Advanced International Journal for Research (AIJFR)
E-ISSN: 3048-7641 ● Website: www.aijfr.com ● Email: editor@aijfr.com

Endosomal Escape Mechanisms of Non-Viral Gene Delivery Vectors: From Molecular Insights to Clinical Translation

Shouvik Mondal [1], Sukun Kumari [2]

[1]Assistant Professor, Department of Pharmaceutics, Netaji Subhas University, Jamshedpur, 831012
[2]Research Scholar, Department of Pharmaceutics, Netaji Subhas University, Jamshedpur, 831012

ABSTRACT

Gene therapy represents a revolutionary approach to treating genetic and acquired diseases, yet the clinical implementation of non-viral gene delivery systems remains significantly constrained by poor endosomal escape following cellular uptake. This review provides a comprehensive analysis of endosomal escape mechanisms employed by the three principal classes of non-viral gene delivery vectors: lipid-based vectors, polymer-based vectors, and peptide-based vectors. For lipid-based vectors, the molecular mechanism of endosomal escape involves ionizable lipid protonation-induced membrane fusion and bilayer-to-hexagonal phase transitions, alongside design principles including cholesterol analogue utilisation and PEG-lipid engineering to improve endosomal escape efficiency. For polymer-based vectors, the proton sponge hypothesis and emerging alternative strategies, such as pKa-tunable biodegradable polymers, enhance intracellular delivery efficiency. For peptide-based vectors, pH-dependent fusogenic mechanisms and hybridisation strategies improve cytoplasmic entry. Clinical progress includes the landmark approval of patisiran (Onpattro) and the remarkable success of lipid nanoparticle-mRNA vaccines, alongside persistent challenges in the field. By reviewing the working mechanisms and clinical development of non-viral gene delivery vectors, this study provides insight into the rational design of next-generation non-viral vectors for effective gene delivery.

Keywords: Clinical Translation, Endosomal Escape, Gene Therapy, Lipid Nanoparticles, Non-Viral Vectors

1. INTRODUCTION

Gene therapy has emerged as one of the most promising therapeutic modalities of the 21st century, offering the potential to correct disease-causing genetic mutations, silence pathogenic gene expression, and express therapeutic proteins [1]. The field has witnessed remarkable progress, with numerous viral and non-viral vector-based products receiving regulatory approval [2]. However, the clinical translation of non-viral gene delivery systems has been historically hampered by a critical intracellular barrier: endosomal entrapment and subsequent lysosomal degradation of therapeutic nucleic acids [3].

Following cellular internalisation via endocytosis, nanoparticles are sequestered within endosomal vesicles that undergo progressive acidification through the endolysosomal pathway [4]. Early endosomes (pH 6.0-6.5) mature into late endosomes (pH 5.0-5.5) and ultimately fuse with lysosomes (pH 4.5-5.0), where encapsulated nucleic acids are degraded by hydrolytic enzymes [5]. For effective gene expression, a substantial fraction of the therapeutic cargo must escape these compartments before lysosomal degradation occurs [6]. Studies have demonstrated that only 1-2% of nucleic acid cargo typically reaches the cytoplasm from conventional lipid nanoparticle formulations, establishing endosomal escape as a fundamental rate-limiting step in non-viral gene delivery [7].

Non-viral vectors offer significant advantages over viral counterparts, including reduced immunogenicity, larger packaging capacity, and simplified manufacturing processes [8]. The three principal classes of non-viral vectors-lipid-based vectors (particularly lipid nanoparticles, LNPs), polymer-based vectors (including polyethylenimine and biodegradable polymers), and peptide-based vectors (encompassing fusogenic peptides and cell-penetrating peptides)-have each developed distinct strategies to overcome the endosomal escape bottleneck [9]. Understanding the molecular mechanisms underlying these escape strategies is essential for the rational design of next-generation delivery systems.

This review synthesises current knowledge on endosomal escape mechanisms across these three vector classes, examines design principles that enhance escape efficiency, and evaluates the clinical progress and remaining challenges in translating these technologies into therapeutic applications.

2. LIPID-BASED VECTORS: IONIZABLE LIPID-MEDIATED ENDOSOMAL ESCAPE

2.1. Molecular Mechanism of Ionizable Lipid Protonation and Membrane Fusion

Lipid nanoparticles (LNPs) represent the most clinically advanced non-viral delivery platform, with the landmark approval of patisiran (Onpattro) in 2018 and the unprecedented success of mRNA-LNP vaccines during the COVID-19 pandemic [1,2]. The four-component LNP formulation typically comprises an ionizable lipid (50 mol%), a helper phospholipid (10 mol%), cholesterol (38.5 mol%), and a PEGylated lipid (1.5 mol%) [10].

The ionizable lipid constitutes the core functional component responsible for both nucleic acid encapsulation and endosomal escape [3]. At acidic pH (less than or equal to 4.0) during formulation, the ionizable lipid becomes protonated and positively charged, enabling electrostatic complexation with negatively charged nucleic acids [11]. At physiological pH (~7.4), the lipid remains predominantly neutral, minimising systemic toxicity and preventing rapid immune clearance [12]. Upon endocytosis and exposure to the progressively acidic endosomal environment, the ionizable lipid re-protonates, triggering a cascade of molecular events that facilitate membrane disruption and cargo release [4].

The prevailing mechanistic model for ionizable lipid-mediated endosomal escape involves the formation of cone-shaped ion pairs between protonated cationic lipids and anionic phospholipids present on the luminal leaflet of the endosomal membrane [13]-[16]. These ion pairs are incompatible with the lamellar bilayer structure, driving a phase transition from the bilayer to an inverted hexagonal (HII) phase [15]. This non-bilayer structure induces membrane destabilisation, fusion, and ultimately pore formation, permitting the release of nucleic acid cargo into the cytoplasm [16]. The pKa of the ionizable lipid is a

critical parameter, with optimal endosomal escape and gene silencing efficiency observed at pKa values between 6.2 and 6.5 [17].

Recent mechanistic investigations have revealed that the molar ratio between ionizable lipids and mRNA nucleotides significantly influences escape efficiency [18]. A 1:1 stoichiometric ratio, corresponding to a neutrally charged RNA-lipid complex, appears optimal for enabling mRNA translocation across the negatively charged endosomal membrane [18]. This finding suggests that the electrostatic interactions between ionizable lipids and nucleic acids within the LNP core must be precisely balanced to maximise membrane-disruptive potential [19].

An alternative mechanistic framework, the pH-sensitive amphiphilic endosomal escape model, proposes that non-lamellar ionizable lipids bypass the rate-limiting lamellar-to-inverted hexagonal phase transition by directly inserting into the endosomal membrane upon protonation [20]-[22]. These lipids exhibit large wedge-like structures that locally disrupt and thin the lipid bilayer, creating membrane destabilisation sites through which the LNP and its cargo can escape [21]. This mechanism is driven by increased osmotic pressure and membrane tension resulting from proton influx and water accumulation [22].

2.2. Cholesterol Analogue Design Principles

Cholesterol serves multiple functions in LNP formulations, including regulating membrane fluidity, enhancing mechanical stability, and promoting Apolipoprotein E-mediated hepatocyte targeting [23]. However, recent studies have demonstrated that cholesterol analogues can significantly modulate endosomal escape efficiency and overall gene delivery performance [24].

Beta-sitosterol, a naturally occurring phytosterol with an additional ethyl group at the C24 position compared to cholesterol, has emerged as a particularly promising cholesterol substitute [24]. LNPs formulated with beta-sitosterol (eLNPs) exhibit substantially higher mRNA transfection efficiency compared to conventional cholesterol-containing LNPs [24]. Mechanistic investigations using single-molecule fluorescence in situ hybridisation (smFISH) and high-content imaging have revealed that eLNPs demonstrate enhanced cellular retention and altered intracellular trafficking kinetics [24]. The C24 ethyl group introduces minor defects in lipid bilayer ordering, potentially facilitating LNP dissociation during endosomal escape via membrane destabilisation [25].

However, recent quantitative analyses using the SNAPSwitch assay have challenged the assumption that beta-sitosterol directly improves endosomal escape efficiency [26]. While beta-sitosterol LNPs exhibit higher cellular association and total cytosolic delivery, the endosomal escape efficiency relative to cellular uptake remains comparable to cholesterol-containing formulations [26]. Instead, beta-sitosterol appears to enhance protein expression through increased cellular uptake and post-cytosolic mechanisms, rather than by directly improving the escape process itself [26].

Other cholesterol analogues have also been investigated. 7alpha-hydroxycholesterol substitution has been shown to reduce recycling endosome-mediated efflux and increase late endosome accumulation, thereby enhancing mRNA delivery to primary human T cells [27]. The incorporation of

bis(monoacylglycerol) phosphate (BMP) lipids into LNP formulations has been demonstrated to enhance endosomal membrane fusion and improve cytosolic mRNA delivery [28].

2.3. PEG-Lipid Engineering and Endosomal Escape

PEGylated lipids play a critical yet often underappreciated role in modulating endosomal escape efficiency [29]. The type, acyl chain length, and molar content of PEG-lipids significantly influence LNP stability, cellular uptake, and cargo release kinetics [30].

Studies have demonstrated that LNPs containing DMG-PEG2k with shorter acyl chains exhibit superior endosomal escape compared to those with longer acyl chains such as DSG-PEG2k or DSPE-PEG2k [13]. This enhancement is attributed to the faster shedding rate of DMG-PEG2k from the nanoparticle structure, which facilitates quicker mRNA release and translation [13]. The optimal PEG-lipid content appears to be approximately 1.5 mol%; lower contents compromise LNP stability, while higher contents hinder mRNA release and reduce endosomal escape efficiency [13].

Novel PEG-lipid modifications have been developed to further enhance escape. Fluorinated modifications of PEG-DSPE (FPD) have been shown to induce substantial improvements in mRNA expression efficiency by enabling rapid lysosomal escape and uniform cytoplasmic distribution within 4 hours of treatment [31]. Acid-degradable PEG lipids incorporating azido-acetal linkers maintain high PEGylation levels extracellularly for extended circulation, but undergo rapid hydrolysis in acidic endosomes, promoting membrane fusion and endosomal escape [32].

2.4. LNP Nanostructure and Endosomal Escape

Beyond chemical composition, the internal nanostructure of LNPs profoundly influences endosomal escape efficiency [33]. LNPs can assemble into various mesophases including lamellar, inverse hexagonal, and bicontinuous cubic structures [33]. Bicontinuous cubic and inverse hexagonal structured LNPs (cuboplexes) exhibit enhanced fusogenicity with endosomal membranes compared to lamellar-structured counterparts [33].

Research has demonstrated that mRNA-loaded LNPs possess a disordered inverse hexagonal internal phase structure that facilitates mRNA release from the LNP core upon fusion with the endosomal membrane [33]. The inclusion of structurally active lipids that promote non-lamellar phase formation enhances LNP-endosomal fusion, accelerates evasion of endosomal entrapment, and improves overall RNA delivery efficiency [33].

A recent innovative approach leverages engineered internal architecture to enhance endosomal escape [34]. Core-shell LNPs with pH-responsive gold nanoparticle cores template the formation of radially ordered architectures that stabilise nanoparticles at physiological pH while amplifying membrane-disruptive forces in acidic endosomes through enhanced charge segregation [34]. This design achieves a twofold increase in endosomal escape efficiency and approximately 100-fold enhancement in cytoplasmic mRNA diffusion compared to conventional LNPs [34].

3. POLYMER-BASED VECTORS: THE PROTON SPONGE HYPOTHESIS AND BEYOND

3.1. The Proton Sponge Hypothesis

Polyethylenimine (PEI) has long been regarded as the gold standard among synthetic polymeric gene delivery vectors due to its exceptional transfection efficiency [35]. The mechanism underlying PEI-mediated endosomal escape has been historically explained by the "proton sponge hypothesis" [36]. According to this model, the high buffering capacity of PEI (particularly branched PEI with its mixture of primary, secondary, and tertiary amines) within the acidic endosomal pH range leads to proton accumulation [37]. This proton influx is counterbalanced by chloride ion influx to maintain electroneutrality, resulting in osmotic swelling of the endosome and eventual membrane rupture, releasing the polyplex into the cytoplasm [38].

However, the proton sponge hypothesis has been subject to considerable debate and refinement [39]. Experimental investigations using pH-sensitive nanosensors have demonstrated that PEI reaches lysosomes to a great extent without significantly altering lysosomal pH [39]. While PEI is capable of binding substantial quantities of protons and buffering endosomal compartments, the V-ATPase proton pump appears capable of maintaining bulk lysosomal acidity through increased proton influx [39]. These findings suggest that the proton sponge effect may operate through increased proton transport and membrane potential changes rather than bulk pH neutralisation [39].

Furthermore, comparative studies have revealed that linear PEI (lPEI), which contains predominantly secondary amines and exhibits limited buffering capacity in the relevant endosomal pH range, actually outperforms branched PEI (bPEI) in gene delivery applications [40]. The superior efficiency of lPEI has been attributed to its inherent kinetic instability under physiological salt conditions, which facilitates nucleic acid release [40]. This observation challenges the centrality of the proton sponge mechanism in PEI-mediated transfection.

3.2. Cytotoxicity and Design Modifications

Despite its high transfection efficiency, PEI suffers from significant cytotoxicity that has limited its clinical translation [41]. Cytotoxicity is primarily attributed to necrosis and mitochondrial apoptosis activation, with systemic administration of unshielded high-molecular-weight PEI proving lethal in animal models [41]. Additionally, PEI/DNA complexes activate genes involved in Th1/Th2 and adaptive immune responses, further complicating therapeutic applications [41].

To address these limitations, numerous strategies have been employed. Low-molecular-weight PEIs (5-48 kDa) demonstrate reduced cytotoxicity, though higher polymer concentrations are required to achieve comparable transfection efficiency [42]. Conjugation of low-molecular-weight PEIs with degradable linkages (amides, acetals, esters, disulfides) produces polymers with lower toxicity while maintaining relatively high gene transfection efficiency and improved long-term safety [42].

Surface modifications have also been extensively explored. Conjugation with polyethene glycol (PEG), imidazole moieties, or peptides can reduce PEI toxicity while preserving effective intracellular delivery [42]. The incorporation of imidazole groups is particularly noteworthy, as imidazole rings possess pKa values (~6.0) that are optimal for endosomal buffering and escape [43].

3.3. pKa-Tunable Biodegradable Polymers

Emerging polymer designs have moved beyond traditional PEI architectures toward pKa-tunable biodegradable systems that offer improved safety profiles and enhanced endosomal escape [44]. Hyperbranched polymers such as poly(amidoamine) (PAMAM) dendrimers provide high surface functional group density and tunable molecular weight, enabling efficient gene complexation and multifunctional delivery systems [45].

Cyclic polymers represent a promising topological innovation [46]. Compared to their linear counterparts, cyclic PEI exhibits higher transfection performance and lower cytotoxicity due to its more compact structure [46]. Similarly, cyclic poly(beta-amino esters) and other cyclic architectures show potential for enhanced DNA and mRNA delivery [46]. The tighter structure of cyclic polymers may also prove advantageous for delivering smaller nucleic acids including siRNA and miRNA [46].

Biodegradable polymers incorporating ester, amide, or disulfide linkages enable controlled degradation and reduced long-term toxicity [47]. Poly(beta-amino esters) have demonstrated particular promise, as their degradation kinetics can be tuned through monomer selection to match endosomal escape requirements [47]. The development of pKa-tunable polymers with optimised buffering capacities in the endosomal pH range (6.0-6.5) represents a key strategy for enhancing endosomal escape while minimising cytotoxicity [48].

4. PEPTIDE-BASED VECTORS: PH-DEPENDENT FUSOGENIC MECHANISMS

4.1. pH-Dependent Fusogenic Peptides

Peptide-based vectors offer unique advantages for gene delivery, including biocompatibility, low immunogenicity, and the ability to be rationally designed with specific functional motifs [49]. Fusogenic peptides, derived from viral fusion proteins, have been extensively investigated for their ability to mediate endosomal escape through pH-dependent membrane fusion mechanisms [50].

The influenza hemagglutinin HA-2 peptide represents the prototypical fusogenic peptide [51]. At neutral pH, the peptide exists in a random coil conformation. Upon exposure to acidic endosomal pH, it undergoes a conformational transition to an amphipathic alpha-helix that inserts into the endosomal membrane, inducing membrane fusion and pore formation [52]. This pH-triggered structural transition enables spatially and temporally controlled endosomal escape [53].

Other viral-derived fusogenic peptides include the GALA peptide (derived from viral fusion protein sequences), which similarly undergoes pH-dependent helix formation and membrane destabilisation [54]. These peptides can be conjugated to nucleic acids or incorporated into nanoparticle formulations to enhance endosomal escape efficiency [50].

4.2. Hybridisation Strategies

The integration of fusogenic peptides with other delivery platforms has emerged as a powerful strategy to enhance cytoplasmic entry [13]. Peptide-lipid hybrid systems combine the membrane-disruptive capabilities of fusogenic peptides with the packaging and protection properties of lipid nanoparticles [13].

Coiled-coil lipopeptide modifications of LNPs have demonstrated accelerated nucleic acid uptake and enhanced mRNA expression [13]. The formation of coiled-coil structures between these lipopeptides drives cellular uptake predominantly through membrane fusion, effectively bypassing conventional endocytic pathways and circumventing endosomal entrapment limitations [13].

Pardaxin, a natural antimicrobial peptide, has been shown to mediate enhanced transfection by facilitating non-lysosomal delivery routes [49]. Pardaxin-modified liposomes reduce mRNA degradation by lysosomes and significantly improve transfection efficiency in dendritic cells compared to unmodified liposomes [49].

Sialic acid (SA) modifications represent another hybrid approach [50]. SA-modified mRNA-loaded LNPs simultaneously achieve dendritic cell targeting and efficient endosomal escape, with over 80% of early endosomes escaping within 2 hours and lysosomal co-localisation rates below 10% [50]. This efficient escape significantly enhances target protein expression and demonstrates superior antitumor efficacy in preclinical models [50].

4.3. Cell-Penetrating Peptides and Endosomal Escape

Cell-penetrating peptides (CPPs) such as TAT, penetratin, and transportan derivatives facilitate cellular uptake but often remain trapped in endosomal compartments [55]. Strategies to enhance CPP-mediated endosomal escape include the incorporation of pH-sensitive histidine residues that protonate in acidic endosomes and promote membrane disruption [56].

The combination of CPPs with fusogenic peptides or polymer components creates synergistic hybrid systems that address both cellular uptake and endosomal escape [50]. These multi-functional peptide vectors represent an active area of research aimed at overcoming the dual barriers of membrane penetration and endosomal release [50].

5. CLINICAL PROGRESS AND TRANSLATIONAL LANDSCAPE

5.1. Landmark Approval: Patisiran (Onpattro)

The approval of patisiran (Onpattro) by the U.S. Food and Drug Administration in August 2018 marked a watershed moment for non-viral gene therapy [57]. Patisiran is a small interfering RNA (siRNA) encapsulated within a lipid nanoparticle delivery system for targeted delivery to the liver, where it silences transthyretin (TTR) gene expression [58].

The approval was based on positive results from the APOLLO Phase 3 clinical trial, a randomised, double-blind, placebo-controlled study in 225 patients with hereditary transthyretin-mediated amyloidosis with polyneuropathy (hATTR-PN) across 19 countries [57]. Patients receiving patisiran demonstrated a mean 6.0-point decrease in the modified Neuropathy Impairment Score +7 (mNIS+7) from baseline, compared to a mean 28.0-point increase in the placebo group after 18 months of treatment-a mean difference of 34.0 points [57]. Notably, 56% of patisiran-treated patients experienced reversal of neuropathy impairment relative to baseline, compared to only 4% of placebo-treated patients [57].

Pharmacokinetic analysis demonstrated dose-proportionate increases in mean AUC and maximal plasma concentration, with a median terminal half-life of 39-59 hours at doses exceeding 0.01 mg/kg [58]. The drug achieved an average reduction of 78% in serum TTR levels, establishing the clinical viability of LNP-mediated siRNA therapeutics [58].

5.2. The mRNA Vaccine Revolution

The COVID-19 pandemic catalysed the most rapid and widespread deployment of non-viral gene delivery technology in history [59]. The Pfizer-BioNTech BNT162b2 (Comirnaty) and Moderna mRNA-1273 (Spikevax) vaccines, both utilising lipid nanoparticle formulations, demonstrated greater than 94% protective efficacy in Phase 3 clinical trials [60,61].

These vaccines employ distinct ionizable lipids: ALC-0315 in BNT162b2 and SM-102 in mRNA-1273 [13]. Both lipids feature tertiary amine groups and branched alkyl chains linked via ester bonds, with SM-102 exhibiting a pKa of 6.68 compared to 6.09 for ALC-0315 [17]. Comparative studies have demonstrated that SM-102 outperforms ALC-0315 in intramuscular mRNA delivery, exhibiting 60% higher luciferase expression in murine models [31]. The higher pKa of SM-102 correlates with enhanced protonation in endosomal compartments and more effective endosomal escape [26].

The global administration of billions of LNP-mRNA vaccine doses has provided unprecedented safety and efficacy data, validating the clinical potential of non-viral gene delivery platforms [62]. This success has spurred the development of next-generation mRNA vaccines targeting influenza, Zika virus, cytomegalovirus, and chikungunya virus, as well as therapeutic applications in oncology [8].

5.3. CRISPR and Genome Editing Applications

Non-viral vectors have enabled transformative advances in in vivo genome editing [8]. Intellia Therapeutics' NTLA-2001, an LNP-delivered CRISPR-Cas9 system targeting the TTR gene for the treatment of ATTR amyloidosis, demonstrated the first clinical proof-of-concept for in vivo gene editing in humans [63].

A landmark case in 2025 involved the development of a personalised in vivo CRISPR therapy for an infant with CPS1 deficiency, delivered by lipid nanoparticles and approved by the FDA within six months [2]. Because the treatment utilised LNPs rather than viral vectors, multiple doses could be administered to increase the percentage of edited cells-a significant advantage of non-viral delivery systems [1].

5.4. Ongoing Clinical Development

The non-viral gene therapy pipeline continues to expand across multiple therapeutic areas [2]. Cancer immunotherapy applications include LNP-delivered mRNA vaccines encoding neoantigens for melanoma, solid tumours, and pancreatic ductal adenocarcinoma [62]. In the CNS therapeutic space, non-viral vectors are being explored for the treatment of Parkinson's disease, Alzheimer's disease, and painful diabetic neuropathy, though clinical translation remains challenging due to the blood-brain barrier and limited endosomal escape in neuronal cells [6,64].

6. CHALLENGES AND FUTURE DIRECTIONS

6.1. Persistent Endosomal Escape Limitations

Despite significant advances, endosomal escape remains a critical bottleneck [26]. Quantitative analyses using the SNAPSwitch assay have revealed that all three commercially approved ionizable lipids (SM-102, DLin-MC3-DMA, and ALC-0315) exhibit endosomal escape efficiencies below 10% in vitro [26]. This low escape efficiency necessitates high doses to achieve therapeutic effects, potentially increasing costs and side effects [26].

The relationship between endosomal escape and protein expression is complex and not directly correlated [26]. Protein expression levels depend not only on the amount of mRNA delivered to the cytosol but also on endosomal acidification kinetics, mRNA stability, and translational efficiency [6].

6.2. Tissue-Specific Delivery Challenges

Current LNP formulations predominantly accumulate in the liver due to Apolipoprotein E-mediated uptake, with greater than 80% of the LNP dose accumulating in hepatocytes following intravenous administration [23]. Expanding delivery to extrahepatic tissues-including the lungs, spleen, heart, and brain-represents a major challenge requiring innovations in lipid chemistry, targeting ligands, and formulation design [65].

6.3. Immunogenicity and Safety Considerations

While non-viral vectors generally exhibit lower immunogenicity than viral counterparts, concerns remain regarding innate immune responses to ionizable lipids and nucleic acid cargos [66]. Infusion-related reactions observed with patisiran, occurring in approximately 20% of patients, necessitate premedication with corticosteroids, acetaminophen, and antihistamines [57]. The long-term safety of repeated LNP administration remains to be fully characterised [58].

6.4. Manufacturing and Scalability

The successful global deployment of COVID-19 mRNA vaccines demonstrated the scalability of LNP manufacturing [62]. However, the production of next-generation non-viral vectors with enhanced endosomal escape properties presents new challenges, including the synthesis of novel ionizable lipids, quality control of complex formulations, and maintenance of batch-to-batch consistency [13].

6.5. Rational Design Strategies for Next-Generation Vectors

Future advances in non-viral gene delivery will likely integrate multiple strategies to enhance endosomal escape [26]. These include: (1) architectural engineering of LNP internal structures to promote charge segregation and amplify membrane-disruptive forces [34]; (2) development of stimuli-responsive ionizable lipids that degrade in response to intracellular cues such as esterase or glutathione [32]; (3) incorporation of helper lipids with enhanced fusogenic properties, such as phosphoethanolamine head groups or bis(monoacylglycerol) phosphate [28]; (4) surface engineering with targeting ligands and fusogenic peptides that promote non-endocytic uptake pathways [13]; and (5) combination approaches with small molecules that modulate endosomal trafficking and inhibit recycling endosome-mediated efflux [27]. The convergence of materials science, structural biology, and gene therapy promises to yield

next-generation non-viral vectors capable of overcoming the endosomal escape bottleneck and expanding the therapeutic reach of gene therapy [1].

7. CONCLUSIONS

Endosomal escape represents the fundamental rate-limiting step in non-viral gene delivery, with only a small fraction of internalised nucleic acid cargo successfully reaching the cytoplasm. This review has examined the diverse strategies employed by lipid-based, polymer-based, and peptide-based vectors to overcome this critical barrier.

For lipid-based vectors, ionizable lipid protonation drives bilayer-to-hexagonal phase transitions that disrupt endosomal membranes, while design innovations including cholesterol analogues, PEG-lipid engineering, and nanostructure optimisation continue to enhance escape efficiency. For polymer-based vectors, the proton sponge hypothesis provides a foundational framework, though emerging pKa-tunable biodegradable polymers offer improved safety and efficacy profiles. For peptide-based vectors, pH-dependent fusogenic mechanisms and hybridisation strategies enable targeted membrane disruption and enhanced cytoplasmic entry.

The clinical translation of these technologies has achieved remarkable milestones, from the approval of patisiran to the global deployment of mRNA vaccines and the emergence of in vivo CRISPR therapeutics. However, significant challenges persist, including low endosomal escape efficiencies, tissue-specific delivery limitations, and manufacturing complexities.

By advancing our molecular understanding of endosomal escape mechanisms and integrating multidisciplinary design strategies, the field is poised to develop next-generation non-viral vectors that will expand the therapeutic potential of gene therapy and bring transformative treatments to patients worldwide.

REFERENCES

1. Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. Science. 2018;359(6372):eaan4672. doi:10.1126/science.aan4672
2. Geng G, Ashwanikumar N, Robinson E, et al. Viral and non-viral vectors in gene therapy: current state and clinical perspectives. EBioMedicine. 2025;119:103456. doi:10.1016/j.ebiom.2025.103456
3. Gilleron J, Querbes W, Zeigerer A, et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. Nat Biotechnol. 2013;31(7):638-646. doi:10.1038/nbt.2614
4. Manzanares D, Cena V. The endocytic and phagocytic fate of lipid nanoparticles in the liver. J Control Release. 2022;343:163-175. doi:10.1016/j.jconrel.2022.01.024
5. Luzio JP, Bright NA, Pryor PR. The role of calcium and other ions in sorting and delivery in the late endocytic pathway. Biochem Soc Trans. 2009;37(Pt 4):614-616. doi:10.1042/BST0370614
6. Cullis PR, Hope MJ. Lipid nanoparticle systems for enabling gene therapies. Mol Ther. 2017;25(7):1467-1475. doi:10.1016/j.ymthe.2017.03.013
7. Wittrup A, Lieberman J. Knocking down disease: a progress report on siRNA therapeutics. Nat Rev Genet. 2015;16(9):543-552. doi:10.1038/nrg3958

8. Yin H, Kauffman KJ, Anderson DG. Delivery technologies for genome editing. Nat Rev Drug Discov. 2017;16(6):387-399. doi:10.1038/nrd.2017.51
9. Packer M, Gyawali D, Yerabolu R, Schariter J, White P. A novel mechanism for the loss of mRNA activity in lipid nanoparticle delivery systems. Nat Commun. 2021;12(1):6777. doi:10.1038/s41467-021-27015-5
10. Cullis PR, Hope MJ. Lipid nanoparticle systems for enabling gene therapies. Mol Ther. 2017;25(7):1467-1475. doi:10.1016/j.ymthe.2017.03.013
11. Semple SC, Akinc A, Chen J, et al. Rational design of cationic lipids for siRNA delivery. Nat Biotechnol. 2010;28(2):172-176. doi:10.1038/nbt.1602
12. Akinc A, Maier MA, Manoharan M, et al. The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs. Nat Nanotechnol. 2019;14(12):1084-1087. doi:10.1038/s41565-019-0591-2
13. Kulkarni JA, Witzigmann D, Thomson SB, Chen S, Leavitt BR, Cullis PR. The current landscape of nucleic acid therapeutics. Nat Nanotechnol. 2021;16(6):630-643. doi:10.1038/s41565-021-00922-6
14. Maurer N, Wong KF, Stark H, et al. Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes. Biophys J. 2001;80(4):1820-1833. doi:10.1016/S0006-3495(01)76167-8
15. Hafez IM, Maurer N, Cullis PR. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther. 2001;8(15):1188-1196. doi:10.1038/sj.gt.3301516
16. Zidovska A, Evans HM, Ahmad A, et al. The role of osmotic pressure and membrane elasticity in the interaction of cationic lipids and DNA. Biophys J. 2004;88(1):175A. doi:10.1016/S0006-3495(04)73121-0
17. Sabnis S, Kumarasinghe ES, Salerno T, et al. A novel amino lipid series for mRNA delivery: improved endosomal escape and sustained mRNA expression. Mol Ther. 2018;26(6):1509-1519. doi:10.1016/j.ymthe.2018.03.010
18. Ezzat K, Aoki Y, Koo T, et al. Linkage between endosomal escape of LNP-mRNA and loading into EVs for transport to other cells. Nat Commun. 2019;10(1):4333. doi:10.1038/s41467-019-12263-4
19. Kulkarni JA, Witzigmann D, Chen S, Leavitt BR, Cullis PR. Lipid nanoparticle technology for clinical translation of siRNA therapeutics. Acc Chem Res. 2019;52(9):2435-2444. doi:10.1021/acs.accounts.9b00210
20. Das S, Samanta A, Mondal S, Roy D, Nayak AK. Design and release kinetics of liposomes containing abiraterone acetate for treatment of prostate cancer. Sensors International. 2021 Jan 1;2:100077.
21. Love KT, Mahon KP, Levins CG, et al. Lipid-like materials for low-dose, in vivo gene silencing. Proc Natl Acad Sci USA. 2010;107(5):1864-1869. doi:10.1073/pnas.0910603106
22. Bhattacharyya S, Choudhury P, Kaur P, Kumar H, Mondal S, Sarkar D. Exploring the impact of 3D printing on modern drug delivery systems. Int J Environ Sci. 2025;11(18 Suppl):2281. doi:10.64252/p4gq5e72.
23. Akinc A, Querbes W, De S, et al. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. Mol Ther. 2010;18(7):1357-1364. doi:10.1038/mt.2010.85
24. Patel S, Ashwanikumar N, Robinson E, et al. Naturally-occurring cholesterol analogues in lipid nanoparticles induce polymorphic shape and enhance intracellular delivery of mRNA. Nat Commun. 2020;11(1):983. doi:10.1038/s41467-020-14711-4

25. Medjmedj A, et al. In Cellulo and In Vivo Comparison of Cholesterol, Beta-Sitosterol and Dioleylphosphatidylethanolamine for Lipid Nanoparticle Formulation of mRNA. Nanomaterials. 2022;12(14):2446. doi:10.3390/nano12142446
26. Li B, Lv X, Yang W, et al. Beyond the Endosomal Bottleneck: Understanding the Efficiency of mRNA/LNP Delivery. Adv Funct Mater. 2024;34(29):2404510. doi:10.1002/adfm.202404510
27. So CD, Lokugamage MP, Lando HD, et al. High-throughput in vivo screen of functional mRNA delivery identifies nanoparticles for endothelial cell gene editing. Proc Natl Acad Sci USA. 2018;115(42):E9944-E9952. doi:10.1073/pnas.1811273115
28. Sagi A, et al. Bis(monoacylglycerol)phosphate: A secondary stabilizer and component for efficient endosomal escape in lipid nanoparticles. J Control Release. 2021;330:415-425. doi:10.1016/j.jconrel.2020.12.031
29. Cheng X, Lee RJ. The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery. Adv Drug Deliv Rev. 2016;99(Pt A):129-137. doi:10.1016/j.addr.2015.12.004
30. Hassett KJ, Benenato KE, Jacquinet E, et al. Optimization of lipid nanoparticles for intramuscular administration of mRNA vaccines. Mol Ther Nucleic Acids. 2019;15:1-11. doi:10.1016/j.omtn.2019.03.003
31. Zhang H, et al. Fluorinated PEG-DSPE lipids enhance mRNA delivery efficiency of lipid nanoparticles. J Control Release. 2022;343:72-82. doi:10.1016/j.jconrel.2022.01.010
32. Kulkarni JA, Witzigmann D, Thomson SB, Chen S, Leavitt BR, Cullis PR. The current landscape of nucleic acid therapeutics. Nat Nanotechnol. 2021;16(6):630-643. doi:10.1038/s41565-021-00922-6
33. Leal C, et al. Lipid nanoparticle topology regulates endosomal escape and delivery of RNA to the cytoplasm. Proc Natl Acad Sci USA. 2023;120(27):e2303396120. doi:10.1073/pnas.2303396120
34. Li T, Zhang J, Guo J, et al. Engineered internal architecture of core-shell lipid nanoparticles promotes efficient mRNA endosomal release. Nat Commun. 2026;17:2187. doi:10.1038/s41467-026-69017-8
35. Das S, Mondal S. Formulation and in vitro study of Ibuprofen loaded crosslinked sodium alginate and gellan gum microspheres. Int J Adv Pharm. 2017;6(3):86-90.
36. Behr JP. The proton sponge: a trick to enter cells the viruses did not exploit. Chimia. 1997;51(1-2):34-36.
37. Sonawane ND, Szoka FC Jr, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem. 2003;278(45):44826-44831. doi:10.1074/jbc.M308021200
38. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med. 2005;7(5):657-663. doi:10.1002/jgm.696
39. Benjaminsen RV, Mattebjerg MA, Henriksen JR, Moghimi SM, Andresen TL. The possible "proton sponge" effect of polyethylenimine (PEI) does not include change in lysosomal pH. Ther Deliv. 2013;4(2):267-277. doi:10.4155/tde.13.3
40. Thomas M, Ge Q, Lu JJ, Chen J, Klibanov AM. Cross-linked small polyethylenimines: while still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo. Pharm Res. 2005;22(3):373-380. doi:10.1007/s11095-004-7659-0

41. Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. Mol Ther. 2005;11(6):990-995. doi:10.1016/j.ymthe.2005.03.010
42. Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. J Gene Med. 2005;7(8):992-1009. doi:10.1002/jgm.773
43. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov. 2005;4(7):581-593. doi:10.1038/nrd1775
44. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov. 2005;4(7):581-593. doi:10.1038/nrd1775
45. Dufes C, Uchegbu IF, Schatzlein AG. Dendrimers in gene delivery. Adv Drug Deliv Rev. 2005;57(15):2177-2202. doi:10.1016/j.addr.2005.09.017
46. Wei H, et al. Emerging non-viral vectors for gene delivery. J Nanobiotechnology. 2023;21:204. doi:10.1186/s12951-023-01234-5
47. Lynn DM, Langer R. Degradable poly(beta-amino esters): synthesis, characterization, and self-assembly with plasmid DNA. J Am Chem Soc. 2000;122(44):10761-10768. doi:10.1021/ja001882e
48. Green JJ, Shi J, Chiu E, Leshchiner ES, Langer R, Anderson DG. Biodegradable polymeric vectors for gene delivery. Acc Chem Res. 2009;42(7):971-979. doi:10.1021/ar800248d
49. Torchilin VP. Cell penetrating peptide-modified pharmaceutical nanocarriers for intracellular drug and gene delivery. Biopolymers. 2008;90(5):604-612. doi:10.1002/bip.21169
50. Madani F, Lindberg S, Langel U, Futaki S, Graslund A. Mechanisms of cellular uptake of cell-penetrating peptides. J Biophys. 2011;2011:414729. doi:10.1155/2011/414729
51. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem. 2000;69:531-569. doi:10.1146/annurev.biochem.69.1.531
52. Subbarao NK, Parente RA, Szoka FC Jr, Nadasdi L, Pongracz K. pH-dependent bilayer destabilization by an amphipathic peptide. Biochemistry. 1987;26(11):2964-2972. doi:10.1021/bi00385a024
53. Parente RA, Nadasdi L, Subbarao NK, Szoka FC Jr. Association of a pH-sensitive peptide with membrane vesicles: role of amino acid sequence. Biochemistry. 1990;29(39):8713-8719. doi:10.1021/bi00492a006
54. Plank C, Zauner W, Wagner E. Application of membrane-active peptides for drug and gene delivery across cellular membranes. Adv Drug Deliv Rev. 1998;34(1):21-35. doi:10.1016/S0169-409X(98)00022-9
55. Madani F, Lindberg S, Langel U, Futaki S, Graslund A. Mechanisms of cellular uptake of cell-penetrating peptides. J Biophys. 2011;2011:414729. doi:10.1155/2011/414729
56. Plank C, Zauner W, Wagner E. Application of membrane-active peptides for drug and gene delivery across cellular membranes. Adv Drug Deliv Rev. 1998;34(1):21-35. doi:10.1016/S0169-409X(98)00022-9
57. Adams D, Gonzalez-Duarte A, O'Riordan WD, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. N Engl J Med. 2018;379(1):11-21. doi:10.1056/NEJMoa1716153
58. Urits I, Swanson D, Swett MC, et al. A review of patisiran (ONPATTRO) for the treatment of polyneuropathy in patients with hereditary transthyretin amyloidosis. Neurol Ther. 2020;9(2):301-315. doi:10.1007/s40120-020-00192-6

59. Baden LR, El Sahly HM, Essink B, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. N Engl J Med. 2021;384(5):403-416. doi:10.1056/NEJMoa2035389
60. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. N Engl J Med. 2020;383(27):2603-2615. doi:10.1056/NEJMoa2034577
61. Baden LR, El Sahly HM, Essink B, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. N Engl J Med. 2021;384(5):403-416. doi:10.1056/NEJMoa2035389
62. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines - a new era in vaccinology. Nat Rev Drug Discov. 2018;17(4):261-279. doi:10.1038/nrd.2017.243
63. Gillmore JD, Gane E, Taubel J, et al. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. N Engl J Med. 2021;385(6):493-502. doi:10.1056/NEJMoa2107454
64. Pardridge WM. The blood-brain barrier: bottleneck in brain drug development. NeuroRx. 2005;2(1):3-14. doi:10.1602/neurorx.2.1.3
65. Dilliard SA, Xiong X, Cheng Q, Siegwart DJ. On the delivery of RNA therapeutics: the emerging roles of non-viral lipid-based nanoparticles. ACS Nano. 2023;17(4):3348-3369. doi:10.1021/acsnano.2c12137
66. Mui BL, Tam YK, Jayaraman M, et al. Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of siRNA lipid nanoparticle formulations in non-human primates. Mol Ther Nucleic Acids. 2013;2:e139. doi:10.1038/mtna.2013.66