

The next generation of lipid nanoparticles for *in vivo* engineering and targeted delivery

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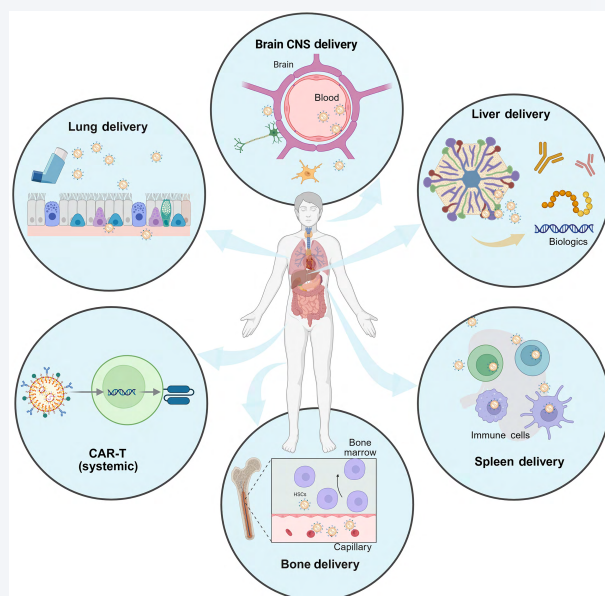
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ABSTRACT: The widespread utilization of messenger RNA (mRNA) vaccines has validated lipid nanoparticles (LNPs) as a clinically relevant platform for nucleic acid delivery. However, their broader applicability is presently limited by a "liver bottleneck", wherein more than 90% of systemically administered particles are sequestered within hepatocytes. Advancements in precise *in vivo* cellular engineering represent the next major challenge, and progress in circumventing hepatic clearance has gained momentum in the treatment of extrahepatic disorders. Numerous next-generation LNPs have been developed, consisting of distinct active components, including biodegradable ionizable lipids, antibody conjugates, and covalent lipids, and employing various targeting strategies such as selective organ targeting, stiffness modulation, and nebulization. In this review, we present a comprehensive overview of these emerging LNP technologies, emphasizing their chemical design and capacity to deliver genetic payloads to targeted tissues. We outline the physiological barriers, such as the blood-brain barrier (BBB) and mucosal surfaces, to elucidate the fundamental principles guiding the design of LNPs for systemic gene editing and immune modulation. We also underscore the benefits and drawbacks of various therapeutic approaches, including ribonucleoprotein (RNP) delivery, *in vivo* chimeric antigen receptor T (CAR-T) cell production, and mRNA-based protein replacement. The primary challenges concerning immunogenicity and manufacturing scalability, as well as prospective directions involving emerging design approaches, are discussed.



KEYWORDS: lipid nanoparticles, extrahepatic targeting, nucleic acid delivery, *in vivo* engineering

1 Introduction

The swift advancement and worldwide deployment of messenger RNA (mRNA) vaccines targeting coronavirus disease 2019 (COVID-19) have elevated lipid nanoparticles (LNPs) to prominence as a clinically validated and pioneering platform for nucleic acid delivery [1–3]. While frequently presented with

conventional liposomes, LNP systems constitute a significant advancement in nanomedicine, distinguished by advanced self-assembly processes and solid internal architectures designed to safeguard delicate genetic payloads and enhance their delivery into cells [4, 5]. Early foundational research demonstrated the potential of lipids as drug delivery vehicles, resulting in the approval of the liposomal drug Doxil in 1995 as the first nanomedicine [6]. Nonetheless, the effective encapsulation and delivery of large, negatively charged nucleic acids necessitated a major shift in lipid chemistry toward ionizable cationic lipids [7, 8].

The pivotal advancement that facilitated clinical translation was the development of ionizable cationic lipids. In contrast to their permanently charged counterparts, these lipids exhibit a neutral

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charge at physiological pH, thereby mitigating toxicity and prolonging circulation time. However, they become protonated in the acidic environment of the endosome, leading to membrane disruption and payload release [4, 9, 10]. Structurally, in contrast to the aqueous core of liposomes, contemporary LNPs feature an electron-dense nanostructured core in which nucleic acids are complexed with ionizable lipids typically adopting inverted micellar or amorphous configurations, stabilized by helper lipids (phospholipids), cholesterol, and polyethylene glycol (PEG)-lipids [5, 11, 12]. This technology resulted in the 2018 Food and Drug Administration (FDA) approval of Onpatro (patisiran), the first LNP-based small interfering RNA (siRNA) therapy for hereditary transthyretin-mediated amyloidosis [1, 7, 13].

Notwithstanding these achievements, a significant constraint of contemporary LNP technology is its intrinsic affinity for the liver. Upon intravenous administration, conventional LNPs rapidly adsorb serum proteins, especially apolipoprotein E (ApoE). This biomolecular "protein corona" facilitates hepatocyte uptake through the low-density lipoprotein (LDL) receptor (LDLR) [14–16], a mechanism that is beneficial for addressing liver diseases but poses a significant obstacle for extrahepatic targets. It is estimated that

more than 90% of systemically administered conventional LNPs accumulate in the liver [16], with only a small percentage reaching other organs such as the lungs, spleen, or bone marrow [11, 17, 18]. This "liver bottleneck" limits the broad adoption of genetic therapies for the treatment of cancer, central nervous system disorders, and infectious diseases occurring outside the liver.

This review examines the "next generation" of LNP technologies aimed at overcoming the liver bottleneck and facilitating precise *in vivo* cellular engineering. We expand beyond conventional hepatic silencing therapies to explore novel frontiers: systemic gene editing, which has evolved from siRNA to clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 RNP complexes; *in vivo* immune engineering, emphasizing methodologies for the direct generation of CAR-T cells within the body; and extrahepatic targeting, involving innovative formulation techniques such as selective organ targeting (SORT) that direct LNPs to the spleen and lungs. Furthermore, we delineate mechanisms to traverse restrictive physiological barriers, including strategies using functionalized lipids to cross the BBB and penetrate mucosal surfaces (Fig. 1). By integrating findings from recent notable studies, we seek to establish a comprehensive framework for the design of LNPs capable of

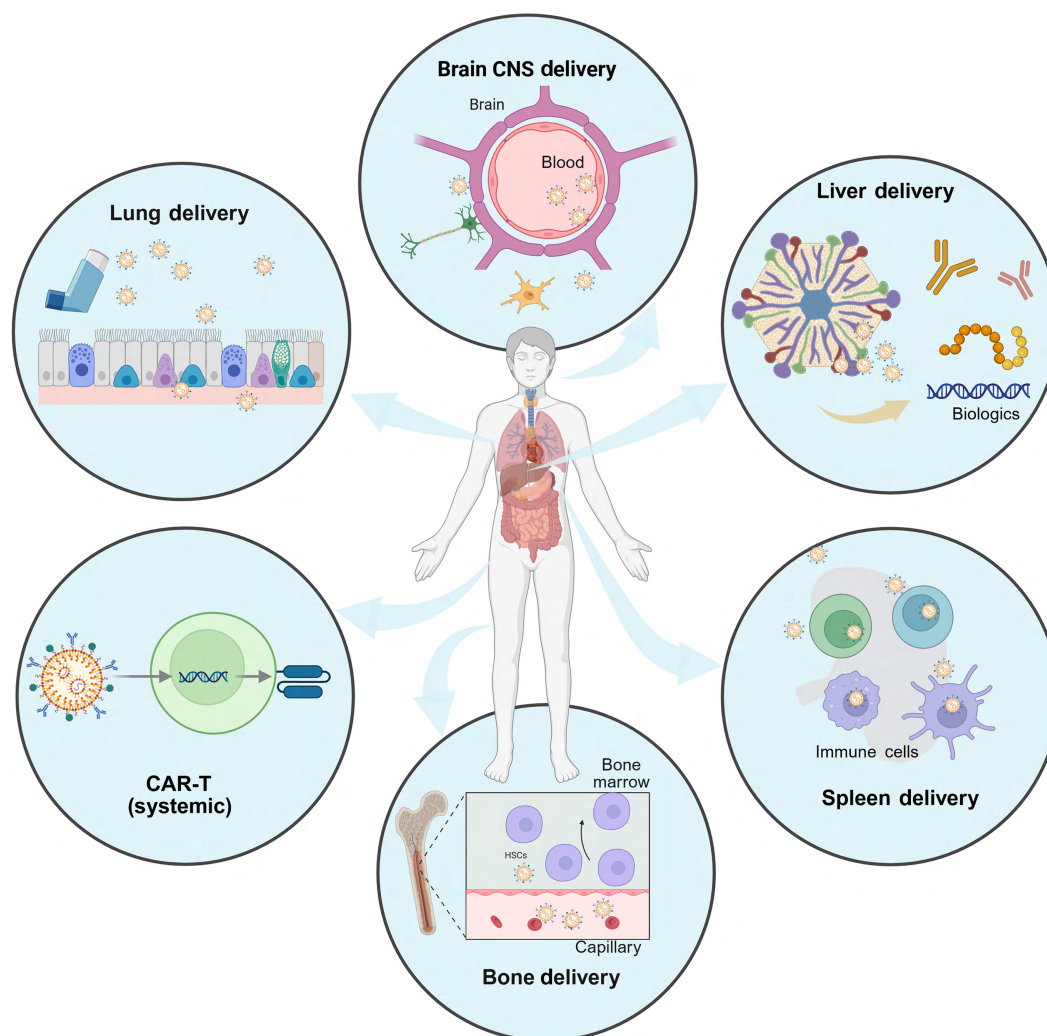


Figure 1 Schematic representation of the expanding therapeutic landscape for LNPs. Moving beyond hepatic silencing, next-generation platforms target six key frontiers. Top: traversing the BBB for CNS delivery; top left: nebulized mucosal delivery to the lung; bottom left: systemic *in vivo* CAR-T cell engineering; bottom: bone marrow homing for HSC editing; bottom right: spleen targeting for immune modulation; and top right: leveraging the liver as an *in vivo* bioreactor for the production of therapeutic biologics.

delivering diverse genetic payloads to specific cell types, thereby maximizing the capacity of nucleic acid therapeutics.

2 In vivo gene editing

2.1 Clinical translation of hepatic editing

The inherent ApoE-mediated tropism established the basis for the first FDA-approved siRNA-LNP therapy (Onpattro) [7, 19]. Since then, the field has quickly expanded to "one-and-done" gene editing therapies. The initial translation of hepatic gene editing into humans was achieved with NTLA-2001, a CRISPR-Cas9 therapeutic candidate for hereditary transthyretin amyloidosis [20]. This formulation utilizes a clinically validated LNP composition to co-deliver Cas9 mRNA and a single guide RNA (sgRNA) directed at the *transthyretin* gene. RNA delivery by LNPs enables transient expression of Cas9 mRNA and guide RNA, resulting in short-lived nuclease activity that reduces the risk of prolonged off-target editing compared with viral vector-mediated expression. A key design feature of these systems is the incorporation of biodegradable ionizable lipids, which promote efficient delivery while ensuring rapid clearance of the Cas9 nuclease, thereby further minimizing prolonged nuclease activity and potential immunogenicity [21, 22]. In a key Phase 1 trial, a single systemic administration of NTLA-2001 (0.3 mg/kg) yielded an average serum transthyretin protein reduction of 87% in patients, with reductions sustained at 28 days

[23]. As illustrated in Fig. 2, this efficacy is driven by the recruitment of ApoE to the LNP surface (Fig. 2(a)), which facilitates targeted hepatocyte uptake and subsequent Cas9-mediated gene disruption that correlates directly with serum protein reduction (Figs. 2(b) and 2(c)). Nonetheless, the therapy is dependent upon functional LDLR, which may restrict its applicability in patient groups with receptor deficiencies [23, 24]. Furthermore, temporary increases in D-dimer levels were noted post-infusion, validating the use of prophylactic premedication with glucocorticoids and antihistamines to alleviate proinflammatory responses [23].

Adenine base editors were developed to mitigate the risk of double-strand breaks associated with Cas9 nucleases by enabling precise single-nucleotide conversions (A·T to G·C) without introducing double-strand breaks [25–27]. This technique was applied to inhibit proprotein convertase subtilisin/kexin type 9 (PCSK9), a crucial regulator of LDL cholesterol. The preclinical formulation employs an LNP encapsulating mRNA encoding an adenine base editor variant and a guide RNA targeting PCSK9 [25, 28]. By precisely modifying the splice donor site of PCSK9, the therapy facilitates exon skipping and nonsense-mediated decay without inducing double-strand breaks, theoretically enhancing the safety profile regarding chromosomal translocations [25]. In cynomolgus monkeys, a single infusion (3.0 mg/kg) resulted in a sustained ~ 90% reduction of blood PCSK9 levels and ~ 60% decrease in LDL cholesterol lasting over 8 months [25]. Although off-target editing remains a theoretical concern for highly active

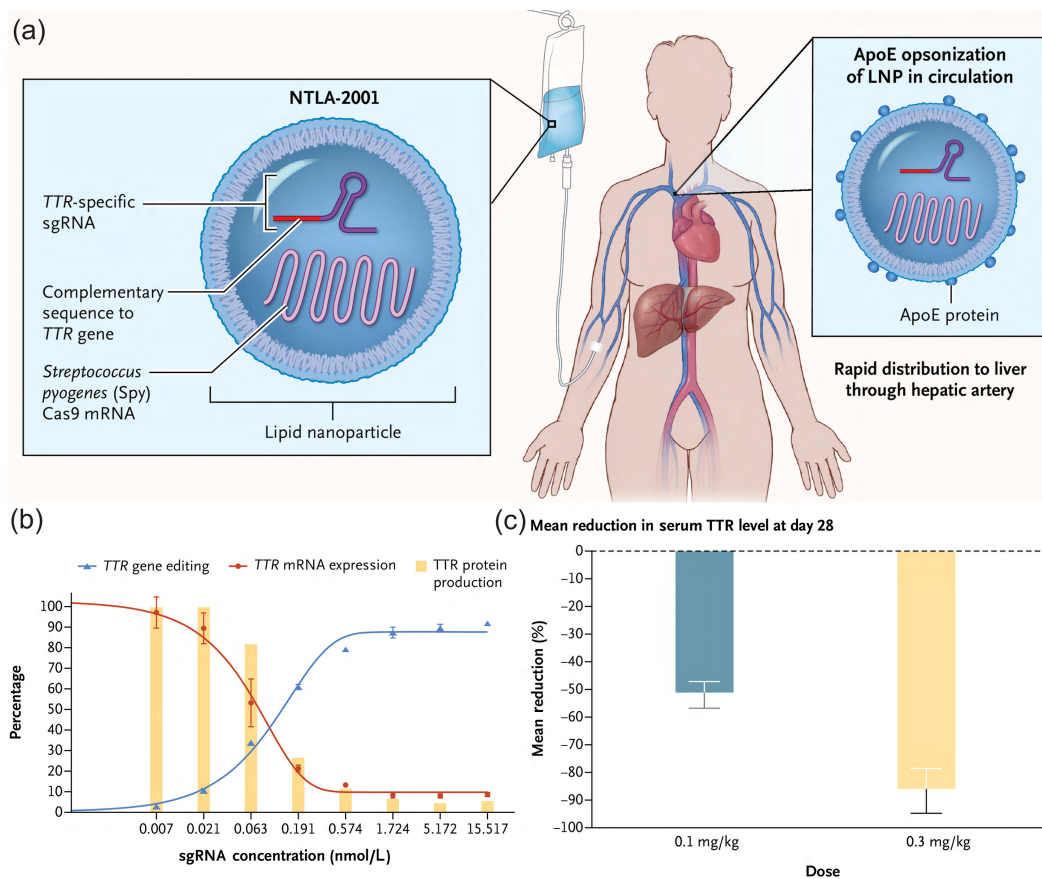


Figure 2 Clinical validation of systemic CRISPR-Cas9 gene editing (NTLA-2001). (a) The LNP formulation encapsulates Cas9 mRNA and a *transthyretin*-specific sgRNA. After intravenous infusion, LNP binds ApoE in the circulation and directs it to the liver via the LDLR for hepatocyte-specific uptake. (b) Preclinical *in vitro* potency data showing that high sgRNA concentrations boost *transthyretin* gene editing (blue line) and suppress transthyretin protein production. (c) Clinical Phase 1 results showing the mean percentage reduction in serum transthyretin protein levels at day 28 in 0.1 and 0.3 mg/kg patients. The LNP-CRISPR platform was validated in humans with a deep mean reduction of 87% at 0.3 mg/kg. Reproduced with permission from Ref. [23], © Massachusetts Medical Society 2021.

enzymes, comprehensive screening in human hepatocytes revealed editing exclusively at the intended target site [25].

To bypass reliance on endogenous ApoE/LDLR pathways, "active" targeting approaches have been incorporated into LNP design. A structure-guided design integrated a trivalent N-acetylgalactosamine (GalNAc) ligand onto the surface of the LNP through a PEG-lipid linker [24, 29]. GalNAc exhibits high-affinity binding to the asialoglycoprotein receptor (ASGPR), which is highly expressed on hepatocytes, thereby offering an orthogonal uptake pathway independent of ApoE/LDLR [24, 29]. Unlike conventional LNPs, which failed to produce significant editing in LDLR-deficient non-human primates, GalNAc-LNPs targeting angiopoietin-like 3 (ANGPTL3) attained a mean liver editing of 61% and an 89% decrease in blood ANGPTL3 protein levels [24]. However, the synthesis of tailored GalNAc-lipids increases manufacturing complexity, and the potential for immune responses to the ligand-conjugated lipid in humans necessitates prolonged monitoring [24, 30].

To enhance the safety and efficacy of hepatic gene editing, new lipid libraries have been evaluated to discover formulations with increased potency and biodegradability. A combinatorial library of bioreducible lipids was synthesized by incorporating disulfide bonds within the hydrophobic tails. The lead lipid, 306-O12B, was engineered for rapid degradation in the reductive intracellular environment, thus promoting cargo release and mitigating toxicity associated with lipid accumulation [31, 32]. In a direct comparison targeting *Angptl3* in mice, 306-O12B LNPs achieved a median editing rate of 38.5%, whereas the benchmark DLin-MC3-DMA (MC3) LNPs reached only 14.6% at the equivalent dosage [31].

Given these achievements, clinical translation faces persistent obstacles. Immunogenicity poses a challenge, as LNP surface components may induce anti-PEG antibodies or complement activation-related pseudo-allergy, which, alongside anti-drug antibodies against the cargo, potentially restrict the efficacy of repeated dosing [33, 34]. Hepatotoxicity is characterized by transient elevations in liver enzymes, attributed to the substantial influx of lipids and exogenous mRNA into hepatocytes [1, 25]. Furthermore, reliance on endogenous ApoE adsorption may result in variability in therapeutic efficiency among different patient populations due to heterogeneous lipid profiles or receptor expression levels [16, 35].

2.2 Ribonucleoprotein delivery

Delivery of CRISPR/Cas9 as RNP complexes provides several safety advantages relative to mRNA-based delivery. Whereas mRNA delivery depends on the translational system of the host cell, RNP delivery provides immediate genome editing activity upon cytosolic entry, followed by rapid intracellular degradation of the Cas9 protein [36–39], thereby shortening active exposure time and mitigating the risk of off-target editing and prolonged immune activation [40–43]. These properties make RNP delivery particularly attractive for therapeutic genome editing, as it allows transient and tightly controlled nuclease activity, which is generally considered safer than prolonged expression from viral vectors or plasmid DNA. However, encapsulating RNPs into LNPs presents biophysical challenges due to the substantial size of the Cas9 protein (~160 kDa), its susceptibility to denaturation in acidic formulation buffers, and its reduced charge density relative to nucleic acids, which complicates electrostatic condensation [41, 42, 44].

A key obstacle to RNP encapsulation is the incompatibility of

Cas9 proteins with the acidic buffers (generally ~ pH 4.0) employed in conventional microfluidic LNP formulation. Exposure to the acidic conditions can destabilize the Cas9 protein, leading to partial denaturation and aggregation that ultimately compromise genome editing activity [44]. To address this, researchers developed a "supplemental component" strategy by incorporating a permanently cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) into a conventional ionizable lipid formulation utilizing the dendrimer lipid 5A2-SC8 [44]. The persistent positive charge of DOTAP facilitates the electrostatic encapsulation of the negatively charged RNP complex in neutral buffers, maintaining protein integrity. While the 5A2-DOT-50 formulation demonstrated effective editing in mouse lungs following intravenous injection, the formulation of RNPs in standard acidic citrate buffer led to particle aggregation and a total loss of editing efficacy [44]. However, the incorporation of permanently cationic lipids can increase toxicity risks, specifically by inducing pulmonary thrombosis, and modify the protein corona in ways that may be undesirable for certain target tissues [16, 45].

One alternative to altering the formulation conditions is to engineer the cargo to endure extreme environments. To mitigate the instability of Cas9 during the organic solvent/aqueous mixing phase, a thermostable variant from *Geobacillus stearothermophilus* (GeoCas9) was developed, resulting in "iGeoCas9". This thermostable Cas9 protein variant can better tolerate the physicochemical stress during the typical LNP formulation process and exhibits over 100-fold greater genome editing efficiency than the wild type SpyCas9 enzyme [42]. When encapsulated in LNPs employing acid-degradable PEG-lipids, iGeoCas9 RNPs preserved activity while regular SpyCas9 RNPs suffered from aggregation and reduced efficacy [42], accomplishing substantial genome editing in the liver (31%) and lung (19%) [42]. The tissue-selective capabilities of iGeoCas9 RNP-LNPs were demonstrated through rational lipid formulation design (Fig. 3). Chen and colleagues engineered a formulation containing the biodegradable ionizable lipid BP lipid 312 (46.0 mol%), which achieved 37% editing efficiency in the liver with preferential uptake by hepatocytes (34%), macrophages (54%), and endothelial cells (75%). This shift in organ tropism from liver to lung was primarily driven by alterations in the cationic lipid component, which modulates protein corona formation and subsequent receptor-mediated uptake pathways. While this study successfully broadened the protospacer adjacent motif (PAM) compatibility of GeoCas9, a potential limitation regarding this approach remains the dependence on engineered Cas9 variants, which may restrict applicability if specific PAM requirements are not satisfied for a designated genetic target compared to the canonical SpyCas9 [42, 46].

While the majority of CRISPR research emphasizes gene knockout, accurate gene correction necessitates homology-directed repair (HDR) via the concurrent delivery of a DNA donor template. To address this, a dendrimer-based LNP system was optimized by tuning the ratios of Cas9 mRNA, sgRNA, and a single-stranded DNA donor template within the particle. This "all-in-one" formulation, employing the specific ionizable lipid 4A3-SC8, accomplished > 20% HDR-mediated correction of a mutated fluorescent reporter gene in tumor xenografts [47]. Control groups administered solely Cas9 mRNA and sgRNA exhibited only non-homologous end joining (NHEJ) indels, confirming the effective co-delivery of the donor DNA [47].

LNP-encapsulated RNPs are especially beneficial for tissues

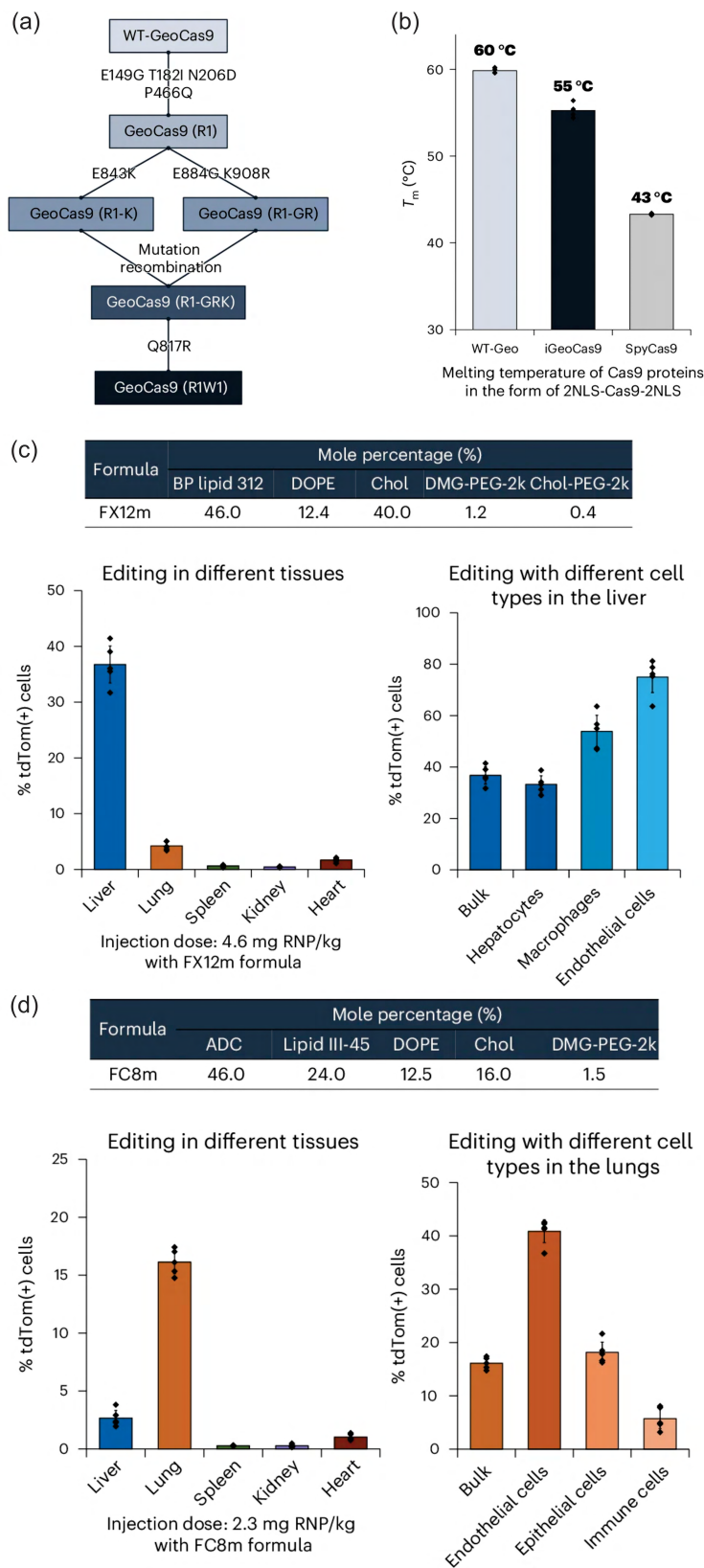


Figure 3 Engineering thermostable iGeoCas9 for tissue-selective RNP delivery via LNPs. (a) GeoCas9 was directed evolutionarily from wild-type (WT-GeoCas9) to intermediate variants to the active iGeoCas9 (R1W1). (b) Melting temperature comparison that shows iGeoCas9 (55 °C) is more thermostable than SpyCas9 (43 °C), allowing compatibility with standard LNP formulation conditions. (c) BP lipid 312 as the ionizable lipid in FX12m formulation for liver-targeted editing in various tissues and cell types. Systemic (4.6 mg RNP/kg) liver editing efficiency was 37% with preferential transfection of hepatocytes, macrophages, and endothelial cells in Ai9 tdTomato reporter mice. (d) Lung-targeted FC8m editing with Lipid III-45 and cationic lipid ADC. Intravenous injection (2.3 mg RNP/kg) caused 16% lung editing with endothelial (41%), epithelial (18%), and immune (6%) cells tropism. Reproduced with permission from Ref. [42], © Chen, K. et al. 2024.

where prolonged expression presents significant safety concerns, such as the ocular region. For instance, an "incisive delivery system" was created utilizing LNPs to encapsulate RNPs for intrastromal injection [48], which effectively edited reporter genes in a mouse aniridia model. However, therapeutic correction of the pathogenic *Pax6* gene was below the detection limit *in vivo*. A significant finding was that repeated dosing enhanced editing efficiency in *ex vivo* embryonic cortical neurons without notable toxicity, highlighting the advantages of titratable dosing feasible with the non-viral, transient RNP method [48].

As genome editing technologies continue to evolve, including the development of base editors and prime editors [49], optimizing LNP platforms for the delivery of large protein-RNA complexes will remain an important challenge for next-generation CRISPR therapeutics.

3 In vivo immune engineering

3.1 From *ex vivo* to *in vivo*

Ex vivo CAR-T cell therapies, including FDA-approved products like Kymriah and Yescarta, have transformed the management of hematologic malignancies. Despite their capacity to achieve sustained remission, widespread application is limited by the existing manufacturing model, which entails a complicated, centralized procedure: leukapheresis, *ex vivo* genetic modification (usually through viral transduction), expansion, and re-infusion. This workflow is resource-intensive, expensive, and challenging to scale, often restricting patient access [23, 50, 51]. Furthermore, lymphodepletion is required to create a favorable cytokine environment (homeostatic space) for the engraftment and expansion of adoptively transferred cells by depleting endogenous immune cells that act as cytokine sinks. In contrast, *in vivo* genetic engineering precludes the need for lymphodepletion as it relies on the presence of endogenous T cells for modification [52–56]. Recent studies demonstrate that targeted LNPs can generate functional CAR-T cells *in vivo* without lymphodepletion, achieving tumor control in humanized mice and B cell depletion in non-human primates with doses as low as 0.3 mg/kg [57]. Consequently, a paradigm shift is occurring towards "*in vivo*" engineering, wherein engineered nanocarriers administer nucleic acids directly to circulating T cells to generate functional CAR-T cells *in situ*, circumventing *ex vivo* manipulation [51–53].

A major obstacle to *in vivo* genetic engineering is that resting T lymphocytes are refractory to transfection compared to hepatocytes or cancer cells [58]. To tackle this issue, Billingsley et al. first identified a novel lipid (C14-4) that significantly surpassed the industry-standard lipid MC3 in transfecting primary human T cells [58]. Subsequently, they employed an orthogonal design-of-experiment (DOE) methodology to optimize the molar ratios of C14-4 LNP excipients, identifying formulation B10 [54]. Unlike the lipid discovery phase, the B10 mechanism relied on optimizing excipient ratios, specifically increasing the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and reducing cholesterol, rather than altering the pK_a [54]. The optimized B10 LNP formulation attained substantial CAR expression in primary T cells while markedly diminishing cytotoxicity in comparison to electroporation [54]. Nonetheless, the translation of these particular formulations to systemic *in vivo* administration demands additional mitigation of off-target hepatic uptake to improve bioavailability to circulating T cells [58, 59].

While oncology continues to be the principal focus, *in vivo* engineering has been expanded to address non-malignant conditions such as cardiac fibrosis. Rurik et al. established an *in vivo* platform employing CD5-targeted LNPs that encapsulate mRNA encoding a CAR targeting fibroblast activation protein (FAP) [53]. This rational design utilized the transient characteristics of mRNA. In contrast to cancer, which necessitates enduring T cells to avert relapse, the treatment of fibrosis requires primarily a transient decrease in activated fibroblasts to resolve symptoms without impairing future wound healing [53, 60]. As illustrated in Fig. 4, the CD5-targeted LNPs bind to T cells to facilitate endocytosis and translation of the FAP-CAR mRNA (Fig. 4(a)). This approach yields a distinct population of FAP-CAR+ T cells in the spleen (Fig. 4(b)), which successfully migrate to the heart to reduce collagen burden and reverse fibrosis (Fig. 4(c)) [53]. The therapeutic effect is inherently transient due to the non-integrating nature and eventual degradation of the mRNA payload [52, 53]. While advantageous for fibrosis, this transience may necessitate frequent administration for chronic ailments or cancers that demand continuous surveillance [52, 59].

Alongside lipid-based systems, polymeric nanoparticles have also shown the feasibility of *in situ* T cell engineering. Parayath et al. employed biodegradable poly(β -amino ester) nanoparticles coated with polyglutamic acid to shield the polymer's positive charge, thereby minimizing non-specific uptake [61, 62]. Anti-CD8 antibodies were first conjugated to the polyglutamic acid polymer and then electrostatically adsorbed onto the cationic poly(β -amino ester) core to promote receptor-mediated endocytosis specifically into cytotoxic T cells [61]. Repeated systemic delivery of these nanocarriers led to sufficient *in situ* transfection to induce tumor regression [61, 63]. Despite surface shielding, a considerable fraction of the administered dose accumulated in the liver and spleen, underscoring the lasting challenge of off-target biodistribution in *in vivo* methodologies [1, 61].

3.2 Antibody-guided LNP design

Antibody-mediated LNP design is a crucial strategy for transfecting hard-to-transfect cell populations such as T lymphocytes and hematopoietic stem cells, where passive ApoE-mediated uptake proves ineffective [63, 64]. This method enhances receptor-mediated endocytosis by functionalizing the nanoparticle surface with monoclonal antibodies or antibody fragments [65]. The structural format of the targeting ligand is a crucial design parameter in antibody-targeted LNPs. Intact antibodies containing the Fc domain can induce non-specific uptake by Fc-receptor-expressing cells (such as macrophages) independent of the target antigen, resulting in accelerated clearance, inflammation, and off-target effects [51, 65, 66].

Recent strategies have employed antibody fragments devoid of the Fc region to address this issue [59]. For example, an "Ab-LNP" platform was demonstrated utilizing reduced antibody fragments derived from F(ab')₂ intermediates produced through enzymatic cleavage of complete antibodies that target CD3, CD5, or CD7 [59]. The fragments were reduced to reveal hinge-region thiols and conjugated to maleimide-functionalized PEG-lipids on the surface of the LNP [51, 59]. This orientation-specific conjugation ensures that the antigen-binding site is directed outward, thereby minimizing Fc-mediated clearance [51]. In a direct comparison, anti-CD3 and anti-CD7 LNPs demonstrated enhanced transfection of circulating T cells in mice relative to the clinical standard MC3-

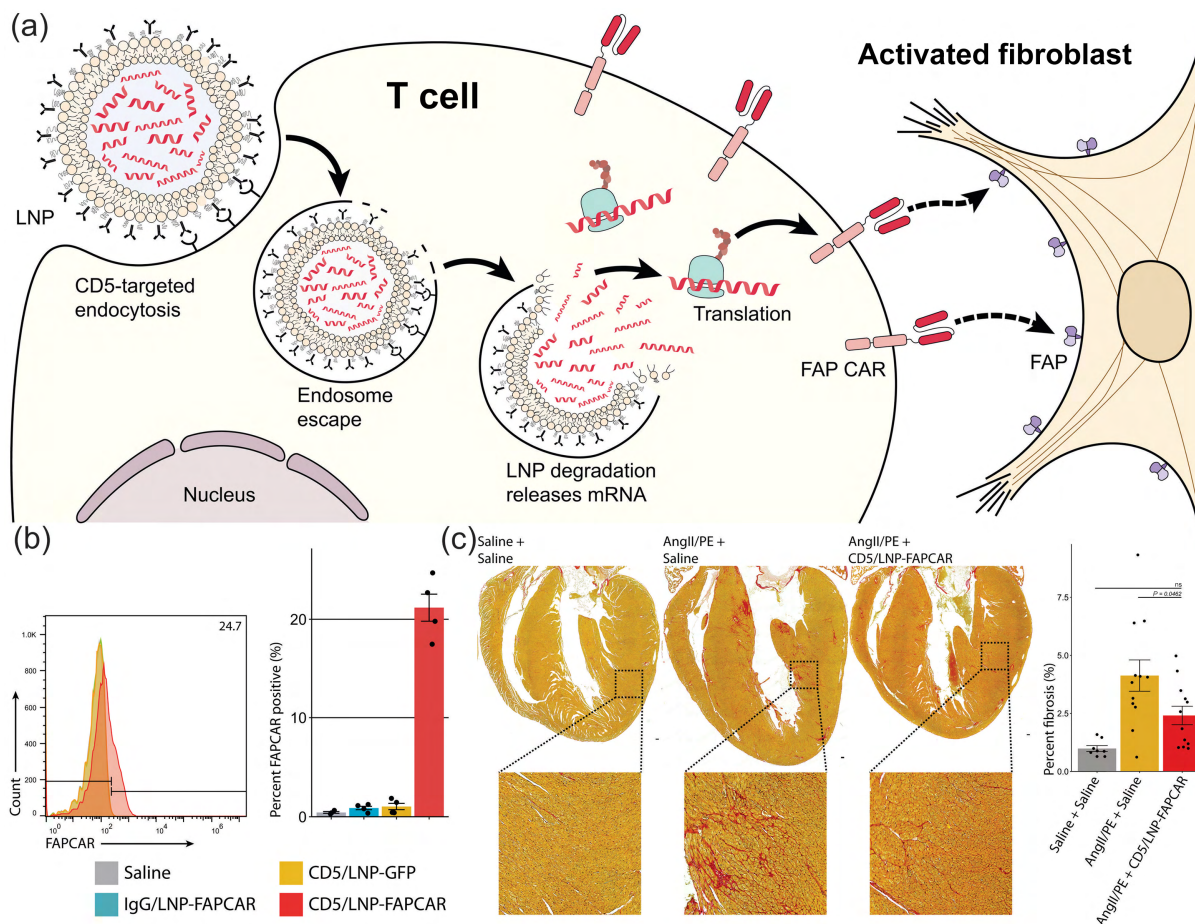


Figure 4 *In vivo* generation of transient CAR-T cells using CD5-targeted LNPs to treat cardiac fibrosis. (a) Schematic for CD5 antibody-targeted LNPs undergo endocytosis by T cells, subsequently escaping the endosome, releasing mRNA, and inducing transient translation of an FAP CAR. The resultant FAP-CAR-T cells then recognize FAP-expressing activated fibroblasts. (b) Representative flow cytometry and quantification demonstrating the *in vivo* induction of FAP-CAR expression in T cells following CD5 LNP FAP-CAR administration within an AngII/PE-induced cardiac injury model. (c) Histological analysis (picrosirius red staining) and quantification demonstrating diminished ventricular fibrosis subsequent to CD5 LNP FAP-CAR treatment relative to controls. Reproduced with permission from Ref. [53], © Rurik, J. G. et al. some rights reserved; exclusive licensee American Association for the Advancement of Science 2022.

LNP and untargeted controls [59]. Nonetheless, even with the Fc region eliminated, antibody-conjugated LNPs demonstrated increased hydrodynamic diameters (~190 nm compared to ~115 nm for unconjugated), which may restrict tissue penetration in certain scenarios [51].

Typical antibody targeting depends on strong affinity binding to any accessible receptor. Distinguishing between active and inactive receptor conformations can enhance therapeutic indices [67]. To accomplish this, Dammes et al. designed LNPs to specifically target the high-affinity conformation of the $\alpha 4 \beta 7$ integrin, a receptor essential for leukocyte migration to the gut in inflammatory bowel disease. They employed a recombinant fusion protein (MAdCAM-1-D1D2-Fc) connected to the LNP through a secondary antibody linker [68]. The conformation-sensitive LNPs selectively delivered siRNA payloads to gut-homing leukocytes in a murine colitis model, circumventing cells that express the resting integrin [67]. This precision decreased the likelihood of systemic immune suppression, a prevalent adverse effect of indiscriminate integrin blockade [67].

An alternative application of antibody targeting is to access the protective bone marrow niche for the treatment of hematologic malignancies such as multiple myeloma. Tarab-Ravski et al. engineered anti-CD38 targeted LNPs for the delivery of siRNA

designed to target myeloid cell leukemia 1 (MCL1) or cytoskeleton-associated protein 5 (CKAP5) [69]. The LNPs were functionalized with anti-CD38 antibodies through thiol-maleimide conjugation [69]. The anti-CD38 LNPs exhibited markedly improved uptake and therapeutic efficacy in multiple myeloma cells in the bone marrow of orthotopic xenograft mice relative to isotype control LNPs [69].

Most recently, the synergy between rational lipid design and surface functionalization was demonstrated by Hunter and colleagues [56], who developed a targeted LNP platform for the treatment of autoimmune diseases. By combining a novel biodegradable ionizable lipid (Lipid 829) with anti-CD8 targeting antibodies, they achieved a critical uncoupling of potency from toxicity [56]. As shown in Fig. 5, this formulation drastically reduced off-target hepatic accumulation, a persistent issue with benchmark lipids like ALC-0315, while maintaining high-efficiency transfection of CD8⁺ T cells in the spleen. This "immune reset" strategy successfully depleted pathogenic B cells in non-human primates, validating the clinical potential of antibody-guided, cell-specific *in vivo* engineering.

While offering the enhanced specificity provided by antibody conjugation, this class of techniques encounters unique challenges. Upon entering the bloodstream, LNPs rapidly adsorb serum

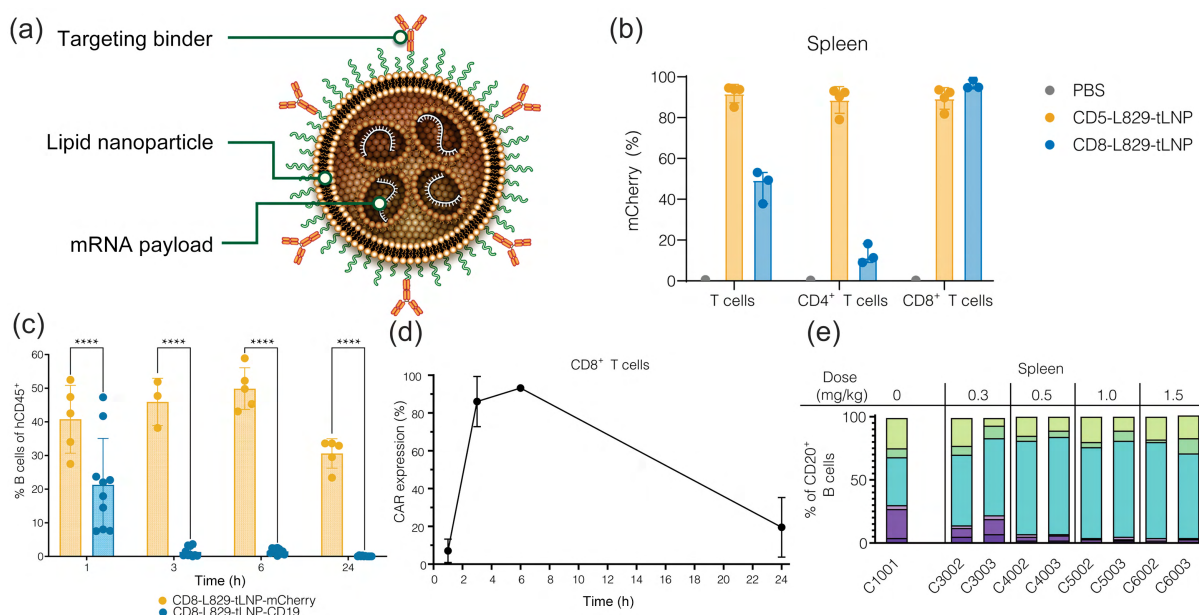


Figure 5 Targeted LNPs for specific *in vivo* engineering of CD8⁺ T cells. (a) Schematic of an antibody-conjugated and L829-formulated LNP encapsulating an mRNA payload. (b) In humanized NSG mice, CD8-targeted LNPs deliver an mCherry reporter preferentially to CD8⁺ T cells in the spleen compared with CD5-targeted control LNPs and PBS. (c) In NSG-PBMC mice, a single intravenous dose of CD8-LNP encoding an anti-CD19 CAR induces rapid depletion of human splenic B cells within hours versus an mCherry control LNP. (d) Kinetics of CAR expression on splenic CD8⁺ T cells following LNP-mediated CAR delivery. (e) Following LNP delivery of an anti-CD20 CAR mRNA regimen in cynomolgus monkeys, the splenic B cell compartment at recovery is enriched for naïve/transitional phenotypes. Reproduced with permission from Ref. [56], © Hunter, T. L. et al. some rights reserved; exclusive licensee American Association for the Advancement of Science. 2025.

proteins, forming a protein corona that masks the conjugated antibodies and inhibits their binding to target receptors [70, 71]. Moreover, the conjugation of biological ligands to LNPs necessitates extra manufacturing processes (such as reduction, conjugation, and purification) that complicate quality control and scalability in comparison to "self-assembling" lipid-only formulations [51, 67]. Finally, the presence of exogenous proteins (antibodies) on the surface of LNPs can provoke the formation of anti-drug antibodies upon repeated administration, potentially resulting in accelerated clearance or hypersensitivity reactions [52].

3.3 Preclinical validation

The progress of *in vivo* engineering from proof-of-concept to therapeutic application requires stringent preclinical validation in immunocompetent and disease-specific animal models. Recent studies have progressed beyond basic reporter gene expression to demonstrate functional therapeutic results, including tumor regression, sustained B-cell depletion, or cardiac fibrosis reversal [53, 59, 61].

A fundamental restriction of mRNA-based *in vivo* CAR-T therapies is the transient nature of expression, requiring repeated administration [52, 53]. To achieve sustained remission using a single-administration strategy, the LNP platform was engineered to co-encapsulate minicircle DNA encoding the CAR and mRNA encoding the SB100x transposase [55]. The surface of this formulation is functionalized with T-cell specific binders, specifically an anti-CD3 single-chain variable fragment (scFv) and an anti-CD7 nanobody [55]. The biological mechanism depends on the transient expression of transposase, which facilitates the stable integration of the CAR sequence from the minicircle DNA into the T cell genome [55]. A single intravenous administration of LNPs generated durable CAR-T cells that persisted and achieved effective tumor suppression. Critical controls lacking the transposase

exhibited only transient CAR expression and were unable to effectively control tumor growth [55]. Nevertheless, genomic integration presents the potential risk of insertional mutagenesis, requiring comprehensive long-term safety evaluations of genotoxicity before human application [52].

Most targeted LNPs depend on antibody conjugation, which complicates both manufacturing and stability. An alternative approach for T cell targeting involves employing lipid chemistry directly to steer tropism [72]. Inspired by cardiolipin, Zhang and colleagues synthesized a collection of cardiolipin-mimetic phosphoramidate lipids. The lead lipid, PL40, generated LNPs characterized by a distinctive polygonal morphology and elevated rigidity [72]. These mechanical and chemical properties promoted preferential uptake by T cells rather than macrophages. Furthermore, the cargo consisted of circular RNA (circRNA) rather than linear mRNA, selected to improve stability and prolong protein expression [72]. PL40-based LNPs encoding a CAR targeting urokinase plasminogen activator receptor (uPAR) efficiently produced senolytic CAR-T cells *in vivo*, resulting in a reduction of liver fibrosis and alleviation of rheumatoid arthritis symptoms [72]. However, key efficacy studies were performed in humanized immunodeficient NCG mice, and owing to species-specific variations in lipid uptake receptors, the observed T-cell tropism necessitates validation in non-human primates [72].

Significant challenges exist in the implementation of preclinical validation methods that must be addressed. While most studies depend on rodent models, LNP biodistribution and protein corona formation exhibit considerable interspecies differences between mice, non-human primates, and humans. For example, variations in vascular fenestrations and serum protein composition mean that the ApoE-mediated uptake pathway, while predominant in mice, may not accurately forecast biodistribution in primates [73]. Furthermore, repeated administration of LNPs, especially those

conjugated with antibodies or containing immunogenic PEG-lipids, may induce the formation of anti-drug antibodies. These antibodies can trigger accelerated blood clearance, alter biodistribution, or provoke hypersensitivity reactions upon subsequent doses [33, 34].

4 mRNA-mediated protein replacement therapy

4.1 The liver as a bioreactor

The liver exhibits a remarkable capacity for protein synthesis and secretion, rendering it an optimal target for mRNA therapeutics aimed at restoring deficient proteins or secreting therapeutic antibodies. By leveraging the preferential accumulation of LNPs in hepatocytes through the previously described ApoE-LDLR pathway [74–76], researchers can transform the liver into an *in vivo* bioreactor [1, 74]. This approach provides a notable advantage over recombinant protein therapies by accessing the host's natural post-translational modification machinery and enabling sustained plasma protein expression [77, 78], thereby mitigating the rapid clearance typically associated with bolus protein injections [78, 79].

Although ionizable lipids are the main contributors to mRNA encapsulation and endosomal escape, recent research has emphasized the essential function of helper lipids in adjusting the internal architecture of LNPs to optimize hepatic transfection [15, 80]. For instance, through systematic screening, Sarode et al. demonstrated that the helper lipid 1,2-dierucoyl-sn-glycero-3-

phosphoethanolamine (DEPE), which possesses longer unsaturated chains compared to standard DOPE, promoted the formation of homogeneous core/multi-lamellar internal structures [15]. This particular morphology markedly improved ApoE binding affinity relative to other helper lipids [15]. As illustrated in Fig. 6, the study initially established these structure-activity relationships using a library of diketopiperazine (DKP)-based lipids. Screening revealed that ester-linked lipids with specific spacer lengths (such as DKP-14) were highly potent, but the substitution of standard DOPE with DEPE provided the most significant enhancement in protein expression. Leveraging these design principles, the optimized formulation, comprising the novel ionizable lipid GA-16 and helper lipid DEPE, generated human ornithine transcarbamylase (OTC) expression levels of approximately 4200 ng/mg in mice, significantly outperforming the clinical benchmark MC3 [15]. Crucially, this formulation exhibited therapeutic efficacy in a knockout mouse model and sustained expression in rats, with a dose of 0.3 mg/kg maintaining therapeutic OTC levels for 2–3 weeks [15].

The concept of the "liver bioreactor" can also be leveraged to transiently express mitogens to expedite tissue repair following acute injury, a context where sustained protein expression could be oncogenic [78]. Rizvi et al. adopted nucleoside-modified mRNA-LNPs to predominantly deliver hepatocyte growth factor (HGF) and epidermal growth factor (EGF) to hepatocytes [78]. This approach induced a fast, transient regenerative response (protein

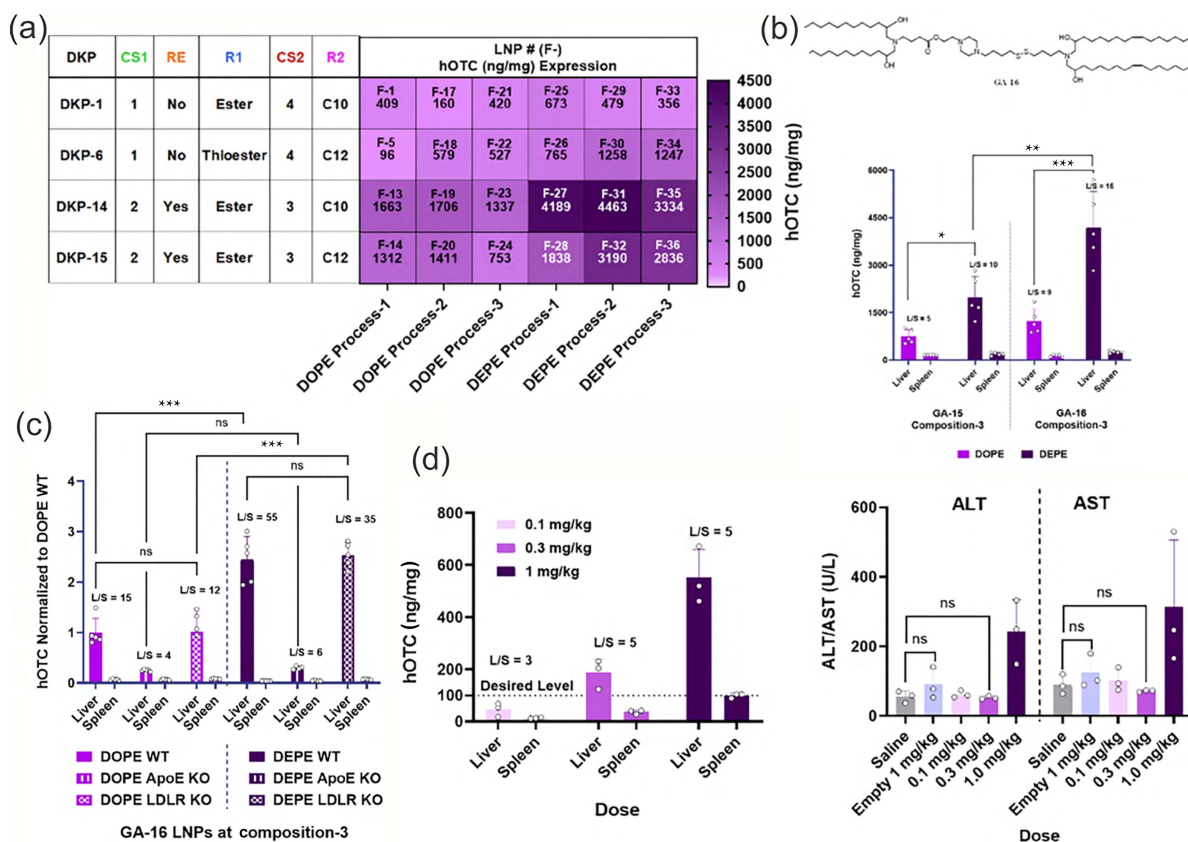


Figure 6 Structural optimization and screening of biodegradable lipids for hepatic protein replacement. (a) Chemical formulation of the DKP ionizable lipid library, incorporating variable carbon spacers and ester/thioester linkages. (b) *In vivo* comparison of GA-15 and GA-16 LNPs showing maximal human OTC protein expression with GA-16/DEPE. (c) Human OTC expression from GA-16 LNPs formulated with DOPE or DEPE in wild-type, ApoE-knockout, and LDLR-knockout mice. (d) Dose-dependent human OTC expression and liver enzyme levels in rats, with 0.3 mg/kg achieving the desired expression without elevating ALT or AST. Reproduced with permission from Ref. [15], © Sarode, A. et al. Advanced Materials published by Wiley-VCH GmbH 2025.

expression lasting ~ 3 days) that repaired liver damage while avoiding the risks associated with viral integration [78]. In a murine model of acetaminophen-induced acute liver injury, a single administration of HGF/EGF mRNA-LNPs markedly expedited hepatic regeneration, evidenced by the rapid recovery of necrotic regions and the normalization of serum alanine aminotransferase (ALT) levels within 48 h [78].

In addition to metabolic enzymes and growth factors, the liver and spleen can be engineered to produce complex immunotherapeutic agents, such as bispecific T cell engagers (BiTEs), which are challenging to manufacture and exhibit limited half-lives when used as recombinant proteins [61]. Huang et al. formulated LNPs utilizing a piperazine-based ionizable lipid (IC8) for the delivery of mRNA encoding a B7H3×CD3 bispecific antibody [81, 82]. These organs served as a reservoir, continually secreting the bispecific antibody into the circulatory system. The *in situ* generation of the protein elicited robust antitumor activity in murine models of leukemia and melanoma, demonstrating superior efficacy compared to direct administration of the recombinant protein, an effect likely attributable to improved pharmacokinetic properties [81].

Facilitating the repeated dosing necessary for protein replacement therapies (such as for hemophilia or anemia) requires optimizing ionizable lipid biodegradability to prevent toxic accumulation [10]. Cornebise et al. structurally refined this class of lipids to identify "Lipid 29", a squaramide-based ionizable lipid containing branched ester tails [83]. In non-human primates, LNPs formulated with Lipid 29 delivering human erythropoietin (EPO) mRNA demonstrated a 6-fold increase in the area under the serum human EPO concentration-time curve relative to the ethanolamine-based benchmark (Lipid 1) [83]. While biodegradable lipids like SM-102 are known to reduce hepatic accumulation following repeat administration, Lipid 29 specifically demonstrated that modifying the lipid headgroup to increase mRNA binding affinity significantly enhances potency [10].

Nevertheless, employing the liver as a bioreactor presents specific challenges that must be addressed. Repeated administration of LNPs may result in subclinical hepatic injury, evidenced by elevated ALT and aspartate aminotransferase (AST) levels, or lipid accumulation, even when formulated with biodegradable lipids [84]. Furthermore, repeated administrations of PEGylated LNPs may elicit the production of anti-PEG antibodies (IgM/IgG), resulting in the accelerated clearance of subsequent doses by the mononuclear phagocyte system (MPS) and a consequent reduction in therapeutic effectiveness [22, 85]. Ultimately, the efficacy of the bioreactor is contingent upon the sinusoidal architecture of the liver. In fibrotic or cirrhotic livers, fenestrations may be absent due to capillarization, and hepatocyte functionality may be impaired, thereby potentially obstructing LNP uptake and subsequent protein synthesis [30].

4.2 Mucosal and pulmonary transfection

Inhalation enables non-invasive, localized delivery of mRNA directly to the airway epithelium. However, pulmonary delivery encounters distinctive physiological barriers distinct from those of systemic administration, such as the complex branching of airways, the viscoelastic mucus layer, alveolar macrophage clearance, and the susceptibility of lung tissue to inflammation [86, 87]. A significant impediment is that the substantial shear forces produced by vibrating mesh nebulizers frequently compromise standard LNP structures, thereby inducing payload leakage and aggregation [88, 89].

To mitigate colloidal instability during nebulization, nebulizable LNPs were engineered through the substitution of cholesterol with β -sitosterol and an increased molar proportion of PEG-lipid [89]. β -sitosterol imparted a polyhedral morphology to the LNP that enhanced endosomal escape, while a high surface density of PEG conferred steric stabilization against shear stress during aerosolization [89, 90]. The optimized nebulizable LNPs prevented aggregation and improved mRNA retention relative to controls following nebulization, whereas conventional cholesterol-based LNPs aggregated and experienced a loss of payload [89]. *In vivo*, nebulizable LNPs effectively delivered cystic fibrosis transmembrane conductance regulator (CFTR) mRNA to the lungs of cystic fibrosis knockout mice, enabling the expression of therapeutic protein while avoiding the off-target hepatic expression observed with systemic controls [89]. However, a high PEG content, although essential for maintaining stability during nebulization and facilitating mucus penetration, can impede cellular uptake by hindering protein corona formation (the "PEG dilemma"), thereby requiring a judicious balance between stability and transfection efficiency [10, 89].

An alternative to steric stabilization with PEG involves utilizing electrostatic repulsion to inhibit aggregation during nebulization [87]. A charge-assisted stabilization approach was implemented by integrating a negatively charged peptide-lipid conjugate (DSSC-DOPE) into the LNP [87]. As illustrated in Fig. 7(a), this modification enhanced the magnitude of the surface charge, imparting significant electrostatic repulsion between particles that prevented aggregation under shear force, avoiding the necessity of high PEG concentrations that could impede cellular uptake [87]. The charge-assisted stabilization LNPs retained their integrity following nebulization (Fig. 7(b)) and demonstrated a 20-fold increase in mRNA expression within the lungs compared to clinical standard SM-102 LNPs [87]. Importantly, inhaled LNPs predominantly transfected dendritic cells (DCs) (Figs. 7(c) and 7(d)), inducing strong mucosal IgA and systemic IgG responses against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in mice, and achieved effective mRNA delivery in pigs and dogs. However, the stability provided by charge is influenced by ionic strength. The LNPs necessitated nebulization in low-salt buffers like 0.1× phosphate-buffered saline (PBS), as physiological salt concentrations screened electrostatic repulsion and induced aggregation [87].

For conditions impacting the pulmonary vasculature or interstitial tissues that are inaccessible to inhaled aerosols, intravenous administration with lung-targeted LNPs is necessary [1, 91]. Recent research has shown that minor chemical modifications to the lipid tail can significantly alter tropism, shifting it from the liver to the lung [91, 92]. A library of N-series lipidoids, characterized by amide linkages within their hydrophobic tails, was synthesized, in contrast to the ester bonds present in conventional liver-targeting O-series lipids [91, 92]. This chemical modification altered the composition of the protein corona, notably enhancing the adsorption of fibrinogen β and gamma chains, thereby promoting uptake by pulmonary endothelial cells [86, 91]. The leading candidate, 306-N16B LNP, selectively localized to the lung following intravenous administration, whereas its ester analog preferentially targeted the liver. This formulation was employed to deliver *Tsc2* mRNA in a mouse model of lymphangioleiomyomatosis (LAM), resulting in a substantial reduction in tumor burden [91]. Notably, although effective for endothelial targeting,

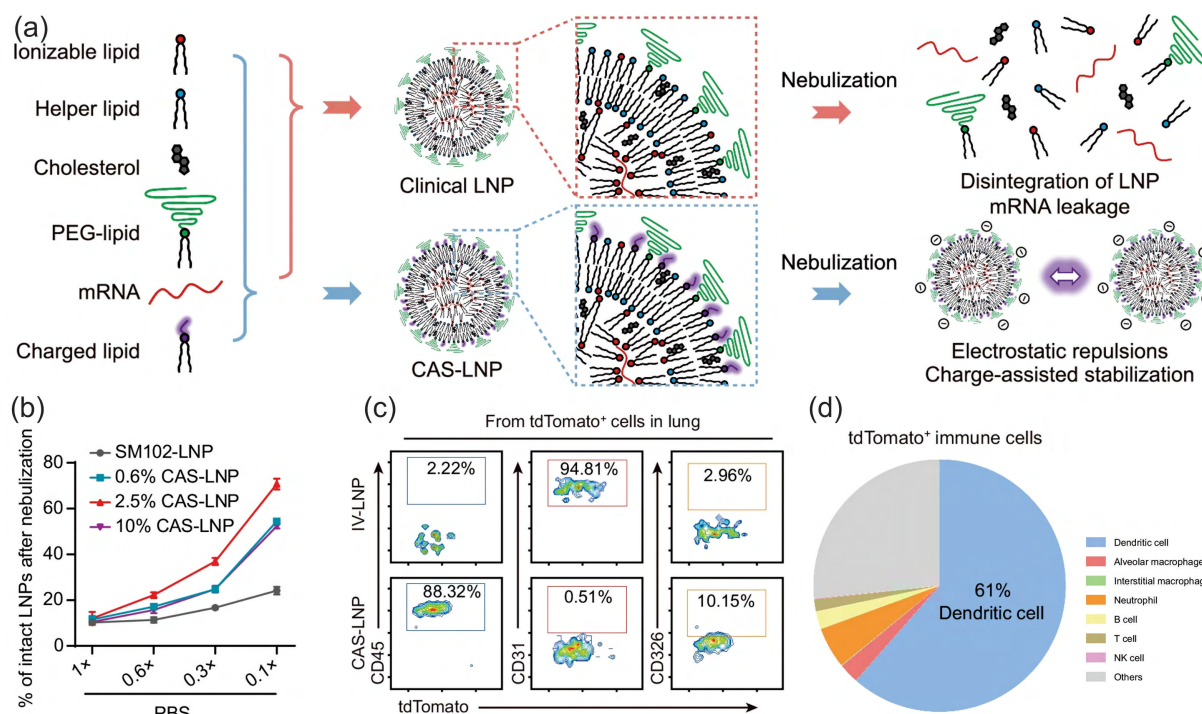


Figure 7 Charge-assisted stabilization enables nebulized mRNA delivery to lung DCs. (a) Schematic comparison of neutral and charge-assisted stabilization LNPs. A negatively charged peptide-lipid conjugate prevents LNP aggregation and mRNA leakage during high-shear nebulization through electrostatic repulsion. (b) Stability analysis showing that LNPs retain more particles than SM-102 LNPs after nebulization in low-ionic strength buffer. (c) Representative lung cell tropism flow cytometry plots. Inhaled LNPs target CD45⁺ immune cells, while LNPs target CD31⁺ endothelial cells. (d) Quantitative distribution of transfected immune cells shows that inhaled LNPs target DCs (61%), enabling robust mucosal vaccination. Reproduced with permission from Ref. [87], © Liu, S. et al. 2024.

systemic administration proves less efficient in reaching other lung cell types (such as epithelial cells) compared to direct inhalation [45, 91].

A significant limitation of cationic and ionizable lipids in the lung is their propensity to induce acute inflammation (reactogenicity), which may be fatal in models with pre-existing pulmonary conditions [93]. To separate potency from immunogenicity, non-inflammatory LNPs were engineered by integrating ursolic acid, a naturally occurring triterpenoid, into phosphoramidate-based LNPs [93]. Mechanistically, ursolic acid activates the vacuolar-ATPase (V-ATPase) complex on endosomes, thereby promoting rapid acidification and protonation of the ionizable lipid [93]. This promotes effective endosomal escape while avoiding the extensive membrane disruption that generally activates pro-inflammatory pathways such as nuclear factor kappa B (NF- κ B) and reactive oxygen species (ROS) generation [93]. The LNPs reached a fortyfold increase in pulmonary protein expression relative to ALC-0315 LNPs, while significantly reducing the secretion of pro-inflammatory cytokines (interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α)) [93]. However, the inclusion of a fifth component elevates manufacturing and quality control demands relative to conventional four-component formulations [94].

5 Overcoming the BBB

5.1 Rational mechanism-based targeting advances

Recent advancements in LNP technology have shifted from empirical screening toward a more systematic, rational mechanism-driven design approach [95, 96]. Beyond passive accumulation,

current research leverages specific physicochemical and biological mechanisms, including *in situ* protein corona engineering [97], mechanical stiffness modulation, and lipid phase topology, to achieve precise organ and cell-type specificity [72, 98].

A major step forward in targeting hematopoietic stem cells (HSCs) in the bone marrow was accomplished by utilizing *in situ* chemical reactivity on the surface of LNPs [99]. In contrast to typical LNPs that depend on non-covalent adsorption of serum proteins (such as ApoE), "covalent lipids" contain reactive groups capable of forming covalent bonds with targeting ligands or functional biomolecules on the LNP surface. Lian et al. incorporated "covalent lipids" featuring N-hydroxysuccinimide (NHS) esters into LNP formulations [99]. The reactive headgroups establish covalent bonds with particular serum proteins following injection, thereby modifying the particle's biological identity [99]. The customized reactivity enables the LNP to covalently bind specific serum proteins. Notably, proteomics indicated that ApoE was significantly enriched and essential for bone marrow homing. This suggests a novel function for ApoE in bone marrow transport, distinct from its canonical role in hepatic clearance, that facilitates LNP uptake by bone marrow resident cells, including HSCs and T cells [99]. Optimized bone marrow-homing LNPs facilitated effective gene editing within HSCs in a murine model [99]. A key control involved utilizing hydrolyzed lipid analogs (where the reactive group was deactivated), which reverted the tropism to the liver, confirming that chemical reactivity and subsequent covalent corona formation were the primary drivers of bone marrow targeting [99]. While the use of highly reactive electrophiles such as NHS esters presents a theoretical risk of non-specific protein haptenization and neo-antigen formation, initial studies indicated

that these formulations were well-tolerated upon repeated administration, maintaining therapeutic efficacy without overt induction of anti-drug antibodies [34, 100].

Although surface chemistry is a well-established targeting parameter, recent research has emphasized the vital influence of LNP mechanics (stiffness) in determining cellular internalization pathways [101]. Lee et al. demonstrated that adjusting the elastic modulus of LNPs can modulate their internalization efficiency and tumor penetration [101]. The study employed 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)-based LNPs encapsulating an alginate core and tuned their stiffness by modulating calcium (Ca^{2+})-mediated cross-linking of the alginate matrix, thereby increasing rigidity without changing surface ligands [101]. While softer LNPs exhibited enhanced cellular uptake in both breast cancer cells and macrophages (preferentially via membrane fusion in cancer cells), stiffer LNPs demonstrated prolonged retention in stiff tumors, highlighting how mechanical properties influence internalization mechanisms and biodistribution [101]. Mechanistically, this modulation exploits the finding that cancer cells preferentially employ membrane fusion for LNP uptake, whereas macrophages predominantly utilize receptor-mediated endocytosis for soft particles [101]. Consequently, soft LNPs demonstrated markedly increased uptake in breast cancer cells (such as MCF-7) and achieved rapid tumor accumulation with deeper penetration relative to their stiffer counterparts, which exhibited prolonged retention [101]. However, the dependence on metal ions (Ca^{2+}) to regulate stiffness within the alginate core may present stability challenges within the systemic circulation due to potential ion exchange or competition with serum chelators [101].

Advancing beyond rational design, artificial intelligence (AI), and deep learning have been utilized to elucidate complex structure-function relationships that determine organ tropism [95, 96]. Witten et al. introduced a deep-learning methodology, "lipid optimization using neural networks", to design ionizable lipids for targeted lung delivery [95]. By training on a dataset comprising diverse lipid structures and their transfection activity, these predictive models can evaluate immense chemical spaces. For example, a recent deep learning framework predicted highly potent candidates from a virtual library of 1.6 million lipids, which is a scale inaccessible to empirical screening [95]. The model identified novel lipids, FO-32 and FO-35, which exhibited enhanced pulmonary mRNA delivery to the conducting airways and alveoli outperforming conventional benchmark lipids [95]. Similarly, the development of platforms like AGILE (AI-Guided Ionizable Lipid Engineering) has demonstrated how deep neural networks, trained on combinatorial lipid libraries, can rapidly identify cell-specific preferences for lipid tail lengths and head groups to optimize extrahepatic mRNA delivery [102].

5.2 Central nervous system disease applications

The delivery of nucleic acid therapeutics to the central nervous system (CNS) is significantly limited by the BBB, as typical LNP formulations predominantly accumulate in the liver and lack inherent brain-targeting properties [103, 104]. A promising approach to facilitate BBB crossing entails synthesizing ionizable lipids functionalized with small molecules capable of traversing the brain, including neurotransmitters or nutrient analogues [105, 106]. Demonstrating the versatility of this approach, Ma et al. synthesized lipidoids derived from tryptamine (NT1) that, when doped into standard LNP formulations, facilitated the delivery of a broad

spectrum of cargos [105]. By incorporating tryptamine-derived NT1-lipidoids into conventional lipid formulations, they successfully delivered three distinct cargo classes, small molecule antifungal (amphotericin B), antisense oligonucleotides (ASOs), and Cre recombinase protein, across the BBB via intravenous administration (Fig. 8). Notably, this approach achieved functional gene silencing (50% tau mRNA reduction) and genome recombination in neurons without requiring targeting ligands or physical BBB disruption, demonstrating that chemical structure alone can drive BBB penetration when specific neurotransmitter scaffolds are incorporated into the lipid design [105].

Another method exploits endogenous transporters or receptors present on brain endothelial cells [104]. For instance, Wang et al. developed a library of 72 BBB-crossing lipids through the conjugation of small molecules such as L-DOPA, D-serine, and tryptamine to amino lipids [104]. The key formulation, incorporating the lipid MK16 (derived from the γ -secretase inhibitor MK-0752), demonstrated extensive mRNA expression in neurons and astrocytes across the mouse brain after intravenous administration, markedly surpassing the performance of FDA-approved lipid standards such as MC3 [104]. Mechanistically, this brain entry was mediated by caveolae- and γ -secretase-dependent transcytosis [104]. Similarly, Sela et al. employed an AI-driven graph neural network to identify acetylcholine and nicotine as the leading candidates for conjugation to PEG-lipids [107]. Other recent models, such as TransMA, integrate both 3D geometric and 1D atomic sequence information to predict LNP transfection efficiency with high accuracy [108]. Acetylcholine-conjugated LNPs demonstrated a 3.6-fold enhancement in brain accumulation relative to non-targeted LNPs, with predominant tropism for neurons following systemic administration [107]. Nevertheless, although effective, these strategies continue to lead to substantial accumulation in peripheral organs such as the liver and spleen [104, 107].

Building on the success of small-molecule functionalization, recent work has demonstrated the power of combinatorial strategies that integrate specific lipid chemistry with surface engineering. Cao et al. developed a library of ionizable lipids incorporating SR-57227, a high-affinity ligand for the serotonin (5-HT₃) receptor, to facilitate active transport across the BBB [106]. The lead formulation (OS4) significantly outperformed FDA-approved benchmarks (MC3, ALC-0315) in brain accumulation. To further enhance transcytosis, the authors conjugated the cell-penetrating peptide Tat to the LNP surface, creating the "OS4T" platform. This hybrid design achieved widespread mRNA expression across neurons, astrocytes, and microglia. Crucially, when applied to a therapeutic context, OS4T LNPs encapsulating mRNA encoding engineered interleukin-12 (IL-12) successfully modulated the immune microenvironment in an orthotopic glioblastoma model, significantly extending survival compared to controls (Fig. 9) [106].

Surface engineering utilizing peptides provides a versatile approach to selectively target specific receptors on the BBB, including the transferrin receptor (TfR) and the nicotinic acetylcholine receptor (nAChR) [109]. Han et al. utilized thiol-maleimide click chemistry to conjugate peptides, including RVG29 (targeting nAChR) and T7 (targeting TfR), to the surface of the LNP [109]. RVG29-functionalized LNPs markedly enhanced neuronal transfection *in vivo* relative to untargeted LNPs, which were predominantly sequestered by endothelial cells [109]. In a

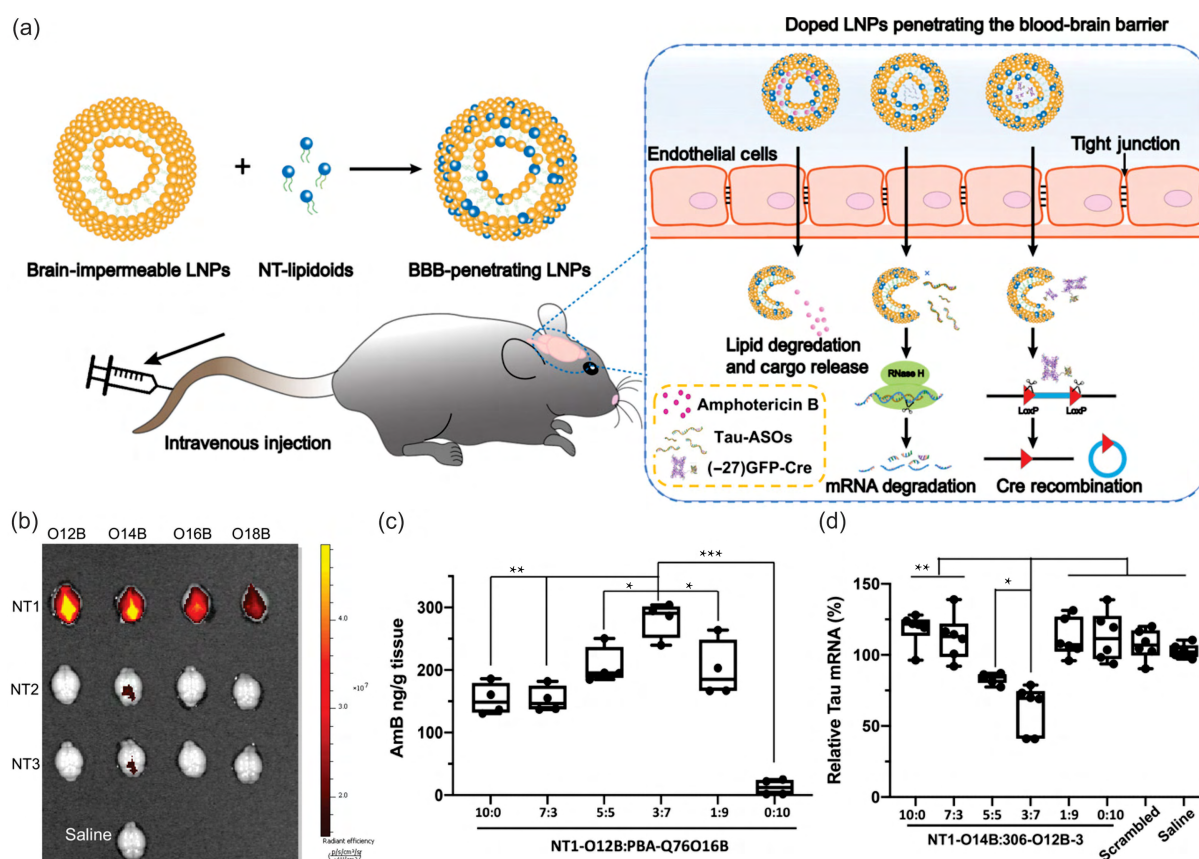


Figure 8 Neurotransmitter-derived lipidoids facilitate the delivery of diverse cargos across the BBB. (a) Brain-impermeable LNPs are doped with NT-lipidoids to penetrate the BBB and release small molecules, ASOs, and Cre proteins. (b) Tryptamine-derived lipidoids (NT1) cross the BBB one hour after intravenous injection, whereas phenethylamine (NT2) and NT3 derivatives do not. (c) Brain tissue AmB concentration quantification shows that optimizing NT1-lipidoids improves delivery efficiency. (d) Compared to scrambled controls, systemic administration of Tau-ASOs formulated with NT1-doped LNPs significantly knocks down brain *Tau* mRNA. Reproduced with permission from Ref. [105], © Ma, F. et al. some rights reserved; exclusive licensee American Association for the Advancement of Science 2020.

stroke model, a dual-targeting LNP was engineered utilizing the ionizable lipid A2-3O14, which naturally attracts a vitronectin-enriched protein corona targeting inflamed endothelium, in conjunction with an astrocyte-specific peptide (AS1) [110]. This formulation effectively silenced multiple EGF-like-domains 10 (*MEGF10*) in astrocytes, thereby reducing synaptic phagocytosis and promoting functional recovery [110]. However, peptide conjugation can increase particle size, which may impede diffusion within the brain parenchyma [109]. Furthermore, protein corona formation within the bloodstream can mask targeting ligands, thereby diminishing their binding affinity to BBB receptors [109, 111].

To effectively overcome the biological limitations of the BBB, physical disruption techniques employing focused ultrasound (FUS) in conjunction with microbubbles/nanobubbles have been integrated with LNP delivery [112–114]. Low-frequency FUS induces the oscillation of microbubbles administered intravenously, transiently mechanically disrupting endothelial tight junctions and generating pores to facilitate LNP entry [113]. Shumer-Elbaz et al. demonstrated that FUS-mediated opening of the BBB facilitated the delivery of mRNA-loaded LNPs to the brain parenchyma and glioblastoma tumors [113]. They observed approximately a 12-fold increase in protein expression relative to non-FUS controls, demonstrating that standard, non-targeted LNPs can effectively transfect brain tissue when the BBB is physically disrupted [113]. Similarly, Ediriweera et al. utilized magnetic resonance imaging

(MRI)-guided FUS to deliver calcium-phosphate LNPs carrying superoxide dismutase 1 (SOD1) ASOs, successfully reducing SOD1 protein levels in the cortex of an amyotrophic lateral sclerosis (ALS) mouse model [112]. While effective, this method requires balancing acoustic pressure to avoid microhemorrhages and necessitates precise timing of LNP administration due to the temporary nature of the BBB opening [112, 113]. Furthermore, the method necessitates specialized equipment (such as MRI or stereotactic guidance), which restricts its throughput relative to solely chemical targeting approaches [112, 113].

Notwithstanding these advancements, the delivery of LNPs to the CNS remains difficult. Even with the inclusion of brain-targeting moieties, the liver and spleen persist as the principal clearance organs, sequestering the majority of the administered dose [103, 109, 110]. Furthermore, interspecies variations in BBB transporter expression and epitope homology (such as TfR) between mice and humans complicate the clinical translation of ligand-based targeting approaches [115].

6 Precision extrahepatic targeting strategies

6.1 Retargeting the immune system

The spleen acts as the body's primary blood filter and a central hub for immune surveillance, populated by high densities of B cells, T cells, DCs, and macrophages. Redirecting LNPs from the liver to

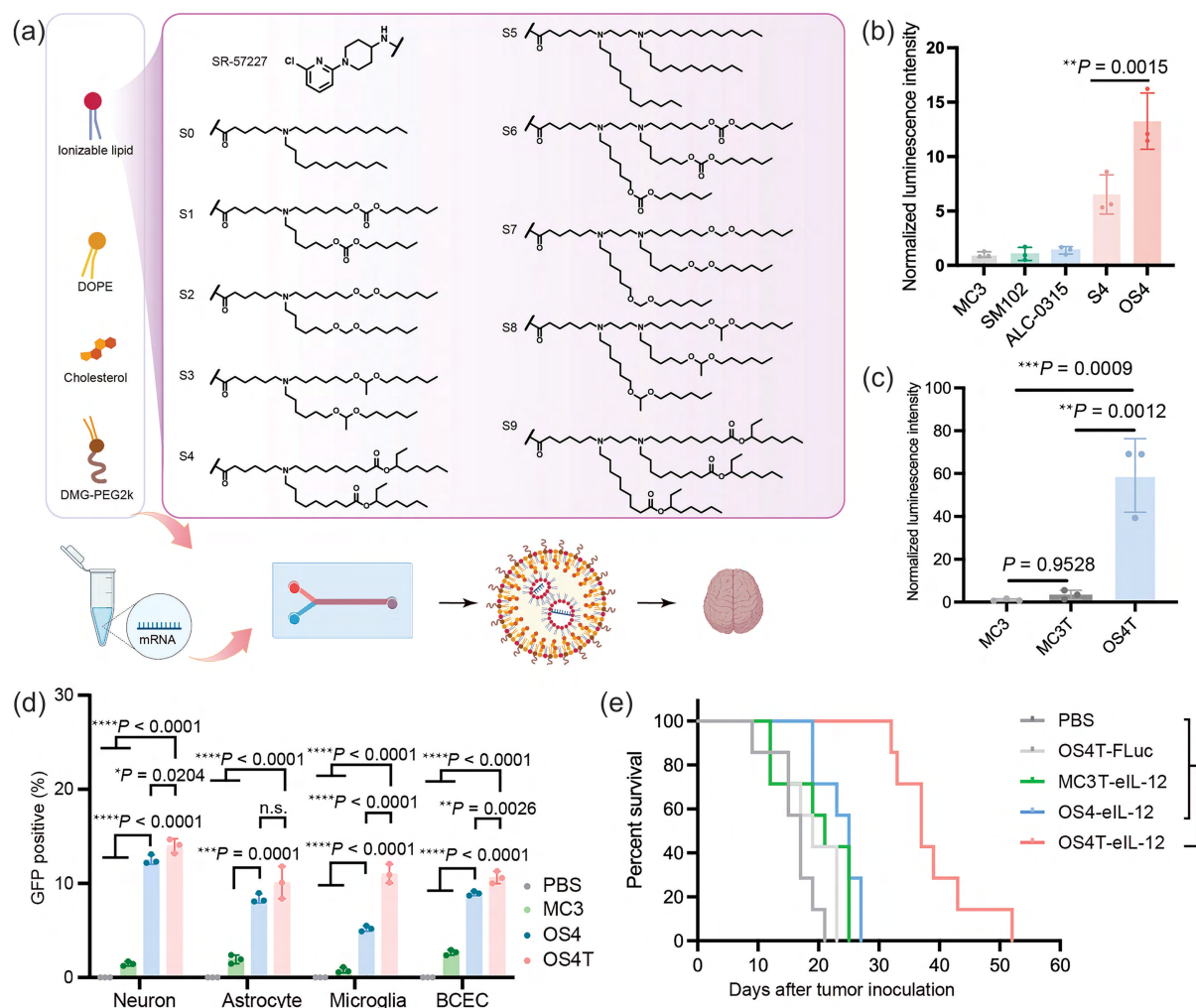


Figure 9 Synergistic design of neurotransmitter-derived lipids and peptide conjugates for glioblastoma therapy. (a) SR-57227-derived lipid library (S0–S9) chemical structures with a 5-HT3 receptor ligand headgroup for BBB crossing. (b) Systemic administration of the optimized lipid (S4) and its refined formulation (OS4) results in significantly higher brain delivery efficiency than clinically approved lipid benchmarks (MC3, SM-102, ALC-0315). (c) The OS4 LNP surface engineered with the Tat peptide (OS4T) improves brain delivery compared to the non-peptide OS4 control and Tat-modified MC3. (d) Flow cytometry analysis of brain single-cell suspensions shows that OS4T LNPs transfect neurons, astrocytes, microglia, and brain capillary endothelial cells with GFP. (e) Compared to controls, systemic treatment with OS4T LNPs encoding engineered IL-12 significantly extends median survival in an orthotopic glioblastoma mouse model. Reproduced with permission from Ref. [106], © Cao, D. et al. some rights reserved; exclusive licensee American Association for the Advancement of Science 2025.

the spleen is critical for applications including mRNA vaccines, cancer immunotherapy, and *in vivo* CAR-T cell engineering [12, 50, 59]. Strategies to shift tropism from the liver to the spleen involve modulating the physicochemical properties of the LNP (endogenous targeting) or functionalizing the surface with affinity ligands (active targeting) [16, 65, 94, 116, 117].

A key approach to reduce liver targeting and improve splenic uptake involves the addition of anionic lipids. While this strategy generally modulates surface charge and protein corona composition [94, 118], specific lipids like phosphatidylserine (PS) actively recruit splenic macrophages by mimicking the "eat-me" signals exposed on the outer leaflet of apoptotic cells and senescent red blood cells [86]. By emulating this physiological clearance mechanism, PS-containing LNPs facilitate robust phagocytosis via specific receptors such as T-cell immunoglobulin and mucin domain-containing protein 4 (Tim4) [94, 119]. For instance, Luozhong et al. demonstrated that incorporating 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) into a standard MC3-based LNP formulation

shifted expression from the liver to the spleen and lymph nodes [116]. The PS-LNPs exhibited robust luciferase expression in these tissues, markedly surpassing that of LNPs formulated with a non-biomimetic anionic lipid phosphatidic acid (PA). This finding indicated that the observed splenic tropism was attributable to specific biological recognition of the phosphoserine group rather than solely to negative charge [116]. As illustrated in Fig. 10, while PA-based LNPs shifted some accumulation to the spleen due to negative charge, the PS-LNP formulation achieved superior specificity for secondary lymphoid organs, including the superficial cervical lymph nodes, at both high and low dosages [116]. Furthermore, flow cytometry confirmed that this tropism is driven by enhanced uptake in monocytes and macrophages, which recognize the PS moiety as a biomimetic "eat-me" signal. In a related study utilizing lipidoid-based LNPs, LoPresti et al. demonstrated that the complete substitution of the neutral helper lipid DOPE with anionic lipids (such as PS) shifted organ tropism from the liver to the spleen [94]. Mechanistically, this transition is

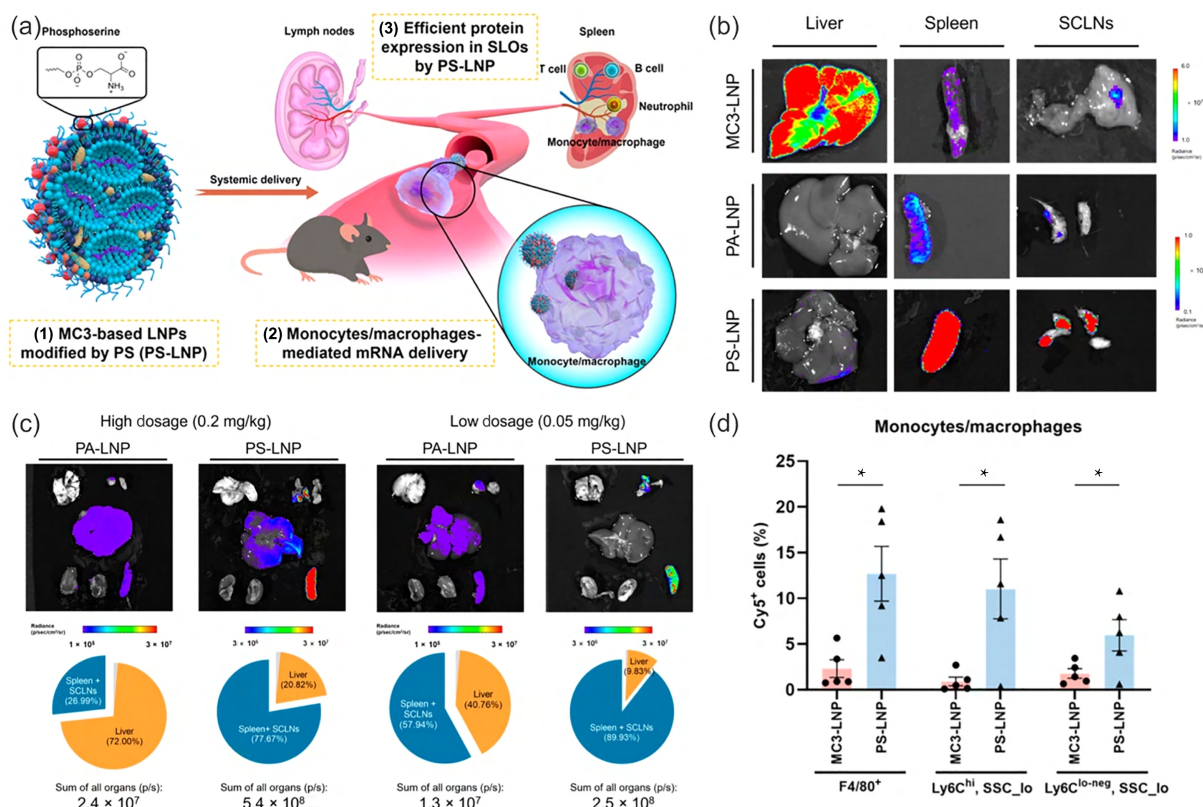


Figure 10 Phosphoserine-modified LNPs redirect mRNA delivery from liver to secondary lymphoid organs via monocytes/macrophages. (a) PS-modified MC3 LNPs (PS-LNPs) mimic apoptotic-cell PS to recruit monocytes/macrophages and transport mRNA to secondary lymphoid organs with systemic administration. (b) Bioluminescence imaging 6 h post-intravenous injection of luciferase mRNA-loaded LNPs reveals PS-LNPs significantly reduce hepatic expression and increase spleen and subcutaneous lymph node luciferase signal compared to MC3-LNPs or PA-LNPs. (c) Quantification of PS-LNPs at high (0.2 mg/kg) and low (0.05 mg/kg) doses suggests dose-independent retargeting to the spleen and lymph nodes, while PA-LNPs are predominantly distributed in the liver. (d) $\text{Cy}5^+$ cells in splenic monocyte/macrophage populations show enhanced PS-LNP uptake compared to MC3-LNPs in F4/80^+ , $\text{Ly}6\text{C}^{\text{hi}}$, and $\text{Ly}6\text{C}^{\text{lo-neg}}$ subsets. Reproduced with permission from Ref. [116], © American Chemical Society 2022.

associated with a reduction in the LNP's apparent pK_a and enhanced uptake by splenic immune cells [16, 94].

Although effective in targeting phagocytes, anionic LNPs frequently exhibit decreased overall protein expression levels relative to liver-tropic formulations [94, 116]. An alternative approach for spleen retargeting involves substituting structural cholesterol with bile acids, which are naturally occurring cholesterol analogs, to modify the LNP surface characteristics and biodistribution [120]. Substituting cholesterol with cholic acid in a C14-4 ionizable lipid LNP formulation (producing "CA-100" LNPs) significantly altered mRNA delivery. Following intravenous administration in mice, CA-100 LNPs exhibited a spleen-to-liver luminescence ratio of approximately 4, whereas the cholesterol control demonstrated liver predominance (ratio < 1) [120]. This substitution strategy demonstrated generalizability, as it also redirected the tropism of C12-200-based LNPs toward the spleen [120].

Although the aforementioned strategies primarily focus on phagocytes, targeting splenic T cells has traditionally relied upon the use of active targeting ligands, though novel lipid chemistries capable of endogenous T cell tropism are emerging [61, 117]. For example, it was demonstrated that antibody-functionalized C14-4 LNPs effectively transfect T cells to elicit functional effects such as CAR-mediated B-cell aplasia [59]. However, this active targeting strategy encounters certain limitations. For instance, Kheirloom et al. observed that anti-CD3 binding resulted in

temporary T cell activation followed by transient depletion from circulation. This emphasizes the dual nature of employing activating receptors for targeting, where ligand engagement triggers downstream biological effects beyond binding [65].

6.2 Selective organ targeting (SORT) technology

The SORT platform represents a formulation-based approach to bypass liver accumulation [118]. Developed by the Siegwart laboratory, SORT enhances the conventional four-component LNP system by incorporating a fifth component, a "SORT molecule", to systematically tune organ tropism [118, 121]. The mechanistic basis is that the physicochemical properties of the LNP (specifically the apparent pK_a), influenced by the chemical identity and molar percentage of the SORT molecule, dictate the recruitment of particular serum proteins (the protein corona). These surface-bound proteins subsequently direct the nanoparticle to specific tissues through endogenous receptor-mediated pathways [16].

A key feature of SORT technology is that the incorporation of permanently charged lipids into a typical liver-targeted LNP (MC3 or 5A2-SC8-based) predictably shifts tissue tropism [118, 121]. As demonstrated in Fig. 11, adding the anionic lipid 18PA (10%–40%) to a standard 5A2-SC8 LNP shifted luciferase mRNA expression exclusively to the spleen [16, 122]. Conversely, the incorporation of the cationic lipid DOTAP resulted in a progressive shift in expression from the liver to the spleen (at ~ 15%) and ultimately to the lung (at 50% or higher) [16, 122]. Elucidating the mechanism

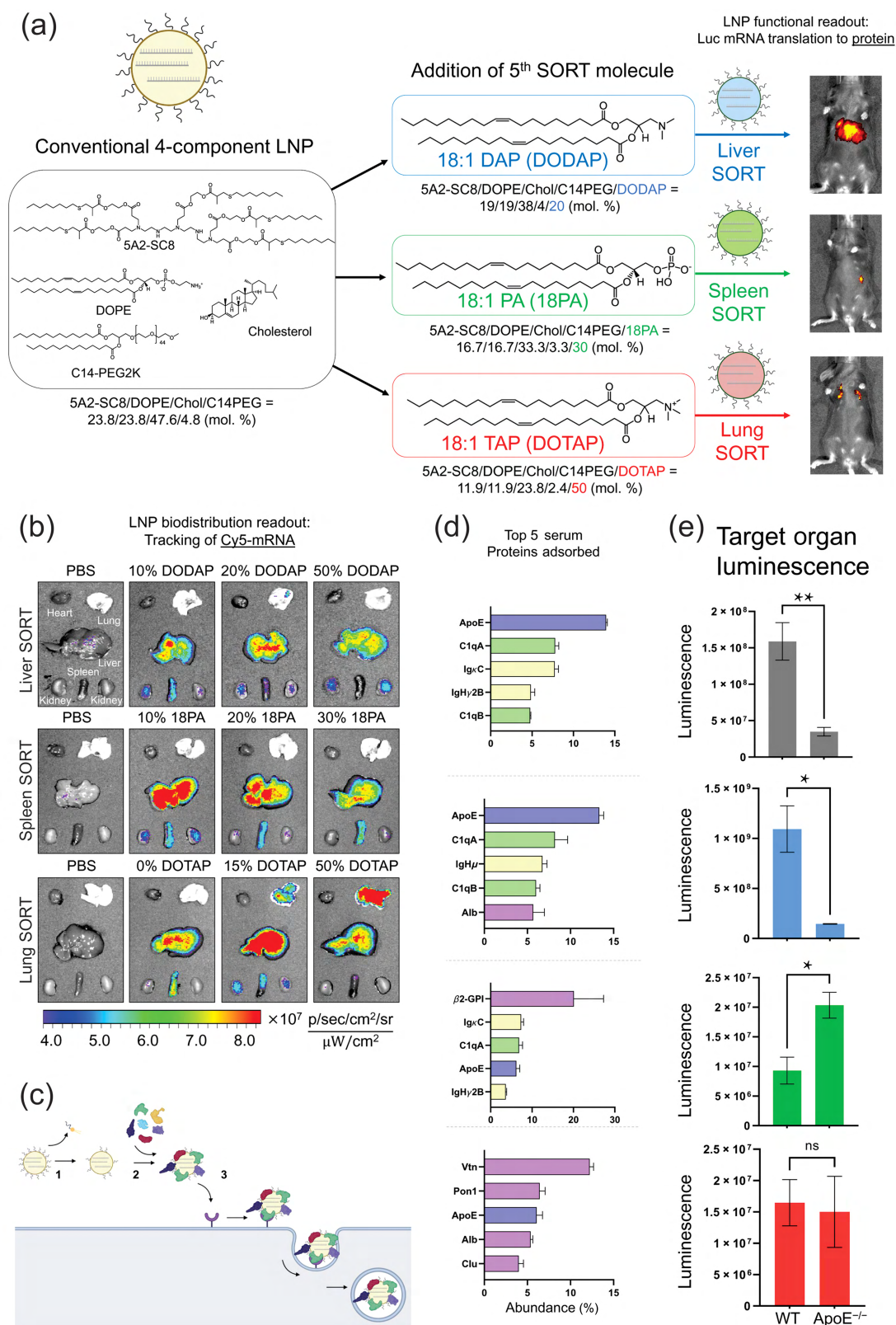


Figure 11 Mechanism and tunability of SORT LNPs. (a) Adding a fifth "SORT molecule" to a four-component liver-targeting LNP creates tissue-specific formulations. (b) Organ tropism is altered by anionic (18PA), DODAP, or DOTAP lipids. In mice, increasing the molar percentage of DOTAP shifts tropism to the lung, while 18PA shifts it to the spleen. (c) Proposed three-step endogenous targeting mechanism: (1) PEG-lipid desorption from the LNP surface post-injection, (2) selective recruitment of tissue-specific serum proteins (ApoE for liver, VN for lung) to form a protein corona, and (3) receptor-mediated uptake by cognate receptors highly expressed in the target. (d) SORT LNPs form protein coronas with specific proteins (VN for lung targeting), driving endogenous receptor-mediated uptake through proteomic analysis. (e) Target organ luminescence quantification shows statistically significant differences in mRNA expression between wild-type and ApoE-knockout mice, confirming tissue tropism by specific protein corona components. Reproduced with permission from Ref. [16], © Published under the PNAS license 2021.

driving this shift, Dilliard et al. demonstrated that the chemical nature of the SORT molecule alters the LNP's apparent pK_a and surface protein adsorption. Specifically, cationic lung-SORT LNPs recruit vitronectin (VN), which interacts with the $\alpha v \beta 3$ integrin receptor abundantly expressed on pulmonary endothelial cells [16, 123].

The SORT platform has been utilized to deliver CRISPR-Cas9 components for therapeutic genome editing in tissues beyond the liver [44, 118]. Utilizing a 50% DOTAP lung-SORT LNP, Cas9 mRNA, and sgRNA targeting *Pten* were administered to mice, achieving an approximate 15% indel frequency across the entire lung tissue [118]. Expanding this platform to immunotherapy, spleen-SORT LNPs incorporating the anionic lipid 18PA generated "*in situ*" CAR-T cells through the delivery of mRNA encoding a CAR to splenic T cells, resulting in tumor regression within a lymphoma model [50].

Despite its utility, the SORT platform encounters challenges in translation. The inclusion of permanently cationic lipids (such as DOTAP) introduces toxicity risks, including acute inflammation and thrombosis, which may narrow the therapeutic window relative to fully ionizable formulations [45, 121]. Additionally, the inclusion of a fifth component can complicate manufacturing and quality control, though four-component variations are being explored. Furthermore, therapeutic efficacy relies on variable endogenous proteins (such as VN), which may result in inconsistent biodistribution among diverse patient populations due to physiological heterogeneity in serum protein levels [16, 86].

7 Conclusion and future perspectives

LNPs are integral to modern genetic medicine, particularly in light of the widespread deployment of mRNA vaccines for COVID-19 and the approval of Onpattro, which have positioned LNPs as a leading non-viral delivery platform [1, 7, 124]. Methods capable of facilitating nucleic acid delivery have been extensively studied. However, progressing from "liver-centric" therapies to precision medicine for extrahepatic diseases, such as cancer, autoimmune conditions, and CNS disorders, necessitates overcoming lingering biophysical and translational challenges [30, 125–127]. Current LNP technology faces limitations that necessitate additional studies to identify innovative solutions. In the following, we outline these challenges and emerging trends in LNP technologies for *in vivo* engineering, emphasizing their chemistry, functionalities, and manufacturing processes [100, 128, 129]. Representative LNP design strategies and therapeutic outcomes are summarized in Table 1.

7.1 Remaining challenges

Traditional development pathways for LNPs rely heavily on empirical screening. However, the combinatorial space, comprising four components at variable molar ratios, is too extensive for exhaustive trial-and-error approaches [95, 130]. Consequently, strategies integrating AI and deep learning are being adopted to predict non-intuitive structural characteristics that influence organ tropism [95, 96]. Recent advancements in deep learning, particularly in pulmonary targeting, demonstrate the capacity of computational methods to identify novel ionizable lipids that exhibit higher efficiency compared to established industry benchmarks [95].

7.2 Emerging trends

This complexity underscores the necessity of moving beyond

traditional empirical screening methods [95]. Consequently, an area of promising research involves developing LNPs capable of actively destabilizing the endosomal membrane through specific phase transitions. This approach seeks to overcome a significant obstacle to therapeutic efficacy, namely the inefficient cytosolic delivery of the payload, which currently ranges from only 1% to 4% [12, 17, 131]. For instance, LNPs could be engineered with internal topologies that shift to non-lamellar inverted hexagonal phases upon acidification, thereby enhancing their interaction with endosomal membranes [80, 122, 131]. Developing processing techniques that enable scalability remains a key future trend. Existing microfluidic mixing techniques frequently encounter challenges in maintaining batch-to-batch consistency in particle morphology [132, 133]. Innovative architectures, such as the parallelized microfluidic device, have the potential to offer new opportunities for increasing production rates to liters per hour while preserving physicochemical properties [132].

Multifunctional materials that separate potency from immunogenicity are especially advantageous for clinical applications in chronic diseases. For example, materials that serve as "silent" or non-inflammatory carriers would offer safety advantages over existing vaccine-optimized formulations [134, 135]. Recent studies have demonstrated that the unique chemistry of the ionizable lipid headgroup determines its interactions with Toll-like receptor 4 (TLR4) [34]. Modulating this recognition is crucial for minimizing reactogenicity and maintaining sustained synthesis of proteins. Further advances are also anticipated in materials that exhibit persistent therapeutic properties beyond transient expression. Payloads capable of facilitating permanent genomic correction or prolonged expression, such as circRNA and plasmid DNA, have drawn increasing interest [72, 136]. Platforms employing minicircle DNA and transposase mRNA have demonstrated the feasibility of achieving stable genomic integration *in vivo*, offering a potential single-dose solution distinct from transient mRNA-only approaches [55]. However, DNA delivery is restricted by the nuclear envelope, which acts as a formidable intracellular barrier [86, 137]. Additionally, the development of such integrating therapies requires careful consideration of the potential risk of insertional mutagenesis [52, 138]. Conversely, strategies relying on repeated administration face the universal challenge of immune recognition. Specifically, developing materials that circumvent the "accelerated blood clearance" phenomenon induced by anti-PEG antibodies continues to be a significant challenge [34, 85, 139].

7.3 Future perspectives

The advancement of LNPs for prospective applications relies on engineering stimuli-responsive properties. Future designs will likely incorporate surface chemistries engineered to selectively recruit endogenous proteins for endogenous targeting, as well as internal topologies that dynamically reorganize in response to endosomal pH [71, 131, 140–142]. In conclusion, overcoming the "liver bottleneck" remains a primary objective [29, 91, 103]. We propose that the synergistic integration of rational chemical design, high-throughput screening, and AI to predict and engineer the bio-nano interface will significantly accelerate their clinical translation [95, 96, 130].

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript.

Table 1 Comparative analysis of key LNP design parameters and therapeutic outcomes

Strategy/LNP class	Key design parameter	Payload	Target/model	Refs.
Immunogenicity modulation	Amine headgroup chemistry (lipids 313, 306)	siRNA	Splenocytes/mice	[34]
BBB crossing	Neurotransmitter-derived lipidoids (NT-lipidoids)	mRNA	Brain/mice	[105]
Bone marrow homing	Covalent lipids and crosslinkers	mRNA/gene editing	Hematopoietic stem cells	[99]
Structural topology	DOPE concentration (10%–49%) to induce inverse hexagonal phase	siRNA	Macrophages/ <i>in vitro</i>	[80]
Payload distribution	N/P ratio and mixing kinetics (cylindrical illumination confocal spectroscopy (CICS) analysis)	mRNA	General characterization	[143]
AI-guided design	Deep learning to identify lipids (FO-32, FO-35)	mRNA	Lung/ferret	[95]
SORT	Charged helper lipids (DOTAP vs. 18PA)	mRNA	Lung vs. spleen	[94]
Lung targeting	N-series lipidoids (306-N16B) with specific tails	mRNA	Lung	[92]
Nebulization	PEG-lipid molar ratio and helper lipid charge	mRNA	Lung	[88]
RNP delivery	Enhancer DNA (enhDNA) in formulation	CRISPR RNP	Neurons/HEK293	[42]
Novel chemistry	Squaramide headgroups (Lipid 29)	mRNA	Liver/mice	[83]
Adjuvanted LNPs	Cyclic/heterocyclic amine headgroups (Lipid 311, 331)	mRNA	Immune cells	[144]
Brain delivery	Orthogonal array optimization of S4 lipid	mRNA	Brain endothelium	[106]
Macrophage targeting	Mannose-cholesterol conjugate	mRNA	Macrophages	[145]
Biodegradable lipids	Diketopiperazine core with ester linkers	mRNA	Liver	[15]
Stability	Self-degradable lipid (ssPalm)/lyophilization	mRNA	General/liver	[146]
Nebulization	Substitution of cholesterol with β -sitosterol	mRNA	Lung	[89]
T Cell engineering	Orthogonal DOE of excipient ratios (formulation B10)	mRNA	T cells	[54]
Phospholipid screening	Phospholipid structure (4A3-SC8 lipid)	mRNA	Spleen/liver	[147]
BBB screening	High-throughput <i>in vitro</i> BBB model	mRNA	Brain	[103]
Polymer-lipid hybrid	Amino-ionizable fatty acid (AIFA) tails	mRNA	Lung	[148]
Endosomal escape	Acid-degradable (AD) linkers in lipids	mRNA	Liver	[149]
Transcriptional response	Helper lipid charge (cationic/neutral/anionic)	mRNA	Lung/spleen	[150]
Cancer targeting	Ionizable lipids (Lipid 15, 30)	CRISPR-Cas9	Head&neck cancer	[151]
Manufacturing	Solvent composition in Impinging Jet Mixer	mRNA	General	[133]
Structure-activity relationship (SAR) study	Carbon spacing in amine core	mRNA	Liver	[152]
T cell activation	Anti-CD3/CD28 conjugation to LNP surface	mRNA	T cells	[51]
Cryopreservation	Buffer selection (Tris/HEPES vs. PBS)	mRNA	General	[153]
Dual-functionality	DOTAP inclusion	mRNA	NK cells	[154]
Safety/toxicity	High cationic lipid content (DOTAP)	mRNA	Lung	[45]
Oral delivery	Plant-derived lipids (digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG))	mRNA	Colon	[155]
Functional de-targeting	Antisense iOligos and Cas9-targeting siRNA	sgRNA, Cas9 mRNA, and siRNA	Lung & splenic endothelial cells	[156]
Mechanism	ApoE-knockout mice	siRNA	Liver/spleen	[14]
Antibody targeting	Anti-CD5 conjugation vs. Maleimide control	mRNA	T Cells	[59]

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

P. H. Z.: Writing – review & editing, writing – original draft,

visualization, resources, investigation, conceptualization. Z. R. Z.: Writing – review & editing. J. C.: Writing – review & editing. W. S.: Writing – review & editing. Z. H. T.: Writing – review & editing. W. J. S.: Writing – review & editing, writing – original draft, supervision, resources, project administration, methodology, investigation, funding acquisition, conceptualization.

Informed consent

Not applicable.

Ethics statement

Not applicable.

Use of AI statement

None.

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