

Red Blood Cell-Derived Extracellular Vesicles for Gene and RNA Therapeutics: Biological, Engineering, and Translational Challenges

Tayyab Shafiq^{1,2,3}, Nawaz Khan^{1,3}, Tehreem Kausar^{1,3}, Waqas Ahmed^{1,3}, Zihao Zhang^{1,2,4}, Yujie Liang⁵, Li Duan¹⁻³

¹School of Biomedical Engineering, Shenzhen University Medical School, Shenzhen University, Shenzhen, People's Republic of China; ²Department of Orthopedics, the First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, People's Republic of China; ³Medical Innovation Technology Transformation Center, Shenzhen Second People's Hospital, Shenzhen, People's Republic of China; ⁴Guangxi University of Chinese Medicine, Nanning, People's Republic of China; ⁵Key Laboratory of Cell and Biomedical Technology, Jining Medical College, Jining, People's Republic of China

Correspondence: Li Duan; Email duanl@szu.edu.cn; yujieliang@mail.jnmc.edu.cn



Abstract: Gene therapy has great prospects of DNA/RNA manipulations and protein modulations. Its use in clinic is, however, stifled by risks of immunogenicity, low target specificity, and adverse effects. The red blood cell (RBC-EVs) extracellular vesicles can serve as a solution to this issue since they are biocompatible, long-term stable, and with low immunogenicity. RBC-EVs permit the accurate delivery of therapeutic cargo to space and time, thus minimizing systemic toxicity. This review presents the most recent developments on the expansion of the use of RBC-EVs to encapsulate the components of mRNA and CRISPR-Cas. Through the addition of the means to address these deficiencies, including stimulus-sensitive release mechanisms (eg, pH- or light-activated systems) and tissue-selective targeting approaches, RBC-EVs can be applied to enable the precise application in genetic diseases, inflammatory diseases, and cancer. Such innovations have the potential to overcome the clinical need and enable the biological complexity of mRNA- and CRISPR-Cas-based agents to provide a powerful delivery platform. Moreover, the review also demonstrates the unprecedented benefits of red blood cell EVs, which include immune evasion, scalability, and universal loading capacity, which can establish them as the next-generation delivery vehicles. Red blood cell EVs have the potential to increase the efficacy of precision medicine by increasing its feasibility. Lastly, we note the potential and translational issues in the provision of red blood cell EV-based mRNA and CRISPR-Cas therapeutic delivery of gene therapy.

Keywords: red blood cell-derived extracellular vesicles, CRISPR-Cas, mRNA therapeutics, targeting strategy, delivery vehicles

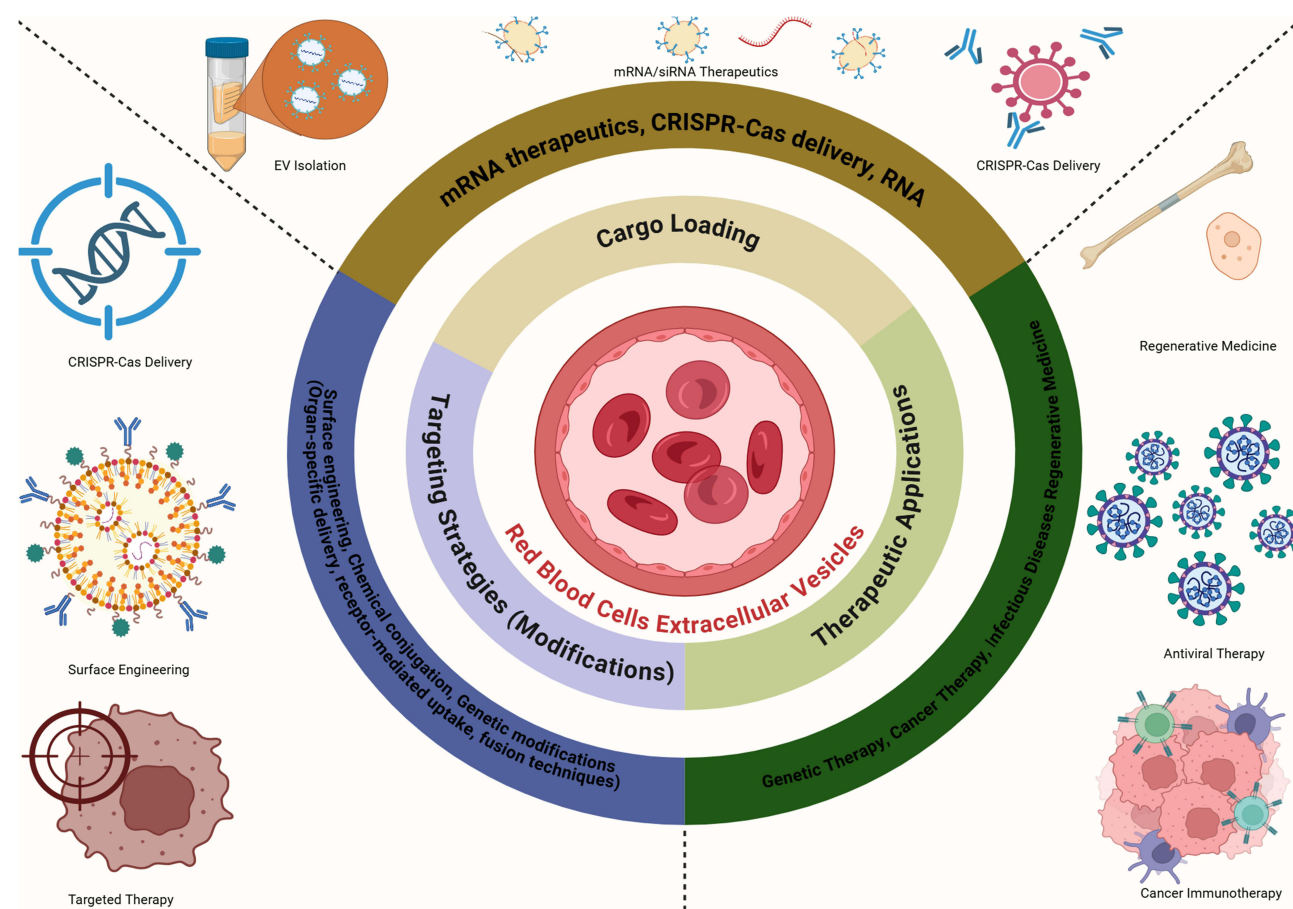
Introduction

Gene therapy has greatly transformed precision medicine as it has the ability to make specific alterations to gene expression and transient production of proteins under control.¹ The techniques are promising when it comes to the treatment of genetic disorders, cancers, as well as infectious diseases. Nonetheless, clinical application has a problem concerning efficacy of deliveries, off-target action, rapid clearance of the body, undesirable immune reaction, and genomic security.²⁻⁴

CRISPR-Cas systems and messenger RNA (mRNA) have been used as a gene-based therapy due to their programmability and the transient activity nature.² Nevertheless, these platforms are highly reliant on delivery systems capable of shielding nucleic acid cargoes, enhance cellular engagements, and minimize immunological toxicities.⁴ Viral vectors are viable, although they create immune response concerns, as well as, insertional mutagenesis and capacity. Conversely, non-viral carrier types, such as lipid nanoparticles, can have challenges in clearing quickly and its incompatibility with the body.⁵



Graphical Abstract



Extracellular vesicles (EVs) have become a potential solution to some of these problems as an up-and-coming biologically inspired approach to delivery. Biocompatibility and cell communication are enhancing their natural membrane structure and their ability to transport different biomolecules.⁶ Of particular interest is the red blood cell-derived extracellular vesicles (RBC-EVs) because they are composed of simple components, do not contain nuclear and mitochondrial DNA, and can be produced in large quantities using easily accessible donor sources.⁷

RBC-EVs offer a flexible method of delivering mRNA, CRISPR-Cas, and targeting ligands and are immune-friendly and can survive in circulation. In order to have a higher precision of timing and location, the recent studies were directed to two complementary approaches: (1) enhancing cargo loading efficiency by engineering membranes, and (2) creating release systems sensitive to certain stimuli and allowing context-dependent activation of therapeutic release, eg, of pH or light-based activation.^{8,9}

In this review, we critically analyze how RBC-EVs can deliver mRNA and CRISPR-Cas-based therapies. We consider the latest developments in engineering that would improve the encapsulation, stability, and activities of EV cargo on the go. We also discuss ways to target and stimulate release using ligands. Preclinical biodistribution, gene-editing efficacy, immune response, and preclinical data are evaluated in different disease models. The continued challenges such as scalable production and in vivo real-time monitoring are some of the challenges that we note as significant obstacles to clinical use. Finally, we describe a clinical implementation plan of RBC-EV-based gene therapies by incorporating delivery engineering and safety testing progress.

Nucleic Acid Therapeutics: From Mechanism to Clinical Translation

Mechanisms and Delivery Platforms for mRNA Therapeutics

The Development of mRNA Therapeutics

Over the past decade, mRNA-based therapeutics have garnered global attention as a treatment for human diseases, including autoimmune diseases, cancers, and infections.^{10,11} The first clinical trial, which used ex vivo mRNA to transfect dendritic cells (DCs) to stimulate cytotoxic T lymphocytes against cancer, was performed in 2002. The field reached a pivotal milestone in 2020 with the US Food and Drug Administration (FDA) approval of mRNA-based vaccines against COVID-19, an event that revolutionized immunization strategies worldwide.¹²

Mechanism of Action and Delivery Challenges

The therapeutic efficacy of mRNA fundamentally depends on efficient delivery to the cytosol. Cellular uptake of mRNA is initiated by the scavenger receptor-mediated endocytosis, which generally determines the access of mRNA to the transcriptional machinery. However, successful therapy requires mRNA to escape endosomes, a process often characterized by low efficiency in reaching the cytoplasm for protein synthesis. Naked mRNA is highly degraded by extracellular exonucleases and has a very low delivery efficiency. Therefore, encapsulated mRNA exhibits much better protein expression results than its unprotected counterpart.¹³

Delivery Systems: Enhancing Efficacy and Stability

There are various delivery platforms that have been used to overcome the delivery challenges of mRNA therapies such as poor cellular uptake and instability. Two of the most known are lipid nanoparticles (LNPs) and polyplexes,¹⁴ which promote the stability of mRNA and its intracellular delivery. EVs have recently attracted attention as biocompatible, naturally occurring carriers, as EVs are endogenous nanoparticles, which are involved in intercellular communication, and may be targeted to recipient cells whilst delivering and protecting mRNA against enzymatic degradation. To make use of these natural attributes,¹⁵ EVs provide a productive approach to enhancing the efficacy, safety, and specificity of mRNA-based therapies and have demonstrated the potential to serve as a biological carrier.¹³ EVs are natural nanoparticles that can be used to assist intercellular communication and, therefore, can enclose mRNA, offering them protection against enzymatic degradation and delivering them to particular cells. Taking advantage of the intrinsic characteristics of EVs has a lot of potential in enhancing therapeutic outcomes.

Targeting Strategies and Organ-Specific Delivery

Cell-specific targeting is critical in minimizing off-target effects and enhancing mRNA therapy safety profile.⁶ Recent developments (especially with selective organ targeting (SORT) nanoparticles) facilitate more targeted delivery of mRNA or CRISPR-Cas systems to corrective and protein replacement therapy to individual organs such as the liver, lungs, and spleen. There have been considerable advances in hepatic usage, where optimized LNP formulations are under development as organ-targeted therapies.^{7,8} However, most of these developments remain largely hepatic and pulmonary-targeted.⁹ The major gap in demand is the effective and selective methods of cardiac delivery. Research to develop cardiac cell-specific LNPs has increased to overcome this barrier and increase the therapeutic efficacy and safety of mRNA-based therapies.

CRISPR-Cas: Mechanism and Clinical Breakthroughs

CRISPR-Cas system is an adaptive immunological response that is present in both bacteria and archaea; it has been shown to be a potent tool in genome editing.¹⁰ It has two broad classes such as multi-subunit Class 1, single-effector Class 2 which have several types and subtypes that have distinct effector proteins. Among the various classifications of CRISPR-Cas systems, the Class 2 type II has received the most comprehensive study due to its structural simplicity, high programmability and broad adaptable nature. The focus of this review will be on CRISPR-Cas9 and its therapeutic use with particular interest shown towards the problems of delivery and the potential avenue of RBC-EVs as one of the promising alternative vehicles.¹¹ The technology is based on adaptative immune responses of bacteria, which identify and cut alien denatomes, thus, preventing phage infection.¹² Ease of use, high efficiency, and accuracy in targeting have made

CRISPR-Cas9 a top choice in therapeutic genome engineering.¹³ Clinical trials in preclinical stages have shown encouraging results in genetic diseases like beta-thalassemia and sickle cell diseases, which was done by ex vivo editing of patient-derived hematopoietic stem cells.¹⁴ To a more recent development, breakthroughs in in vivo delivery methods have made it possible to deliver the CRISPR components directly to the lesioned tissue by viral vectors as in the case of the treatment of Leber congenital amaurosis, a type of hereditary blindness.¹⁵ These clinical achievements underscore the revolutionizing nature of CRISPR-Cas9 within the field of precision medicine, which can be used to create targeted, long-term, and potentially curative forms of therapy. However, the current challenges, such as immunogenicity, inefficient delivery, and off-target editing, demonstrate the need to explore the fundamental mechanisms and advance delivery technology.

Challenges of CRISPR-Cas to Clinical Translation: Immune Response and Delivery Status

Despite its therapeutic promise, the widespread clinical translation of CRISPR-Cas9 technology remains impeded by several barriers. Key among these are immunogenic reactions to bacterial-derived Cas9 proteins, suboptimal in vivo delivery systems, and the risk of off-target mutagenesis.¹⁶ In particular, the development of safe and efficient delivery platforms capable of targeted in vivo application continues to be a central challenge in CRISPR-based gene therapy.¹⁷ Although preclinical studies and some clinical trials have demonstrated promising efficacy for both CRISPR-Cas9 and mRNA therapeutics, their broader clinical application is still constrained by existing limitations, most notably, the immunogenicity of the CRISPR system and the inadequacy of available delivery technologies. Immunogenicity is a major concern, the Cas9 endonuclease, commonly derived from non-human sources like *Staphylococcus aureus* (SaCas9) or *Streptococcus pyogenes* (SpCas9), can elicit adaptive immune responses in humans.¹⁸ Research has revealed that a sizable section of the population has pre-existing antibodies and T cells specific to Cas9, which could compromise therapeutic efficacy and elevate the risk of immune-mediated adverse events.¹⁹ Additionally, Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) induce type I interferon responses that may provoke inflammation or suppress translational efficiency.²⁰

Delivery issues are equally critical. Without adequate shielding, naked mRNA and CRISPR components frequently fail to reach target cells and are extremely vulnerable to enzymatic degradation in circulation.²¹ Despite their widespread usage and clinical approval (for example, in mRNA COVID-19 vaccines), LNPs are associated with limitations such as potential hepatotoxicity, rapid systemic clearance, and non-specific biodistribution. Furthermore, efficient intracellular delivery necessitates overcoming barriers to cellular uptake, endosomal escape, and, for CRISPR-mediated editing, efficient nuclear import.²²

These limitations underscore the urgent requirement for safer and more efficient delivery platforms. Cell-derived nanocarriers, particularly those derived from RBCs, present a promising alternative due to their extended circulation half-life, inherent biocompatibility, and immune-evasive properties.²³ RBC-derived nanovesicles exhibit strong potential for delivering CRISPR and mRNA therapeutics with reduced immunogenicity and enhanced targeting, as they effectively encapsulate and shield nucleic acid cargo while minimizing immune recognition.²⁴ To put the need for new delivery systems in perspective, a list of the main clinical obstacles related to CRISPR and mRNA treatments, and illustrating how RBC-EVs can get beyond them with their stealth, specificity, and adjustable release, has been summarized in Figure 1.

Delivery Systems for CRISPR-Cas9: Current Strategies and Emerging Solutions

CRISPR-Cas system exhibits significant therapeutic potential. However, its realization necessitates delivery vehicles capable of targeted transport, high efficiency, and minimal toxicity.²⁵ Delivery modality critically governs genome editing efficiency and on-target specificity. CRISPR-Cas9 components, Cas9 protein, and guide RNAs are delivered as ribonucleoprotein complexes or via DNA, RNA, and mRNA formats.²⁶ There are two types of delivery methods, which are generally present. Physical methods include electroporation and microinjection, and viral vectors include lentiviral, adenoviral, and AAV vectors.²⁷ Non-viral approaches encompass plasmid DNA, lipid-based nanoparticles, polymeric nanoparticles, and EVs.²⁸ Physical methods achieve high transfection rates but are predominantly restricted to in vitro applications.²⁸

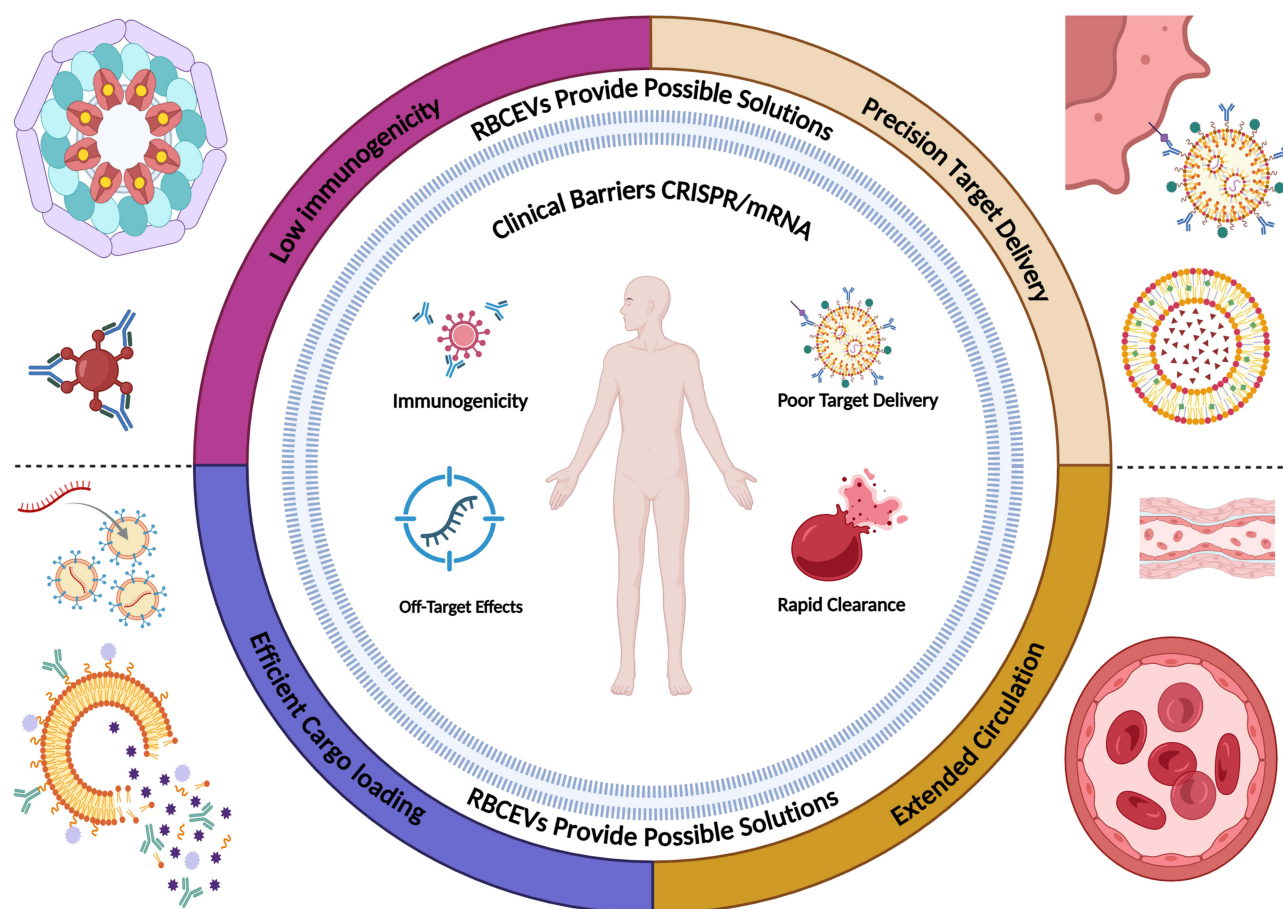


Figure 1 Clinical delivery barriers in CRISPR and mRNA therapeutics and the role of RBC-EVs as biocompatible carriers. The schematic depicts important hurdles to the effective clinical delivery of CRISPR and mRNA therapies, such as fast enzymatic breakdown in circulation, immunological activation, off-target tissue accumulation, inadequate cellular uptake, and endosomal trapping. Current delivery methods, like LNPs and viral vectors, have limited targeting, immunogenicity, and possible safety issues. Because of their inherent qualities, RBC-EVs are offered as a viable substitute delivery method. These include: (1) low immunogenicity and natural biocompatibility; (2) extended circulation made possible by CD47-mediated immune evasion; (3) the ability to load cargo both passively and actively; and (4) the ability to surface functionalize for tissue-specific targeting. All of these characteristics point to RBC-EVs as a cutting-edge platform to get beyond delivery restrictions and improve the accuracy of CRISPR and mRNA treatments.

To date, viral vectors demonstrate superior CRISPR-Cas9 delivery and editing efficacy. AAVs, the most widely used viral vectors, offer advantages including reduced immunogenicity and cytotoxicity compared to alternative viral platforms.²⁹ A significant constraint is the limited AAV packaging capacity (<4.7 kb), hindering delivery of CRISPR-Cas9 components and derived editors.³⁰ For instance, the *Streptococcus pyogenes* Cas9 open reading frame spans ~4.2 kb. Furthermore, CRISPR-Cas9-derived base editors (4.2–5.2 kb) and prime editors (6.3 kb) exceed AAV cargo limit.³ While adenoviral and lentiviral vectors possess larger cargo capacity than AAVs, they present distinct limitations, including pronounced immunogenicity against adenoviral vectors.³⁰

Enhancing the efficacy and safety of targeted genome editing requires refinement of CRISPR-Cas9 delivery strategies.³¹ Engineered platforms, including LNPs, EVs, and viral vectors, are under development for gene therapy. These clinical approaches face major challenges,³² notably safety concerns and rapid clearance by the mononuclear phagocyte system.¹¹ Cell-derived nanovesicles (eg, EVs) may circumvent these limitations and exhibit superior performance characteristics compared to synthetic carriers.¹¹ EVs efficiently encapsulate dsDNA (100 bp – 20 kb) with inherently low immunogenicity.³³ Moreover, EVs induce significantly lower levels of proinflammatory cytokines and attenuated inflammatory responses relative to LNPs.³⁴ Given the limitations of synthetic nanovesicles, EVs represent a highly promising delivery vehicle for CRISPR-Cas9. Their endogenous membrane structure confers protection to nucleic acids and proteins against immune recognition and nucleases, establishing EVs as compelling candidates for therapeutic

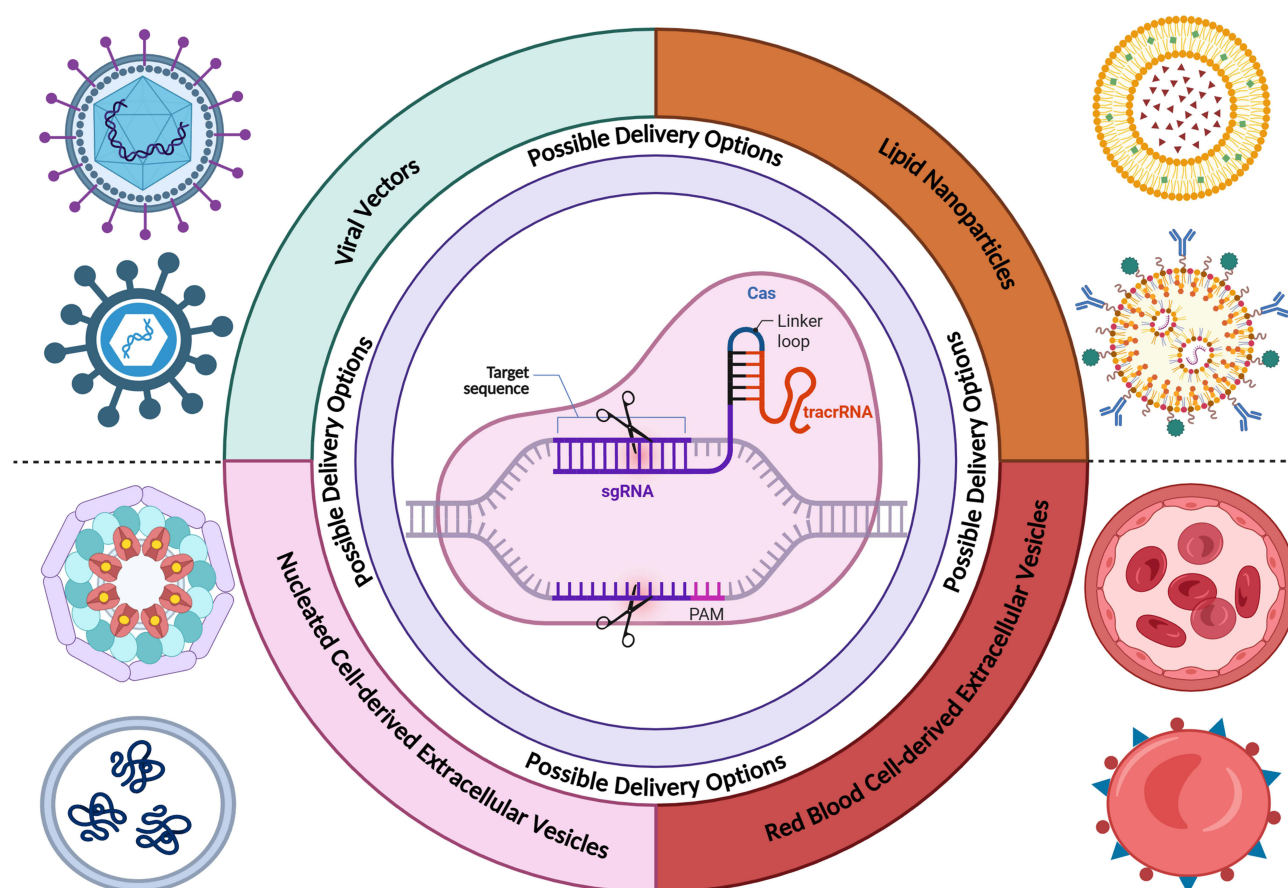


Figure 2 CRISPR-Cas9 Genome Editing Mechanism and Comparative Delivery Platforms. This schematic illustrates the mechanism of CRISPR-Cas9-mediated genome editing. Guided by a single-guide RNA (sgRNA), the Cas9 endonuclease induces a site-specific double-strand break (DSB) in the target DNA. Key delivery platforms for CRISPR-Cas9 components include viral vectors, lipid nanoparticles, EVs derived from nucleated cells, and RBC-EVs.

CRISPR-Cas9 delivery.³⁵ By delivering Cas9 nuclease and guide RNA to specific DNA regions, the CRISPR-Cas9 system makes site-specific genome editing possible.³⁶ The creation of secure and efficient distribution systems is essential to the system's optimal therapeutic use. Several strategies have been investigated to address delivery-related issues, such as viral vectors, LNPs, and RBC-EVs.³⁷ A comparison of different delivery methods and the fundamental process of CRISPR-Cas9 genome editing is shown in [Figure 2](#).

Classification, Functional Diversity, and Therapeutic Advantages of RBC-EVs

Extracellular Vesicles and the Distinctive Features of RBC-EVs

Extracellular vehicles (EVs) are membrane-bound nanoparticles discharged by cells under both physiological and pathological circumstances and are widely divided into exosomes (30150 nm), macrovesicles/ectosomes (1001000 nm), and apoptotic bodies (>1000 nm) in terms of size and biogenesis.³⁵ Exosomes are formed through the endosomal path, and macrovesicles are directly formed through the bud of the plasma membrane, and apoptotic bodies are formed during the process of programmed cell death.¹⁰ In spite of the fact that classification boundaries are sometimes permeable, this review concentrates on red blood cell-derived extracellular vesicles (RBC-EVs), which are mainly ectosomes and exosome-like vesicles with EVs being used to promote intercellular communication through the delivery of proteins, lipids, and nucleic acids that indicate physiological conditions of the parent cell. RBC-EVs are characterized by features that are especially useful in the delivery of genes in comparison to EVs made of nucleated cells, including mesenchymal stem cells. The erythrocytes in their mature forms do not contain nuclear and mitochondrial DNA, and

Table 1 Comparison of Exosomes and RBC-EVs

Feature	Exosomes	Red Blood Cell-Derived EVs (RBC-EVs)
Source	Derived from various cells (eg, stem cells, immune cells, tumor cells)	Derived from enucleated red blood cells (RBCs)
Nucleic Acid Content	mRNA, miRNA, and even nuclear/mitochondrial DNA depending on the cellular source ⁴⁰	Lack of nuclear and mitochondrial DNA, minimizing the risk of unintended donor genetic material transfer and enhancing biosafety. ⁴¹
Immunogenicity	Express MHC I/II and co-stimulatory molecules, posing immune risk ⁴²	Naturally immunologically inert due to lack of MHC molecules and DNA ⁴³
Composition	Heterogeneous in composition, including proteins, lipids, and RNAs that depend on the cell of origin ⁴⁴	A typical lipid bilayer and a repertoire of RBC-derived membrane proteins ⁶
Targeting Capability	Non-specificity necessitates additional engineering for precise targeting ⁴⁴	Chemically modified for targeting while inherently possessing long-circulating CD47 signals ⁴⁵
Loading Capacity	A moderate loading capacity that depends on the method used ⁴²	A loading capacity comparable or superior to alternatives while maintaining structural stability across various loading approaches ⁴⁶
Stability in Circulation	Rapid mononuclear phagocyte system (MPS) clearance and short half-life ⁴⁷	Utilizes CD47-mediated immune evasion contributing to relatively prolonged circulation compared with synthetic nanoparticles ^{48–50}
Production	Low, donor-dependent yield with challenges in upscaling ⁷	Scalable and efficient production from abundant, nucleus-free RBCs ⁷
Isolation and Purification	Multiple techniques exist (eg, ultracentrifugation, SEC, precipitation), but outcomes vary with source and scalability needs ⁵¹	Purified by extrusion, TFF, or centrifugation; Uncomplicated surface simplifies downstream processing, with emerging techniques like immunoaffinity and microfluidics enabling clinical-grade isolation ⁵²
Engineering Flexibility	High engineering flexibility, often necessitating functional validation post-modification ⁴⁷	Capable of surface functionalization without compromising membrane integrity ⁵³
Clinical Translation	Hindered by batch variability, immune risks, and complexity in production ⁴¹	Promising for clinical application due to reproducibility, biocompatibility, and reduced risk of immune activation ⁵⁴

thus, the chances of unwanted donor DNA transfer are minimal.³⁸ Secondly, the RBC-EVs enjoy the advantage of high-density and uniform supplier sources, which allow scalable manufacturing with less batch to batch deviation.³⁹ Whereas EVs produced by nucleated cells can have an intrinsic regenerative signaling, RBC-EVs have biosafety benefits, compositional ease, and translational capability Table 1 gives a comparative overview.

Biogenesis, Physiology, and Functional Properties of RBC-EVs

Red blood cells (RBCs) are clastogenic, end-differentiated cells that are dedicated to the conveyance of oxygen. The biconcave shape (7.5 –8.7 μm diameter) and highly structured membrane-cytoskeleton structure provide them with an unprecedented deformability and mechanical strength so they can circulate through small capillaries.⁵⁵ The phospholipids and cholesterol are major components of the RBC membrane which has a cytoskeletal network of spectrin, ankyrin, band 3, and glycophorin. All these features lead to membrane stability and ion homeostasis during the RBC lifespan.⁵⁶

Even though intact RBCs possess comparatively longer circulation than synthetic nanocarriers, RBC-derived EVs have shorter and yet comparatively longer circulation lifespan in comparison with numerous synthetic nanocarriers, mainly because of diminished immune recognition instead of imitating native RBC longevity.⁵⁷ Significantly, enucleated nature of RBCs removes the risks of vertical gene transfer or uncontrolled growth, which contributes to biosafety of RBC-EVs in therapeutic use.³⁸

Physiological vesiculation helps in the elimination of damaged membrane components and maintenance of RBC integrity, but pathological vesiculation in hemolytic disorders, inflammatory conditions or in blood storage causes the release of more EVs with a different cargo composition. Regarding delivery, these biogenesis pathways affect RBC-EV size, membrane composition, circulation stability, and cargo compatibility.⁵⁷ In addition to their biological origin, RBC-

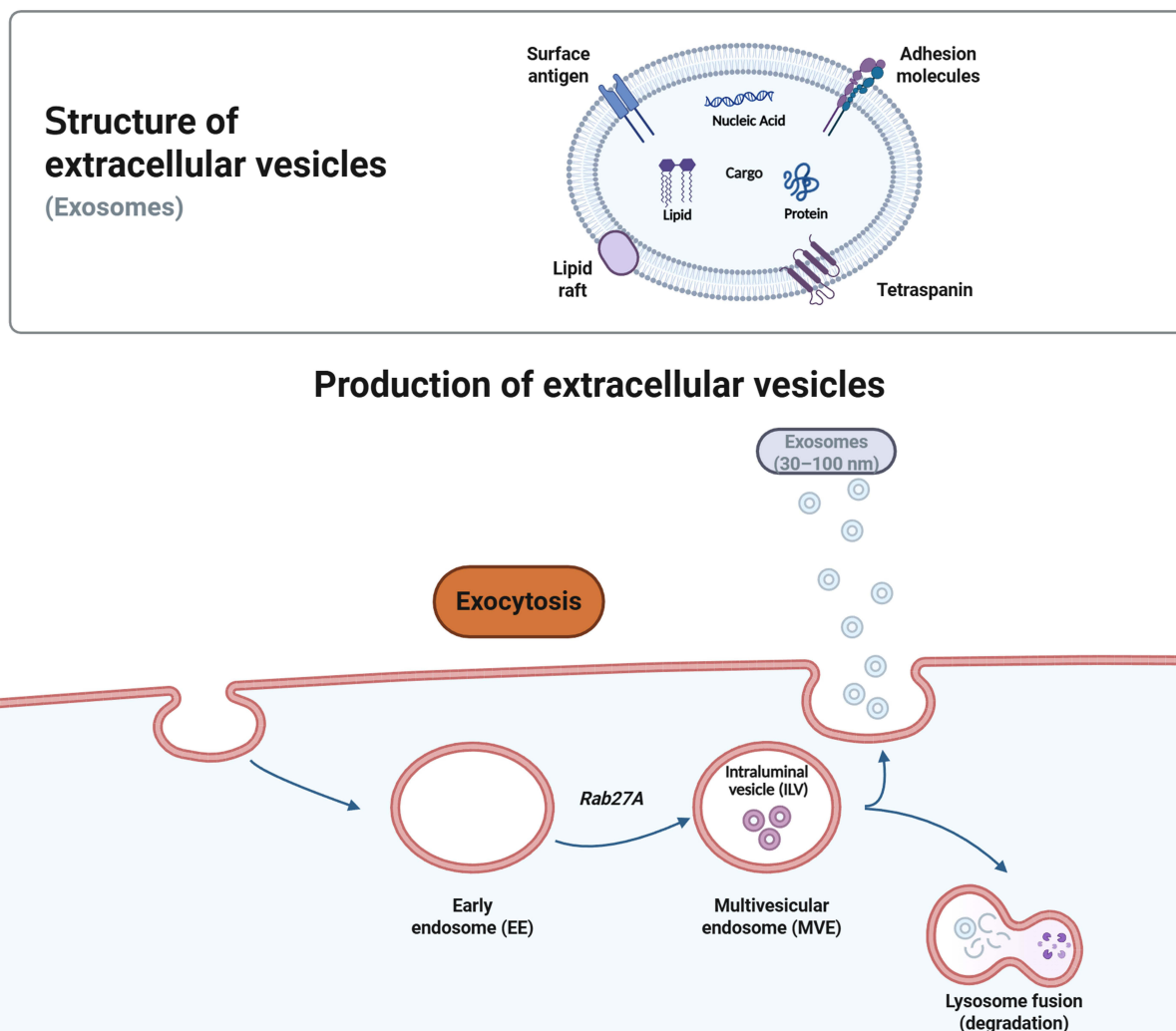


Figure 3 Schematic diagram of EVs biogenesis. The upper panel illustrates the molecular composition of EVs, including lipid rafts, surface antigens, adhesion molecules, and tetraspanins embedded in the membrane, while the lumen contains diverse cargo such as nucleic acids, proteins, and lipids. The lower panel depicts two distinct biogenesis pathways (i) Exosomes (30–100 nm), which originate from the endosomal system through the formation of intraluminal vesicles (ILVs) within multivesicular endosomes (MVEs) and are secreted by exocytosis in a Rab27A-dependent manner; or degraded upon lysosomal fusion; and (ii) Microvesicles (100–1000 nm), which are released directly from the plasma membrane via ectocytosis through outward budding and fission. Together, these pathways generate heterogeneous EV populations with distinct structural and functional properties.

EVs have a variety of functional outcomes due to their cargo, which includes coagulation, inflammation, and intercellular signaling regulation.⁵⁸ Notably, they have immune-compatible and non-integrating properties that promote their use as drug delivery vehicles of therapeutic nucleic acids.⁶ Figure 3 has revealed the Schematic analysis of the molecular composition of EVs and the entire process of EV biogenesis.

Production, Purification, and Quality Control of RBC-EVs

Chemical, physical, and storage-induced methods can be used to increase the production of RBC-EV. The most commonly used calcium ionophores and oxidative stressors are used to cause vesiculation in vitro by facilitating calcium influx and membrane budding. Scalable production is possible with the physical techniques of extrusion and shear stress mimetic systems, which mechanically stimulates the formation of vesicles.⁶ More sophisticated ex vivo engineering approaches like hypotonic dialysis, electroporation, and microfluidic shear stress take advantage of anucleate nature and plasticity of RBCs membrane to allow the generation of vesicles under control without destroying important surface proteins.⁴⁸ Post-purification purification is essential in eliminating the remaining cells, free hemoglobin, and debris.

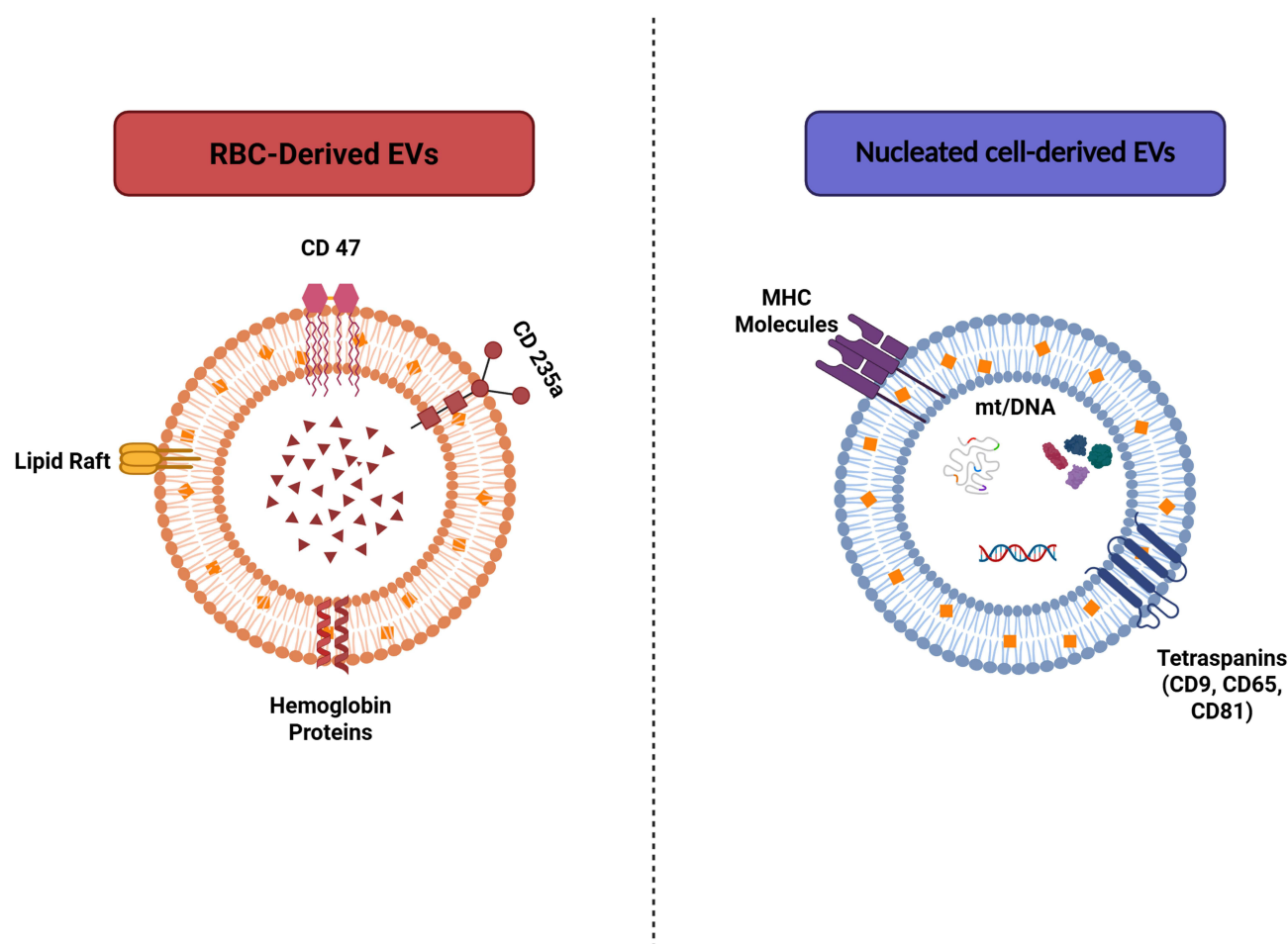


Figure 4 Molecular composition of RBC-EVs. This illustration explained the vesicle membrane of RBC-EVs is enriched with CD235a (glycophorin A), CD47, adhesion molecules, and lipid rafts, reflecting their red blood cell origin. Unlike nucleated cell-derived EVs, RBC-EVs lack nuclear and mitochondrial components, but they carry hemoglobin-related proteins and membrane-associated markers. In contrast, EVs from nucleated cells typically display tetraspanins (CD9, CD63, CD81), MHC molecules, integrins, and growth factor receptors, and their cargo includes DNA, mRNA, microRNAs, mitochondrial proteins, and other regulatory molecules. Additionally, both RBC-EVs and nucleated cell EVs encapsulate diverse bioactive cargo, such as functional proteins and nucleic acids (eg. regulatory RNAs), which collectively contribute to their therapeutic potential and role in intercellular communication.

Typical purification methods are the use of differential and density gradient centrifugation, size-exclusion chromatography, field-flow fractionation, immunoaffinity pull-down using markers on erythrocyte (eg, CD235a), and scalable filtration-based techniques, such as tangential flow filtration. Both methods demand vesicle integrity, yield, purity, and scalability of clinical extent.⁵³ Translational applications are required to be thoroughly characterized and controlled in quality. Nanoparticle tracking analysis and electron microscopy are used to check physical properties, whereas biochemical validation of erythrocyte origin is done by band 3, glycophorin A, and CD47. Clinical grade RBC-EVs also need sterility testing, residual DNA analysis, and functional bioactivity assays as a means of ensuring batch consistency and regulatory compliance.⁵⁹ Figure 4 explains the illustration of the molecular composition of RBC-EVs telling us about the presence of surface markers and the possibility of potential growth factor receptors and cargo load.

Composition, Cargo Loading, and Targeted Delivery Potential

The chemical, physical, and storage-induced techniques can be used to improve RBC-EV production. In vitro induction of vesiculation is done through calcium ionophores and oxidative stressors which stimulate calcium influx and membrane budding.⁵³ Mechanical stimulation of vesicle formation and scalable production is done by physical techniques like extrusion and shear stress mimetic systems.⁶⁰ The most recent ex vivo engineering approaches such as hypotonic dialysis, electroporation, and microfluidic shear stress are based on the anucleate nature and plasticity of RBCs

Table 2 Overview of EV Cargo Loading Strategies

Loading Strategy	Approach	Description	Example Cargo	References
Endogenous Loading	Cell-Based	Cargo (eg. proteins, RNAs) is naturally sorted into EVs during biogenesis by engineered donor cells	miRNA, therapeutic proteins	[65]
Exogenous Loading	Cell-Free	Isolated EVs are externally loaded with cargo using physical or chemical methods	siRNA, drugs, enzymes	[66]
Passive Loading	Exogenous	EVs are incubated with cargo; loading relies on diffusion and hydrophobicity	Curcumin, Paclitaxel	[67]
Active Loading	Exogenous	Membrane is disrupted (electroporation, sonication, extrusion) to introduce cargo	siRNA, DNA, proteins	[68]
Cell-Based Loading	Endogenous	Cells are transfected, incubated, or stimulated to express and sort cargo into EVs	mRNA, protein, miRNA	[69]
Non-Cell-Based Loading	Exogenous	Direct loading into isolated vesicles without cellular involvement	Drugs, peptides, nucleic acids	[69]

membrane that facilitates the controlled production of vesicles by preserving dominant surface proteins.⁶¹ The use of post-production purification is paramount in eliminating remaining cells, free hemoglobin, and debris. Purification methods commonly used are differential or density gradient centrifugation, size-exclusion chromatography, field-flow fractionation, immunoaffinity capture using erythrocyte markers (eg CD235a), and scalable filtration-based methods, such as tangential flow filtration. All the methods demand the balance between vesicle integrity, yield, purity, and clinical scalability.⁶² Table 2 sums up the primary approaches to the introduction of therapeutic molecules into EVs. Translational applications are required to be comprehensively characterized and controlled in terms of quality.⁶³ Nanoparticle tracking analysis and electron microscopy are used to measure physical properties, whereas biochemical validation is used to determine the erythrocyte origin, by measuring band 3, glycophorin A, and CD47. RBC-EVs of clinical grade also need sterility and residual DNA test and functional bioactivity test to verify consistency of batches and regulatory conformity.^{54,64} The general process of EV generation, purification, and therapeutic encapsulation is illustrated in Figure 5.

Technical Challenges and Future Directions

In spite of this potential, RBC-EV-based therapeutics have difficulties associated with vesicle heterogeneity, standardization, and cargo loading efficiency. The development of scalable, GMP-compliant production and purification processes is an important priority.⁴³ However, RBC-EVs could be a promising delivery vehicle because of their bi-safety, compositional easiness, immune compatibility, and scalability. Further refinement of loading efficiency, target specificity, and in vivo performance will be required to drive RBC-EVs into clinical translation.⁷⁰

RBC-EVs: A Non-Viral Delivery Platform for Precision Genome Editing and mRNA Therapeutics

RBC-EVs are an exciting natural delivery system in mRNA therapy treatments and CRISPR gene editing. Their potential as a clinical translationer is due to their outstanding biocompatibility, sufficient sourcing, and lack of nuclear DNA features that are collectively minimizing immunogenicity and oncogenic risks over viral vectors.⁷¹ Most importantly, RBC-EVs can be optimally modified to pack and transport mRNA, CRISPR-Cas ribonucleoproteins (RNPs), and single-guide RNAs (sgRNAs) to cells. This allows the temporal control of genome editing with less off-target effects.⁴ The direct application of intact red blood cells as nucleic acid carriers has also been tested but they are too large to penetrate through tissues and enter cells. Conversely, compared to the others, RBC-EVs preserve important membrane properties of parent RBCs but provide nanoscale size that allows cells to pick them up and distribute across the body, thereby enabling mRNA and CRISPR delivery tools to be used.⁵ Mechanical extrusion-generated nanovesicles of RBC membranes do not resemble naturally released RBC-EVs in terms of biogenesis, cargo, and orientation of membrane proteins.⁴⁹ These disparities can affect biological behavior, functional performance, and specificity. Endogenous RBC-

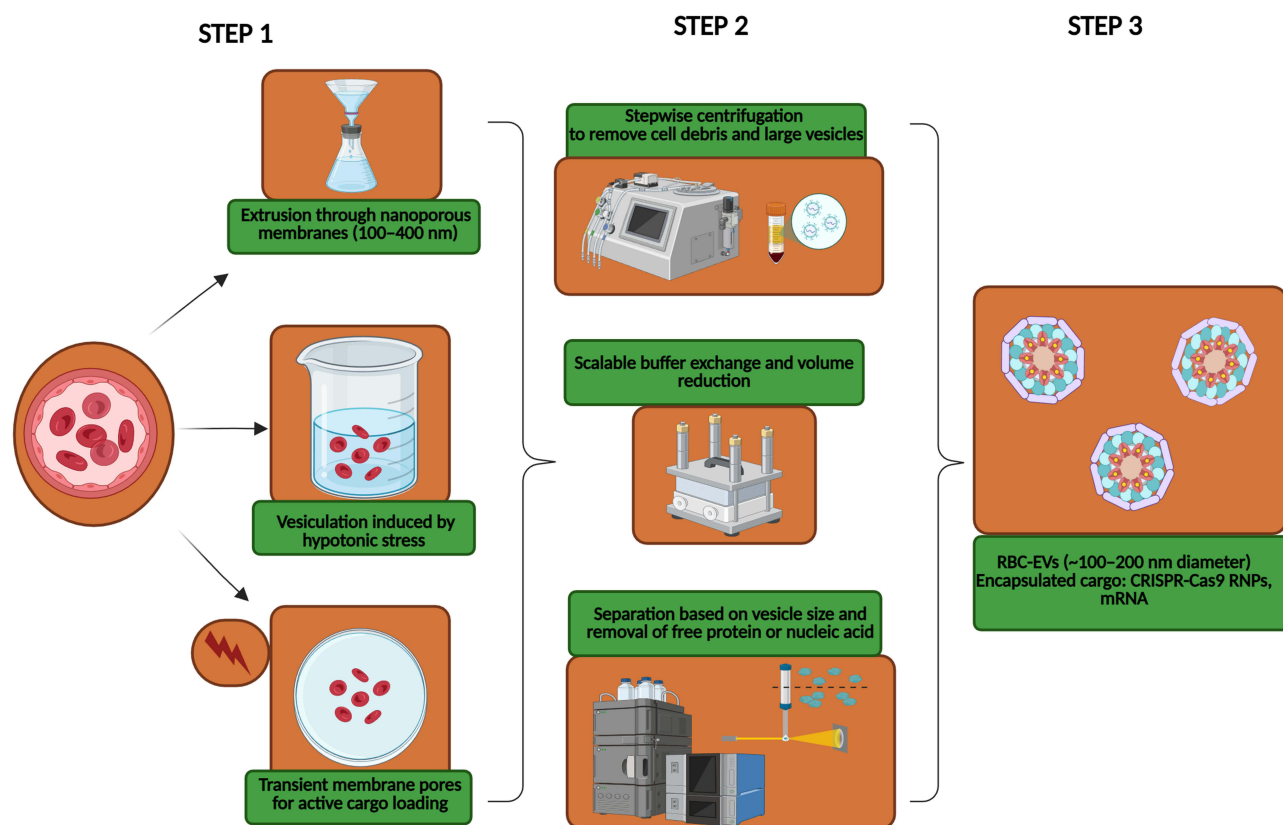


Figure 5 EV generation, purification and encapsulation. Schematic workflow for the generation, purification, and therapeutic loading of RBC-derived nanovesicles (RBC-NVs). **Step 1:** RBCs are treated with different vesiculation-inducing strategies, including extrusion through nanoporous membranes (100–400 nm), hypotonic stress to trigger vesicle budding, and transient pore formation for active cargo loading. **Step 2:** The resulting vesicle mixture undergoes purification and enrichment through sequential processes: stepwise centrifugation to eliminate cell debris and large vesicles; scalable buffer exchange and volume reduction to remove contaminants; and size-based separation techniques to enrich for nanoscale vesicles while removing free proteins and nucleic acids. **Step 3:** Purified RBC-EVs (~100–200 nm in diameter) are loaded with therapeutic cargo- such as CRISPR-Cas9 ribonucleoproteins (RNPs) or mRNA- to generate functional delivery vehicles ready for downstream biomedical applications.

EVs, in contrast, are closer to physiological vesicles formation and can have better-preserved native membrane functions.⁶²

Emerging Extracellular Vesicle Platforms for Therapeutic Delivery

The use of non-viral genome-editing delivery schemes has received significant investigation to circumvent the safety hazards of viral vectors, and extracellular vesicles have been suggested as a biologically naturally available contender that could convey CRISPR-Cas elements.⁵⁰ Owing to the points made in Challenges of CRISPR-Cas to Clinical Translation: Immune Response and Delivery Status, extracellular vesicles (EVs) have received growing popularity. Specifically, red blood cell derived extracellular vesicles (RBC-EVs) have an appealing cellular uptake profile and characteristics that have been described in Challenges of CRISPR-Cas to Clinical Translation: Immune Response and Delivery Status and should be used as delivery vehicles of nucleic acid-based therapeutics.⁶ The high cellular internalization caused by the nanoscale size of RBC-EVs specifically allows delivering it to the cell in complex biological settings.⁶² Recent work has shown that it is possible to reproducibly make RBC-EV mimetics that can load and transfer RNA molecules, including siRNA and mRNA, with activity in vitro and in vivo.^{72,73} The evidence provided herein indicates that RBC-EVs are viable as vectors of genetic payloads, but much of what has been discovered so far is preclinical. To the best of our knowledge, clinical testing of EV-based therapeutics has been primarily conducted on mesenchymal stem cell (MSC)-derived exosomes as opposed to RBC-EVs.⁷⁴ The first-phase clinical trials of MSC-derived exosomes have shown promising safety profiles, which can be used to justify the extended applicability of EV-based delivery systems on the translational scale.⁷⁵ Nevertheless, direct clinical validation of the RBC-EVs has not been reported and this highlights the necessity to undertake additional in vivo efficacy,

biodistribution, and safety studies to advance such platforms into clinical use. Phases I/II are becoming clinical-validation. In another study, exosomes produced by the bone marrow mesenchymal stem cell (MSC) as a treatment in patients with acute respiratory syndrome with severe illnesses treated with COVID-19 were administered.⁷⁶ These 7 patients did not have any dose-related toxicity or adverse events within 28 days after treatment.⁴⁴ Although these early safety results are promising, the sample size is too small to draw conclusive results on translational potential and additional research is undertaken to assess safety and efficacy.

Non-Viral CRISPR Delivery Strategies and the Emerging Potential of RBC-Derived Extracellular Vesicles

Lipid nanoparticles (LNPs) currently represent the most clinically advanced non-viral delivery system for nucleic acid therapeutics; however, their limitations have prompted comparative investigations into alternative platforms such as RBC-derived extracellular vesicles. Non-viral CRISPR/Cas9 delivery strategies have demonstrated significant therapeutic promise in the context of hereditary diseases, particularly through lipid nanoparticle (LNP)-based platforms.^{32,77} Preclinical studies have shown that LNP-mediated delivery of Cas9 mRNA, in combination with guide RNA and repair templates, can achieve functional gene correction in disease models.⁷⁸ For example, correction of a splicing mutation in the fumarylacetoacetate hydrolase gene has been achieved in a mouse model of hereditary tyrosinemia using LNP-delivered Cas9 mRNA together with adeno-associated virus-mediated delivery of sgRNA and repair templates, resulting in partial hepatocyte correction and prevention of disease symptoms.⁷⁹ In parallel, extensive progress has been made in genome-editing therapies for hemoglobinopathies and immunodeficiencies through ex vivo CRISPR/Cas9 editing of hematopoietic stem and progenitor cells, as summarized in recent reviews.^{80,81} These advances underscore the feasibility of non-viral or minimally viral genome-editing approaches for genetic diseases.⁸² Although direct in vivo CRISPR/Cas9 delivery using red blood cell-derived extracellular vesicles (RBC-EVs) has not yet been reported, the intrinsic biocompatibility, scalability, and low immunogenicity of RBC-EVs suggest that they may represent a promising future platform for nucleic acid delivery.⁸³ Further experimental studies will be required to evaluate their suitability for genome-editing applications and to benchmark their performance against established non-viral delivery systems such as LNPs.

Precision-Targeted RBC-EVs for CRISPR-Based Oncogene Modulation and Immune Checkpoint Editing in Cancer

The use of non-viral genome-editing delivery schemes has received significant investigation to circumvent the safety hazards of viral vectors, and extracellular vesicles have been suggested as a biologically naturally available contender that could convey CRISPR-Cas elements.⁵⁰ Owing to the points made in Challenges of CRISPR-Cas to Clinical Translation: Immune Response and Delivery Status, extracellular vesicles (EVs) have received growing popularity. Specifically, red blood cell derived extracellular vesicles (RBC-EVs) have an appealing cellular uptake profile and characteristics that have been described in Challenges of CRISPR-Cas to Clinical Translation: Immune Response and Delivery Status and should be used as delivery vehicles of nucleic acid-based therapeutics.⁶ The high cellular internalization caused by the nanoscale size of RBC-EVs specifically allows delivering it to the cell in complex biological settings.⁶² Recent work has shown that it is possible to reproducibly make RBC-EV mimetics that can load and transfer RNA molecules, including siRNA and mRNA, with activity in vitro and in vivo.^{72,73} The evidence provided herein indicates that RBC-EVs are viable as vectors of genetic payloads, but much of what has been discovered so far is preclinical. To the best of our knowledge, clinical testing of EV-based therapeutics has been primarily conducted on mesenchymal stem cell (MSC)-derived exosomes as opposed to RBC-EVs.⁷⁴ The first-phase clinical trials of MSC-derived exosomes have shown promising safety profiles, which can be used to justify the extended applicability of EV-based delivery systems on the translational scale.⁷⁵ Nevertheless, direct clinical validation of the RBC-EVs has not been reported and this highlights the necessity to undertake additional in vivo efficacy, biodistribution, and safety studies to advance such platforms into clinical use. Phases I/II are becoming clinical-validation. In another study, exosomes produced by the bone marrow mesenchymal stem cell (MSC) as a treatment in patients with acute respiratory syndrome with severe illnesses treated with COVID-19 were administered.⁷⁶ These 7 patients did not have any dose-related toxicity

Table 3 Targeting Strategies Explored in Extracellular Vesicle-Based Delivery Systems

Approach	Mechanism	Target Type	Example	References
Surface ligand conjugation	Surface ligand conjugation	Surface ligand conjugation	Surface ligand conjugation	[84]
EV membrane-mediated	Native surface proteins and lipid composition facilitate internalization	Immune and non-immune cells	Preferential uptake by immune cells	[65]
cellular uptake	Cargo release influenced by pH, redox state, or enzymatic activity	Tumor or inflammatory microenvironments	pH-responsive or enzyme-cleavable systems	[68]
Microenvironment-responsive delivery				

or adverse events within 28 days after treatment.⁴⁴ Although these early safety results are promising, the sample size is too small to draw conclusive results on translational potential and additional research is undertaken to assess safety and efficacy. To overcome challenges related to biodistribution and targeting efficiency, multiple engineering approaches have been developed for RBC-EVs, which are systematically summarized in Table 3.

RBC-EVs: A Revolutionary CRISPR Delivery Platform Driving Safe and Efficient Antiviral Therapy

RBC-EVs have certain safety benefits compared to conventional viral vectors, eg adeno-associated virus (AAV) and lentivirus, when used as antiviral CRISPR vectors.⁷⁷ RBC-EVs are not replicative, and they do not insert genetic material into the host genome, which is why they do not generate the risk of integrational mutagenesis caused by the integration of viral vectors. Moreover, RBC-EVs do not contain viral capsid proteins, which cause neutralizing antibody reactions and overcome repeat dosing.⁸⁵ The short-term expression of CRISPR components as mRNA of Cas9 or ribonucleoprotein complexes further decreases long-term expression of nuclease and minimizes the risk of cumulative off-target genome editing.⁷⁷ Viral vectors can be effective in viral vectors delivery in some settings, but RBC-EVs can provide a safer modality of delivery when antiviral strategy needs immune compatibility and repeated delivery. Such a transient expression profile, together with good circulation characteristics and low immunological clearance, makes them potentially useful as a safer, context-relevant alternative to viral vectors in more specific disease treatments.⁷⁷ Especially in antiviral therapy, a temporal delivery of CRISPR-Cas RNPs or mRNA by RBC-EVs provides a significant benefit of safety because it reduces the chances of genotoxicity and immune response.⁸⁶ The current development of delivery technologies, including LNPs and RBC-EVs, has made preclinical studies with evidence of efficient gene editing to stop viral replication possible.²⁴ This method has the potential of a wide application, including HIV as well as newly developed viral infections.

Engineered RBC-EVs: Triple Advance in Scalable Production, Hybrid Delivery, and Targeted Modification Synergistically Accelerating Clinical Translation

Engineering improvements have recently boosted RBC-EV targeting and effectiveness to a large degree. Extrusion-based production facilitates mass production of homogeneous, cargo-loaded RBC-EVs via pre-loading molecules into red blood cells prior to vesiculation.⁸⁷ To achieve the highest level of intracellular delivery of CRISPR RNPs, hybrid systems based on RBC-EVs and on viral nanoparticles (eg, virus-like particles or lentivirus-derived nanoparticles) have been designed, enhancing the uptake efficiency.⁷⁷ The critical barriers to clinical translation, namely, the functionalization of RBC-EVs with targeting moieties, are overcome by surface engineering, greatly expanding cell-type selectivity, which is a significant obstacle to genome editing therapies.⁵¹ Some of the main methods are the attachment of targeting ligands to deliver to a specific tissue, the functional attachment of functional moieties to enhance selectivity, and the application of stimuli-reactive surfaces (eg, pH- or light-activated) to release upon command.⁸⁸ These specific changes combined with improvements in cargo loading and hybrid delivery systems all promote stability, biodistribution, and therapeutic efficacy, which leads to rapid clinical adoption.⁸⁹ The information regarding the engineering strategies and their functional tasks in the maximization of the delivery outcomes is provided in Figure 6.

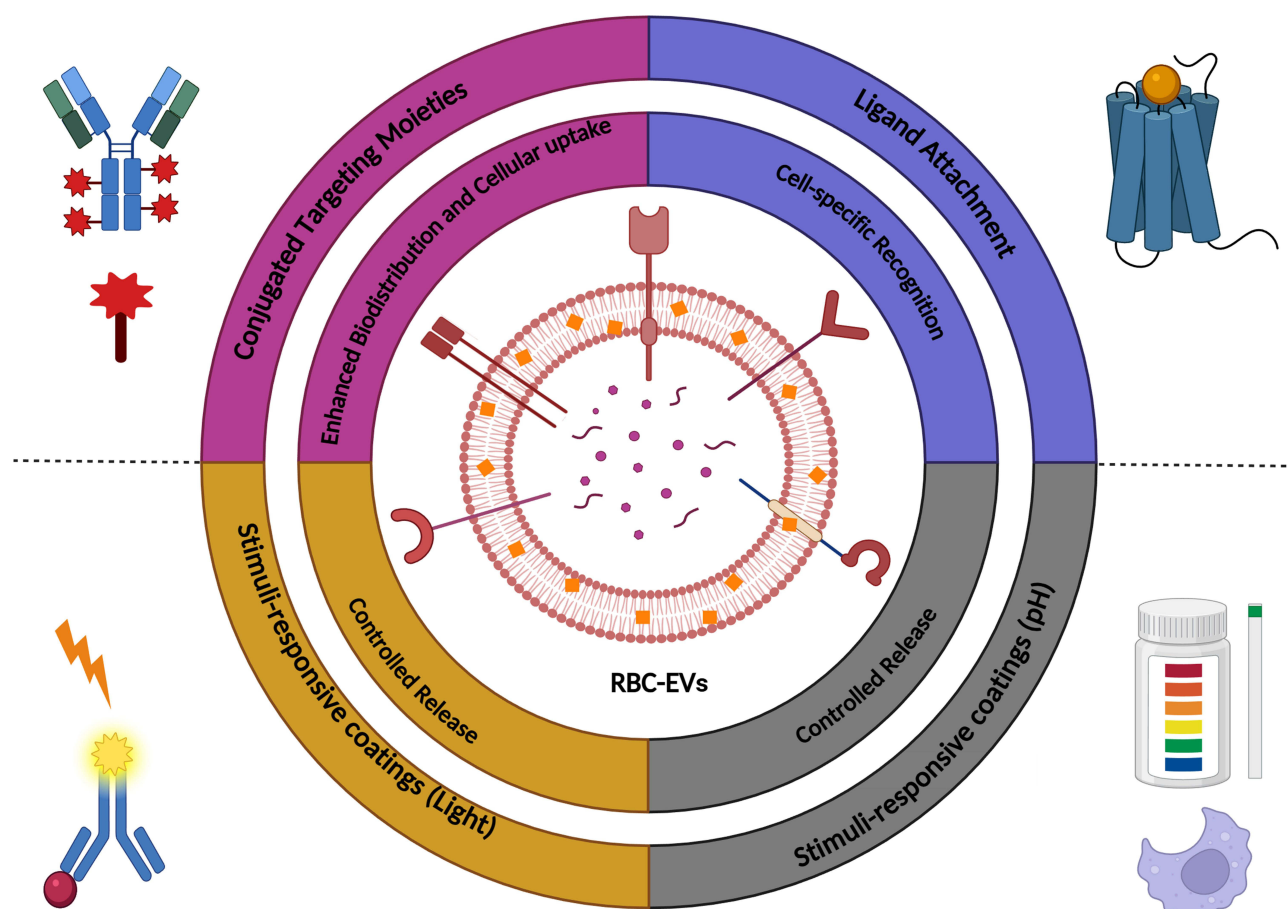


Figure 6 Surface engineering strategies for RBC-EVs. This schematic illustrates strategic modifications to RBC-EVs including conjugated targeting moieties for enhanced biodistribution and cellular uptake, stimuli-responsive coatings (pH/light) for spatiotemporally controlled release, and attached ligands for cell-specific recognition which collectively maximize delivery specificity and therapeutic efficacy in advanced biomedical applications.

RBC-EVs for CRISPR-Cas and mRNA Delivery: Unmet Potential and Translational Hurdles

Promising Attributes

RBC-EVs represent a highly promising delivery platform for CRISPR and mRNA therapies, as explained in RBC-EVs: A Revolutionary CRISPR Delivery Platform Driving Safe and Efficient Antiviral Therapy. However, translating this potential into clinical reality necessitates overcoming several critical translational hurdles. Even with these benefits, some unrealized potentials are still not fully realized. More research is specifically needed in the areas of RBC-EVs' intrinsic targeting abilities and the viability of producing them on a wide scale for therapeutic uses.⁶² To harness the therapeutic potential of RBC-EVs, a concerted effort must be made to address several pivotal translational challenges. Key barriers, including efficient cargo loading, targeted delivery specificity, scalable production, and long-term stability, must be systematically overcome to facilitate their clinical advancement.⁹⁰

Latent Capacity

As explained in the section of 4.4, RBC-EVs are proving to be a very promising delivery system of CRISPR and mRNA-based therapeutics.⁹¹ These properties make RBC-EVs a powerful alternative to be used in systemic delivery, which may surpass significant drawbacks related to traditional delivery vectors, such as lipid nanoparticles and viral systems. Despite such a substantial potential, the entire translational applicability of RBC-EVs is limited by a number of insufficiently studied issues.⁹² The key ones are an incomplete description of their targeting capabilities; how their native tropism can be effectively applied in order to obtain tissue or cell-specific delivery remains unknown.⁹³ Second, there are

considerable technical challenges involved in producing scaled-up production of RBC-EVs of uniform quality and yield that need to be overcome to translate them to clinical use.

Delivery Specificity

One of the major issues of therapeutic delivery is to provide an efficient and cell-specific delivery of mRNA and CRISPR payloads into diseased tissues. Although RBC-EVs naturally avoid immune detection more efficiently than synthetic nanoparticles or virus vectors, their biodistribution and cellular uptake mechanisms should be improved further.⁹⁴ The most important is to reach the target tissues selectively and to decrease the off-target effects. The ongoing attempts to make RBC-EVs targeted or surface-modified are still in an early stage of development and extremely difficult technologically.⁹⁵

Cargo Loading Challenges

Another significant challenge is the efficient loading of therapeutic cargo, including CRISPR-Cas9, mRNA, guide RNAs, or ribonucleoprotein complexes, into RBC-EVs without affecting cargo integrity or vesicle integrity. Traditional loading techniques (eg, extrusion and electroporation) may lead to the vesicle damage or cargo degradation, which decreases the functionality of delivery.⁹⁶ It is important to maintain the vesicle integrity whilst loading, at high efficiency and in a manner that can be scaled and reproducibly. Moreover, clinical-grade RBC-EVs do not have scalable and standardized procedures to obtain them despite the strong source of RBCs. Among them are issues of vesicle heterogeneity, batch-to-batch variability, purification problems, and the necessity to build GMO-compliant processes with consistent cargo loading, quality, purity, and functional delivery capacity.⁹⁷ The effective encapsulation of the cargo is critical to the effective delivery of the CRISPR and mRNA therapy in RBC-EVs. Despite the possible diverse cargo loading into RBC-EVs, it still faces several challenges to address, including loading efficiency, cargo stability, and optimization of the encapsulation process (eg, electroporation, sonication, and extrusion).² Big molecules such as Cas9 protein or CRISPR components can be challenging to encapsulate effectively by these methods, so although mRNA, proteins, and nucleic acids can be readily loaded, this can often lead to low loading outputs or cargo destruction. To ensure therapeutic efficacy, cargo integrity in the formation of vesicles is also important.⁵⁹

Safety & Immunogenicity

Although RBC-EVs exhibit low inherent immunogenicity, their potential to elicit immune responses, particularly upon repeated dosing, requires careful evaluation, as this can be influenced by cargo and surface proteins.⁶⁰ A key safety advantage over viral vectors is the non-integrative nature of EV-delivered cargo, which mitigates risks like insertional mutagenesis. However, comprehensive long-term safety data are still limited.⁹⁸ For example, toll-like receptors (TLRs) and other immune receptors may be activated by mRNA cargo contained in EVs, resulting in an innate immune response and the production of pro-inflammatory cytokines.⁶² Furthermore, immunogenic proteins or lipid alterations on the EV surface can promote complement activation or dendritic cell maturation, which may aid in immunological activation.⁶³ Comprehensive long-term safety data are still few, even though EVs have a significant safety benefit over viral vectors because of their non-integrative nature, which reduces concerns like insertional mutagenesis.⁹⁹ To guarantee safety after repeated administration, the immunogenicity assessment must take into account both the cargo's composition and the EVs' surface features.

Cargo Size Limitations & Biodistribution

Despite their advantages, RBC-EVs exhibit intrinsic limitations related to cargo size and biodistribution. Current evidence indicates that RBC-EVs efficiently encapsulate small RNA species, including siRNA and miRNA (<1 kb), as well as mRNA cargos typically below ~3–4 kb in length.¹⁰⁰ In contrast, larger nucleic acids such as plasmid DNA (>6–8 kb) and oversized mRNA constructs show reduced loading efficiency and heterogeneous encapsulation, likely due to steric constraints during vesicle biogenesis and post-loading methods such as electroporation.¹⁰¹ Furthermore, biodistribution studies suggest that systemically administered RBC-EVs preferentially accumulate in clearance-associated organs such as the liver and spleen, reflecting uptake by the mononuclear phagocyte system.¹⁰² While this profile may benefit applications targeting hepatic or immune-related disorders, it presents challenges for extrahepatic delivery.¹⁰³

Ongoing strategies, including surface ligand modification and membrane engineering, aim to improve tissue-specific biodistribution and overcome these limitations. Beyond safety concerns, physical constraints also limit applications.¹⁰⁴ The size and complexity of CRISPR-Cas systems pose specific constraints. RBC-EVs may have limited capacity to package large Cas proteins or multiple gRNAs simultaneously, potentially restricting applications requiring multiplexed editing or delivery of large gene-editing complexes.¹⁰⁵ While potential solutions such as carrier co-delivery or modular assembly have been proposed, these approaches introduce new complexities in cargo compatibility, delivery efficiency, and systemic distribution, often compromising stability or triggering immune responses.¹⁰⁶ Critical pharmacokinetic parameters – including biodistribution patterns, circulation half-life, and clearance mechanisms (particularly reticuloendothelial system-mediated uptake) – require precise characterization and control.¹⁰⁷ Rapid clearance or off-target accumulation can reduce efficacy and increase toxicity. Surface modifications to prolong circulation and evade macrophage uptake are actively researched but remain technically challenging.¹⁰⁸

Regulatory & Commercial Landscape

RBC-EVs are a unique biological delivery system that is subject to intense regulatory examination for their safety, effectiveness, and characterization. Important requirements for regulatory approval include defining critical quality characteristics (CQAs), developing standardized analytical assays, and proving reproducible clinical-grade production.¹⁰⁹ Cargo-loading efficiency, purity, size distribution, membrane integrity, and stability are some of the most important CQAs for RBC-EVs. Because overloading or underloading might impair delivery performance, specific cargo-loading thresholds must be set to guarantee treatment efficacy. In order to guarantee the end product's safety and uniformity, purity standards must also be established to reduce contamination from cellular debris or other undesirable vesicles. These characteristics are essential for fulfilling regulatory criteria, as is thorough in vivo characterization (such as biodistribution, half-life, and clearance).¹¹⁰

In conclusion, although RBC-EVs offer an attractive natural platform for resolving the delivery issues of CRISPR and mRNA-based therapeutics, concentrated efforts are needed to overcome a number of significant obstacles before their full therapeutic potential can be realized. Targeted delivery, effective cargo loading, scalable GMP manufacturing, immunogenicity evaluation, cargo size restrictions, biodistribution management, and regulatory navigation are a few of these. Since they have a direct impact on the therapeutic efficacy of RBC-EVs, targeted distribution and effective cargo loading are the main bottlenecks among them. To improve the accuracy and efficacy of RBC-EV-mediated delivery, these obstacles must be overcome. Simultaneously, reducing immunogenicity and guaranteeing scalable GMP production will be crucial to RBC-EVs' feasibility for clinical application.^{111,112} As shown in Table 4, RBC-EVs have been investigated as vehicles for delivering genetic cargo, including DNA, mRNA, and siRNA. This highlights preclinical studies investigating the feasibility of loading and delivering nucleic acid cargos using red blood cell-derived extracellular vesicles (RBC-EVs) or RBC-EV mimetic system.¹¹³ The summarized studies primarily focus on proof-of-concept demonstrations of RNA encapsulation, delivery efficiency, and functional expression or gene silencing in in vitro and limited in vivo experimental models. At present, evidence supporting RBC-EV mediated gene delivery remains largely preclinical, and direct therapeutic gene-editing applications, including CRISPR/Cas-based strategies, have not yet been validated for RBC-derived EVs.¹¹⁴ The table, therefore, emphasizes experimental feasibility rather than confirmed disease-level therapeutic efficacy.

Table 4 Preclinical Studies Exploring Nucleic Acid Loading Into RBC-Derived Extracellular Vesicles

Therapeutic Cargo	Loading Method	Target Gene	Disease	Effects	References
mRNA	Electroporation/ passive loading	Reporter or therapeutic proteins	In vitro and in vivo models	Successful mRNA loading and protein expression	[5]
siRNA	Electroporation	Model gene targets	Cell culture models	Efficient gene silencing observed	[62]
RNA oligonucleotides	EV fusion/incubation	Model targets	Proof-of-concept studies	Demonstrated feasibility of nucleic acid delivery	[63]

Conclusion and Future Perspective

RBC-EVs are a disruptive platform of delivering CRISPR/Cas9 systems and mRNA therapeutics. The inherent benefits of low immunogenicity, remarkable biocompatibility, and the high ability to avoid the immune system facilitate high effectiveness in encapsulating and delivering gene-editing cargo into target cells, and being better than synthetic nanoparticles and viral vectors are mentioned in this review. Their effectiveness in a variety of disease models, including cancer, genetic disorders, and infectious diseases, is supported by strong preclinical evidence, which makes RBC-EVs an attractive instrument of precision medicine. But four crucial issues confront the clinical translation: (1) targeted delivery to tissues using engineered ligands (eg, aptamers and peptides) with little to no off-target effects, (2) gentle and high-efficiency cargo loading strategies to retain vesicle integrity and payload stability, and (3) scalable and GMP-conformable manufacturing protocols to maintain batch uniformity. Also, a detailed safety evaluation is still essential to consider the risks of horizontal gene transfer, immunogenicity following repeated dosing, and long-term profiles of biodistribution, which can be effectively overcome through the use of so-called stealth modification (eg PEGylation). To unleash the full therapeutic potential of RBC-EVs, future studies should focus on: (1) The development of novel solutions to challenges to enable selective delivery, including (a) modular targeting designs; (b) next-generation loading approaches (eg membrane permeabilization and RNA dimerizations); (c) microfluidic biomanufacturing; (2) The expansion of therapeutic paradigms, such as multiplexed co-delivery of CRISPR and mRNA cargoes, combination strategies with immune. With the further development of manufacturing and regulatory routes, RBC-EVs will make it possible to perform safe, adjustable genome editing, and gene modulation and transform the treatment of complex diseases. Combined with the strong preclinical validation, scalable manufacturing, and the emerging clinical models, the convergence marks their central involvement in the next-generation precision therapeutics.

Generative Artificial Intelligence

Portions of this manuscript were assisted by OpenAI's ChatGPT (GPT-5 model) for language refinement, organization, and clarity improvement. All scientific content, interpretation, and conclusions were verified and approved by the authors.

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

The authors would like to extend their sincere appreciation to Shenzhen Medical Research Fund (No. D2402009), Natural Science Foundation of Guangdong Province (No. 2025A1515011298), Shenzhen Science and Technology Projects (No. JCYJ 20240813140405008), Medical-Engineering Interdisciplinary Research Foundation of Shenzhen University (No. 2023YG014).

Disclosure

The authors declare that they have no conflicts of interest.

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