drug encapsulation. This fusogenic approach enables a streamlined "mix-and-load" workflow. (**b**) Evaluation of blood-brain barrier (BBB) permeability for the resulting hybrid EVs. The efficiency of BBB uptake and transcytosis can be finely tuned by optimizing the EV-to-cubosome ratio. Scale bars in the fluorescence images represent 200 μm. Reprinted from Nature Communications, Volume 16, Son et al, with permission from Springer Nature.[22]

owing to the EV membrane, and (iii) intrinsic or engineered tissue tropism that can be harnessed for precision delivery.[227–229] However, critical challenges remain. The reproducibility and scalability of membrane fusion processes, especially under GMP conditions, require further optimization. The stability and uniformity of hybrid vesicles must be carefully controlled, as batch-to-batch variation can compromise both efficacy and safety.[147,230] Regulatory considerations are also complex: hybrid EVs may be regarded as new biological-synthetic combination products, necessitating rigorous safety assessment to evaluate both endogenous EV cargo and synthetic components.

Looking forward, the rational design of lipid composition, particularly the balance of helper lipids, cholesterol, and PEG-lipids, together with emerging nanostructures such as cubosomes, is likely to drive the next generation of hybrid EVs systems. Furthermore, integrating programmable loading strategies (RNA-binding domains, sorting motifs) with hybrid platforms could synergistically enhance selectivity, loading efficiency, and translational applicability. Such innovations position hybrid EVs, and especially fusion-based systems, as a leading platform for future mRNA therapeutics in oncology, vaccination, and central nervous system diseases.[22,168,169,231]

Table 12 Registered First-in-Human and Early-Phase Clinical Studies of EV-Mediated mRNA Delivery

Candidate	Clinical Identifier	Indication	EV-mRNA Cargo	Study design/Phase	Public Results
ENDFH	NCT05043181	Homozygous familial hypercholesterolemia (HoFH)	LDLR mRNA-loaded exosome nanoplatform	First-in-human, Phase 1	Not publicly reported
SPOT-mRNA01	NCT06567119*	Skin aging/healthy adults	COL1A1 mRNA-loaded EVs	Randomized, double-blind, placebo-controlled exploratory phase 1	Not publicly reported
SPOT-mRNA03	NCT07188012	Duchenne muscular dystrophy	Muscle-targeted EVs loaded with full-length dystrophin mRNA	First-in-human, open-label, single-arm exploratory study	Not publicly reported
ZZSW-01	NCT07240974	Relapsed/refractory B-cell malignancies	Extracellular vesicle carrying functional CD19 CAR mRNA	Single-center, single-arm, open-label, dose-escalation early-phase 1	Not publicly reported

Clinical Translation Progress: IND-Enabling Studies and Clinical Readiness

The clinical translation of EV-mediated mRNA delivery has transitioned from conceptual proof-of-concept to rigorous Investigational New Drug (IND)-enabling evaluations and early-phase clinical applications.[232] A primary hurdle for regulatory approval is establishing a robust safety, biodistribution, and immunogenicity profile. Recent preclinical milestones have provided compelling evidence in this regard; for instance, fibroblast-derived EVs loaded with VEGF-A mRNA via cellular nanoporation (CNP) demonstrated efficient, dose-dependent protein expression in ischaemic tissues. Crucially, compared to viral vectors (AAV) or synthetic lipid nanoparticles (LNPs), these VEGF-A EVs did not trigger innate or adaptive immune responses even upon serial administration, satisfying a key safety prerequisite for clinical entry.[74]

Furthermore, the development of targeted platforms, such as cardiac progenitor cell-derived EVs (CPC-EVs), has shown that engineered EVs can minimize off-target accumulation in the liver while maximizing mRNA delivery to the heart. RNA-seq analyses of these platforms revealed minimal transcriptomic disruptions, further bolstering their profile as a safe alternative to conventional synthetic carriers.[233] Advancements in large-scale cGMP-compliant manufacturing and sophisticated engineering are now addressing the “potency-to-dose” ratio challenges essential for standardized clinical protocols.[232]

While large-scale efficacy results are still maturing, first-in-human trials in oncology and regenerative medicine are currently validating the safety and dose-tolerance of EV-mRNA candidates. These milestones collectively suggest that EV-based mRNA delivery is moving beyond experimental research toward a clinically viable therapeutic reality, bridging the gap between benchtop innovation and human application (Table 12).

Current Limitations and Future Directions of EVs as Carriers of mRNA: Opportunities and Challenges

EVs offer a biologically attractive platform for mRNA delivery because of their endogenous origin, membrane protection, and potential for tissue-selective interactions. However, despite these advantages, several technical and translational barriers still limit their broader clinical development.

A major challenge is **loading efficiency**. Endogenous loading strategies often result in low and variable mRNA copy numbers per vesicle, whereas exogenous approaches such as electroporation, sonication, or freeze–thaw cycles may compromise vesicle integrity and cargo stability.[234,235] In addition, loading performance is often influenced by mRNA size, formulation conditions, and the physicochemical properties of the carrier system. Although hybrid platforms such as EV–liposome or other engineered vesicle systems have improved encapsulation efficiency, reproducibility remains highly dependent on formulation composition and processing parameters.[15,236,237]

This variability has direct implications for clinical translation and regulatory development. In particular, inconsistent loading and batch heterogeneity complicate dose definition, potency assessment, and the establishment of robust product specifications. These issues are especially important in the context of Chemistry, Manufacturing, and Controls (CMC),

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where batch-to-batch consistency, comparability, and release criteria are essential for IND-enabling development and subsequent clinical evaluation.

Another major limitation is formulation stability. Although the EV membrane can partially protect encapsulated RNA, degradation during storage, transport, and in vivo circulation remains a significant concern. Some engineered EV formulations have shown improved short-term stability, but the long-term preservation of intact and functional mRNA has not yet been sufficiently validated under clinically relevant conditions. This is particularly important for scalable manufacturing and multi-site clinical use, where reproducible storage and transport conditions are required. Approaches such as lyophilization and rational excipient design may improve product robustness, but their effects on EV integrity, cargo retention, and biological activity require careful optimization.[22,238,239]

Scalability and purification also remain unresolved challenges. The production of EV-based mRNA therapeutics at clinically meaningful scale requires not only high-yield upstream manufacturing, but also downstream processes that maintain purity, integrity, and functional consistency. Technologies such as bioreactor-based cell expansion and tangential flow filtration have improved production feasibility, yet the co-isolation of non-EV components and the variability inherent to cell-derived products still complicate standardization. These limitations make it difficult to define a consistent manufacturing baseline suitable for cGMP-compatible production.

In addition, analytical characterization remains an important bottleneck. Conventional readouts such as particle number, protein markers, or total RNA content are useful but insufficient to fully evaluate mRNA-loaded EV products intended for therapeutic use. For clinical translation, more advanced analytical methods will be needed to assess loading efficiency, intact cargo content, vesicle integrity, purity, and functional potency in a standardized manner. Such tools will be essential not only for quality control, but also for comparability assessment when manufacturing processes, donor cells, or formulation conditions are modified.

Despite these challenges, the field continues to advance through multiple engineering strategies. Rational lipid design, programmable loading technologies, and hybrid nanostructures are being developed to improve loading efficiency, endosomal escape, and product stability. At the same time, growing attention is being paid to the integration of these innovations with translational requirements, including CMC development, cGMP-compatible workflows, and clinically relevant formulation design. Therefore, future progress in EV-mediated mRNA delivery will depend not only on improving delivery performance, but also on establishing reproducible, analytically supported, and regulatorily tractable manufacturing frameworks.

Overall, EVs remain a promising platform for mRNA therapeutics, but their successful clinical translation will require a closer alignment between engineering innovation and pharmaceutical development. Addressing loading variability, stability, scalability, and analytical standardization in an integrated manner will be critical for moving EV-based mRNA systems from experimental platforms toward clinically applicable therapeutics.

Conclusions

The landscape of mRNA delivery is undergoing a significant transformation, moving away from simple EV loading toward the use of sophisticated, tailor-made hybrid nanostructures. This evolution, as this review highlights, is thanks to combining programmable RNA-binding proteins with cleverly designed lipid components. This pairing has emerged as a pivotal advancement, solving long-standing issues regarding mRNA loading efficiency and stability. While there are still practical challenges to address—such as inconsistencies between manufacturing batches and size-dependent loading constraints—the development of hybrid structures like EV-LNP fusions and cubosomes offers a clear path toward clinical application. For EV-based mRNA therapies to truly succeed in the clinic, the focus must now shift beyond laboratory-scale validation. Ensuring that these sophisticated designs can be produced reliably and perform consistently is essential. Bridging the gap between engineering breakthroughs and predictable, high-quality therapeutic products remains the next major challenge, and mastering this transition will be what finally establishes EVs as a dependable pillar of modern genetic medicine.

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.

Funding

This work was supported by the National Research Foundation of Korea (NRF), which was funded by the Korean government (MSIT)(2022R1C1C2002949, RS-2026-25475042, and RS-2026-25472341). This research includes results partly supported by the "Gyeonggi Regional Innovation System & Education Project (Gyeonggi RISE Project)", supported by the Ministry of Education and Gyeonggi Province (grant No. 20250093).

Disclosure

The authors report no conflicts of interest in this work.

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