PNAS PERSPECTIVE OPEN ACCESS

Recent advances in nanoparticulate RNA delivery systems

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Edited by Christine Seidman, Harvard Medical School, Boston, MA; received July 12, 2023; accepted August 2, 2023

Nanoparticle-based RNA delivery has shown great progress in recent years with the approval of two mRNA vaccines for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and a liver-targeted siRNA therapy. Here, we discuss the preclinical and clinical advancement of new generations of RNA delivery therapies along multiple axes. Improvements in cargo design such as RNA circularization and data-driven untranslated region optimization can drive better mRNA expression. New materials discovery research has driven improved delivery to extrahepatic targets such as the lung and splenic immune cells, which could lead to pulmonary gene therapy and better cancer vaccines, respectively. Other organs and even specific cell types can be targeted for delivery via conjugation of small molecule ligands, antibodies, or peptides to RNA delivery nanoparticles. Moreover, the immune response to any RNA delivery nanoparticle plays a crucial role in determining efficacy. Targeting increased immunogenicity without induction of reactogenic side effects is crucial for vaccines, while minimization of immune response is important for gene therapies. New developments have addressed each of these priorities. Last, we discuss the range of RNA delivery clinical trials targeting diverse organs, cell types, and diseases and suggest some key advances that may play a role in the next wave of therapies.

The advent of nanoparticle-mediated RNA delivery has helped usher in a new era of biotechnology, providing potential for new therapies for a range of diseases including infectious disease, cancer, and genetic disease. There has been substantial recent preclinical and clinical progress toward this future, but critical hurdles must be overcome to fully harness the capabilities of RNA delivery. In the following sections, we describe recent advances and open questions for four areas of focus for the next generation of RNA delivery systems: 1) optimizing the RNA cargo, 2) enhancing delivery potency, 3) refining targeting strategies, and 4) controlling the immune response. We conclude with an overview of the present and future of clinical development for RNA delivery systems.

RNA Cargo Optimization

RNA constructs used for delivery can be generally divided into two classes: small RNAs that are short enough to be chemically synthesized, and mRNA which must be in vitro transcribed. Chemically synthesized RNAs, including small interfering RNA (siRNA) for gene knockdown and guide RNAs (gRNAs) for CRISPR-based gene editing, can be made with custom nucleotide modifications that enhance stability and activity. siRNA modification is a mature technology that has led to Food and Drug Administration (FDA)-approved products including Onpattro, an siRNA lipid nanoparticle (LNP) drug, and multiple naked ligand-conjugated siRNAs (1). While chemical synthesis of small RNAs has been the primary strategy, enzymatic synthesis of custom-modified siRNA has recently been reported which could possibly be more suitable for manufacturing siRNA at scale (2). Chemical modifications of gRNAs for CRISPR gene editing can enhance editing efficiency (3–7).

For mRNA, in vitro transcription (IVT) is required as opposed to chemical synthesis. For linear mRNA, addition of a 5′ cap and poly-A tail is required and the length of the poly-A tail influences expression (8). Additionally, replacement of uridine with modified variants during IVT (8) and minimization of double-stranded RNA (dsRNA), either via High-Performance Liquid Chromatography (HPLC) purification (9) or more recently via engineered T7 RNA polymerase (10), both decrease innate immune detection of the mRNA. Codon optimization to minimize uridines, whether modified or not, can also improve expression (11). Additionally, both screening-based and computational approaches to codon and UTR optimizations have shown success in improving mRNA expression (12, 13).

RNA can also be circularized, for example via incorporation of a self-splicing intron, which can reduce immunogenicity and increase the duration of peak expression (14–16). These circular RNAs require Internal Ribosome Entry Sites (IRESs) to facilitate translation, since they do not have 5′-cap or poly-A tail, both of which are generally required for efficient canonical RNA translation (17). The extended peak expression of circular RNAs can potentially reduce dosing frequency of chronically dosed mRNAs, and more generally yield a higher quantity of protein per mRNA molecule. More complex RNA shapes can be generated through designed RNA folding, a process termed "RNA origami," which may further facilitate RNA stability, function, and delivery (18, 19).

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Author contributions: J.W., Y.H., R.L., and D.G.A. wrote the paper.

Competing interest statement: Both R.L. and D.G.A. have extremely extensive sets of patents including many in the RNA delivery space. For some examples, see: D.G.A.: US Patent App. 18/080,299 (J.W. is also an author on this patent application) US Patent 11,608,412 US Patent 11,603,396 US Patent 11,459,304 R.L.: US Patent 11,279,928 US Patent 10,933,139.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2307798120/-/DCSupplemental.

Published March 4, 2024.

Another RNA form used for vaccine applications is self-amplifying RNA (saRNA), in which the gene of interest is encoded along with the components of an alphavirus that allows for replication of the mRNA. This amplification leads to higher protein expression per RNA molecule delivered than standard mRNA, thus yielding higher potency per unit dose. One key challenge for saRNA is that the self-amplification machinery is large, generally resulting in a >10-kb construct that may require specialized delivery materials (20). This may potentially be addressed by saRNA-targeted materials design (20–22) or by using trans-amplifying RNA where the replication machinery and gene of interest are split between two separate, smaller RNAs (23). Another challenge is the immunogenicity of viral proteins and dsRNA intermediates that can form during RNA self-amplification. The immunogenicity of saRNA may lend itself well to vaccine applications (24), though saRNA LNPs were recently reported to treat a mouse model of genetic male infertility so their utility is not necessarily limited to vaccines (25).

Finally, a consistent challenge to RNA delivery is its chemical and enzymatic instability (26). One approach to addressing stability has been through a focus on the 2′ OH group. While irreversible 2′ OH modification of mRNA is not feasible as it eliminates translation, reversible 2′ OH modification of mRNA has recently been reported (26). This may allow for stabilization of mRNA, either in vivo to improve expression or during mRNA nanoparticle storage to improve shelf life at room temperature. Improving mRNA nanoparticle stability without requiring a cold chain is particularly important for use in low-resource communities; other recent stabilization strategies include lyophilization (27) and loading into a polymer blend followed by drying (28).

Progress in Nanoparticle Chemistry

RNA delivery nanoparticles must fulfill multiple criteria including RNA encapsulation, cell uptake, and endosomal escape (Fig. 1*A*), and there is a wide array of chemistries that have been reported to enable these functions. Polymers or oligomers including poly(beta-amino esters) (PBAEs) (29–33), charge-alterable reversible transporters (CARTs) (15, 16, 34), cell-penetrating peptides (CPPs) (35, 36), and others have been investigated for mRNA delivery with varying results (Fig. 1*B*). Complexation of RNA with cationic lipids in the form of lipoplexes has also shown recent promising clinical results for cancer vaccination (37). However,

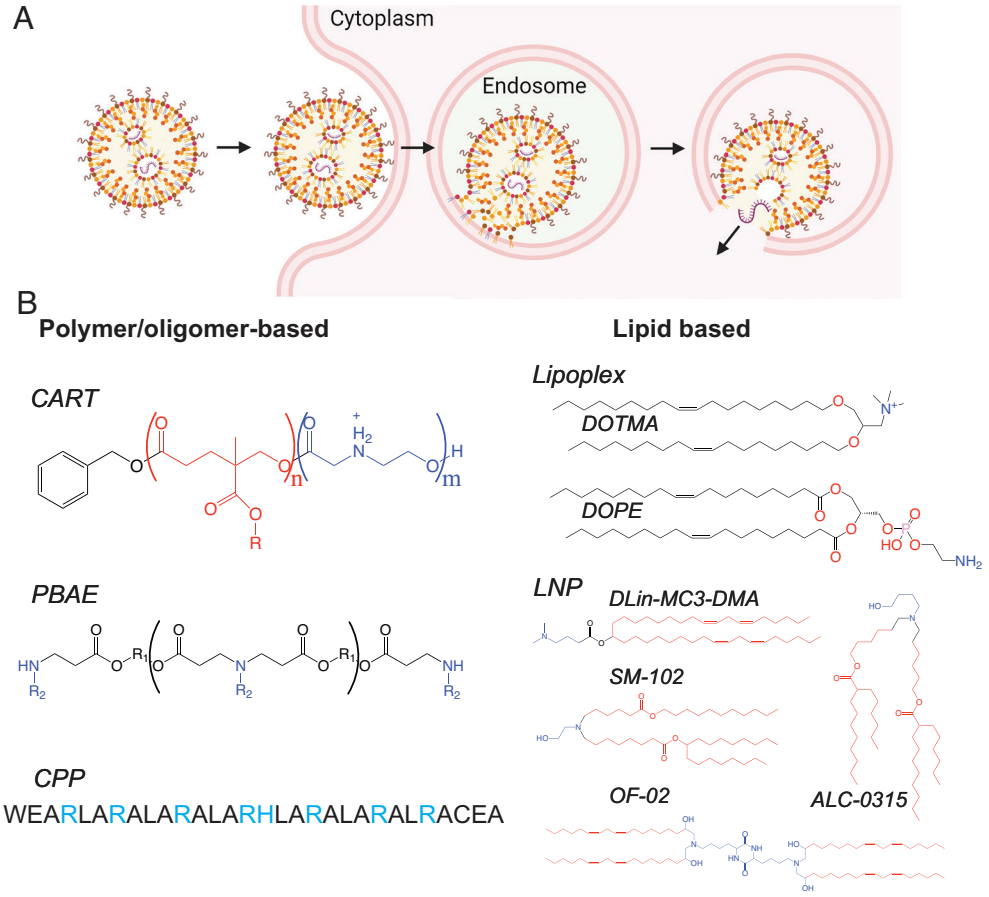

Fig. 1. (*A*) Overview of nanoparticle RNA delivery. RNA must be encapsulated in the nanoparticle, endocytosed, and escape the endosome into the cytoplasm. Generated by BioRender.com. (*B*) Sample of polymeric and lipid-based RNA nanoparticle delivery materials, lipid tails in red and cationic or ionizable components in blue. R groups for charge-altering releasable transporters (CARTs) and poly(beta-amino ester) PBAEs indicate structural flexibility that can be tuned via high-throughput screening; for cell-penetrating peptides (CPPs), structural optionality is not explicitly shown, but hundreds of CPPs for delivery of various cargos have been described (25). Lipid-based delivery can use ionizable lipid-free lipoplexes, such as those containing DOTMA and DOPE, while LNPs contain ionizable lipids with examples given here. DLin-MD3-DMA is FDA approved in a liver siRNA delivery formulation, and SM-102 and ALC-0315 are used in the Moderna and Pfizer-BioNTech COVID mRNA vaccines, respectively. OF-02 is highly potent for liver mRNA delivery lipid and illustrates the structural diversity of ionizable lipids.

the most clinically advanced results have come from ionizable lipid-based LNPs, which are used in the two FDA-approved COVID-19 mRNA vaccines, the FDA-approved liver-targeting siRNA formulation Onpattro, and multiple liver gene editing clinical trials (38).

To understand how delivery materials are identified, it is useful to consider the space of possible materials for a particular class of material. This space can be conceptually visualized as in Fig. 2*A*. Among materials that can be synthesized, only a subset can be synthesized via a high throughput-compatible chemistry and a different, overlapping subset will be useful for mRNA delivery. Here, "useful" denotes delivery potency as well as other relevant parameters for an application of interest such as safety and immunogenicity. A third category, nonidentical but overlapping, is the set of rationally designable materials, which are expected to be potent due to fulfilling known criteria (e.g., containing branched tails and biodegradable groups) and/or resembling potent materials identified previously. Below, we consider in detail the case of ionizable lipids for LNPs and show how past, present, and future ionizable lipid search strategies fit into this paradigm.

The most commonly explored LNPs are generally composed of an ionizable lipid, a helper lipid, cholesterol, and a PEG-conjugated lipid (Fig. 2*B*) (38). The helper lipid [usually, but not always (39), a phospholipid] and cholesterol help stabilize the LNP structure and may impact endosomal escape via altering membrane fluidity, while the PEG-conjugated lipid helps stabilize the LNP against aggregation and reduces macrophage-mediated clearance (38). While PEG-lipid is crucial for LNPs, the commonly used C_{14} anchored PEG-lipid is shed in vivo within ~30 min of intravenous (IV) injection, which helps prevent anti-PEG responses that can inhibit mRNA delivery (40). The ionizable lipid binds the RNA and plays a dominant role in achieving endosomal escape, which makes it the subject of intense research.

Several common features of ionizable lipids include the following: 1) they have an ionizable amine and one or more lipid tails, 2) they incorporate biodegradable groups such as esters (41), carbonates (42), or disulfide bonds (43) into the tails to facilitate faster clearance in vivo (38), 3) they have a cone-shaped overall conformation with a smaller headgroup and wider lipid tails (generally achieved via *cis* unsaturation

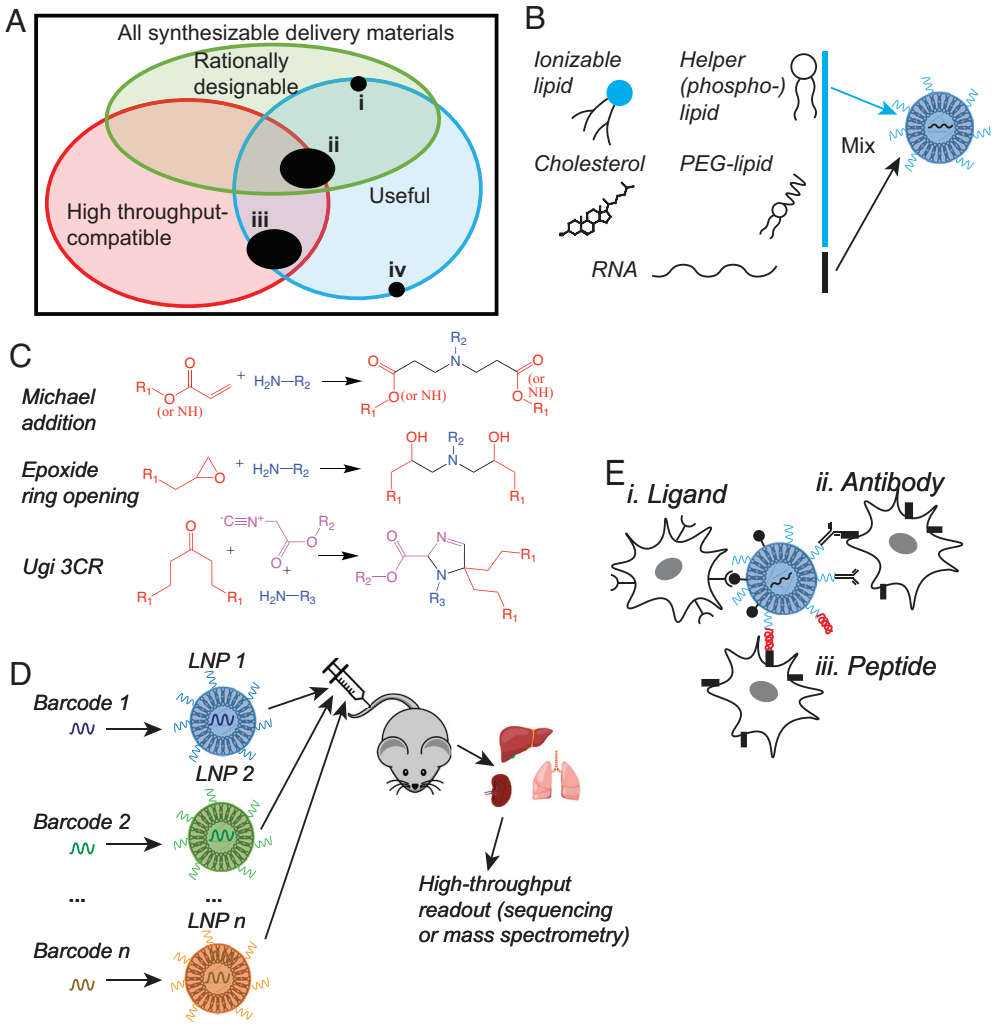

Fig. 2. (*A*) Present and future delivery material discovery strategies. Black ellipses represent ionizable lipid screening efforts with size of ellipse representing the number of lipids screened. (*i*) Small, targeted, rationally designed screen. (*ii*) High-throughput screen. (*iii*) High-throughput machine learning–guided screen. (*iv*) Small, targeted machine learning–guided screen. (*B*) The components of an LNP. (*C*) Prominent multicomponent reactions used to generate ionizable lipid libraries; Michael addition can use an acrylate or acrylamide. (*D*) Barcoded delivery screening. (*E*) RNA delivery nanoparticles can be targeted to specific cell types via (*i*) incorporation of specific ligands, (*ii*) antibody conjugation, or (*iii*) peptide conjugation.

and/or tail branching (44) which assists endosomal membrane disruption and escape, and to a lesser degree 4) they have alcohols near the headgroup that may aid RNA encapsulation (45). An example of rational design using these principles is shown in the report of a small, 10-lipid library that yielded lipid 8 (later renamed SM-102), the ionizable lipid used for the Moderna COVID-19 mRNA vaccine, as well as lipid 5, a top candidate for mRNA delivery to the liver (45). Systematic investigation of alternatives to headgroup alcohols for mRNA binding led to the discovery of squaramide-containing lipid 5 analogues that yielded a greatly increased duration of mRNA expression in nonhuman primates (NHPs) (46). A more recent effort rationally designed a 16-lipid library based on three-tailed DLin-MC3-DMA (MC3) analogues. The top lipid outperformed MC3 and other controls including lipid 5 for mRNA delivery to the liver in mice, showed minimal toxicity in mice and excellent biodegradability in rats, and yielded potent redosable delivery in NHPs (47). This small, targeted search strategy employed in these works is represented in Fig. 2 *A, i*.

One approach that has facilitated ionizable lipid discovery is the use of combinatorial chemistry, which can allow for the generation and evaluation of hundreds or thousands of ionizable lipids due to the combinatorial explosion of lipids generated from a relatively small set of components (48). Two of the most common combinatorial reactions are Michael addition between amines and acrylates or acrylamides (42, 48, 49) and epoxide ring opening reactions between amines and epoxides (50, 51) (Fig. 2*C*).

For example, combinatorial generation via Michael addition of a library of biodegradable carbonate-containing branched-tail lipids identified one that could perform CRISPR gene editing on the mouse lung epithelium via intratracheal delivery (42). Conversely, the lung endothelium was targeted via the top candidate from a small library of Michael addition-based disulfide-containing combinatorial lipid synthesis (52). Other recent combinatorial efforts include Michael addition-based library for vaccines (53) and epoxide ring opening-based libraries for delivery to the placenta via IV delivery for treatment of placental disorders (54), to the fetus via injection into the vitelline vein for in utero treatment of genetic disorders (55), to the brain for base editing (56), to the muscle for vaccines (57), and to T cells ex vivo for T cell engineering (58). Combinatorial lipid synthesis via enzymatic formation of ester bonds is a novel, green, alternative strategy that has yielded a highly potent mRNA vaccine candidate (59). Using an ionizable core of the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and combinatorial addition of multiple tails also produced a potent mRNA vaccine candidate that showed good activity in NHPs (60).

While combinatorial chemistries have tended to have two components, we recently reported a three-component Ugi reaction between an amine, an isocyanide, and a ketone to allow for even greater chemical diversity (Fig. 2*C*) (61). These multicomponent reactions permit the exploration of broader chemical space than one-by-one synthesis, facilitating the identification of more potent lipids. In this case, it yielded the serendipitous discovery of a lipid that activated the STING pathway which enhanced its immunogenicity in an mRNA vaccine context (61). Moving forward, we expect that as more potent ionizable lipids are identified, analyzing the properties of the lipids beyond just simply characterizing the delivery efficiency of the cargo will gain importance as a way to differentiate top candidates. Controlling the immune response to LNPs, and improving the ability to be put into a shelf-stable formulation, are two examples of these other key properties.

Whatever the combinatorial chemical approach used to generate these lipids, they are represented in Fig. 2 *A, ii*: The fraction of lipids that are potent for delivery is usually relatively low, but enough lipids are screened to generate multiple potent candidates. Moreover, the components are often selected to conform to the criteria described above such as incorporation of branched tails and biodegradable groups (41, 42).

As more combinatorial lipid libraries are created and more high-throughput screens are done, a significant amount of training data will become available. Machine learning (ML) applied to such lipid structures may therefore assist identification of improved lipids for delivery. ML holds out the tantalizing possibility of identifying lipids that may not have been predicted by the human expert but are nevertheless potent. Introducing this chemical diversity to the ionizable lipid space may identify more potent lipids with new and unusual properties. ML could be used either to design the components for input into a high-throughput screen (Fig. 2 *A, iii*) or for small-scale, targeted testing like that used to discover SM-102 (Fig. 2 *A, iv*).

Another key challenge to lipid discovery is how best to identify promising formulations from a high-throughput synthesis effort because in vitro screening can be an unreliable predictor of in vivo delivery potency (62). This bottleneck may be resolved by in vivo barcoded delivery screening, in which each member of a library of LNPs receives a unique tag that can be read out in a high throughput manner to quantify successful delivery (Fig. 2*D*) (41, 63–67). For example, a barcoded LNP screen led to the identification of adamantane-containing helper phospholipids that potently targeted T cells in vivo (63, 68), while a different barcoded screening methodology helped identify LNPs for oral delivery (69). The recent development of a barcoded single-cell sequencing-based assay has shown how LNP chemistry affects delivery to different cell subtypes of the liver (67). Additionally, barcoded delivery screening can pair well with machine learning because high-throughput in vivo data can be used to train higher-quality models, while these models can be used to design better screens.

While most research has centered on ionizable lipids, the other components can vary in significant ways. Notably, Siegwart and colleagues reported the combinatorial synthesis of an ionizable phospholipid (iPhos) library via a ring-opening reaction in which the top iPhos showed potent in vivo mRNA delivery (70), as well as zwitterionic amino lipids (ZALs) that eliminate the requirement for a helper phospholipid (71). Both iPhos and ZALs were capable of liver gene editing (70, 71). Even for standard LNPs, helper lipid charge determines whether an intravenously delivered LNP will target the lung, liver, or spleen (39). Cholesterol can also be replaced with analogues, which have been shown in different studies to improve I.V. RNA delivery, nebulized mRNA delivery, and mRNA delivery to T cells (72–74).

Even for a fixed set of components, changing component ratios can dramatically alter delivery potency, usually

in unpredictable ways that may necessitate a design of experiments (DOE)-based approach to formulation optimization for each individual top ionizable lipid (75). For example, DOE formulation optimization was used to improve LNP stability in amniotic fluid, which allowed for mRNA delivery to the fetal lung, liver, and intestines via intra-amniotic injection (76). Last, aside from the iPhos and ZAL work, there has been comparatively little large library screening for the nonionizable lipid components. Such screening offers an alternative opportunity to make improvements in LNPs.

The physical properties of delivery nanoparticles also play a role in delivery. LNP size, which is coupled with their payload capacity (77), influences the delivery of RNA to the liver (78). Another study found that the antibody titer from an intramuscular mRNA vaccine was influenced by LNP size (79), for which a later study suggested that larger LNPs > 200 nm have higher delivery efficiency to dendritic cells (80). Nanoparticle morphology has a less explored relationship to RNA delivery but there are some interesting results. Replacing cholesterol with the analogue β-sitosterol in LNPs improved nebulized mRNA delivery while altering the morphology to a polyhedral shape, which may be related to improved nebulizability (73).

Also, while the presence of bleb structures on LNPs is often avoided in the interest of generating a homogeneous nanoparticle population, it may also lead to enhancement of gene expression (81). Understanding the correlation of function with LNP morphology and structure through conventional and advanced characterization methods (82–87), and developing methods to control the physical properties of LNPs, is therefore another promising avenue for improving RNA delivery.

Receptor-Targeted RNA Delivery

While appropriate selection of ionizable and helper lipids can broadly guide organ specificity to the liver, lung (primarily endothelium), and spleen following I.V. delivery (39), delivery to other organs or specific cell types often necessitates the use of a distinct targeting agent such as a small molecule ligand for a particular receptor (Fig. 2 *E, i*). For instance, LNPs displaying a vitamin A derivative were reported to transfect hepatic stellate cells (HepSC) and lung myofibroblasts, key cell types involved in liver and pulmonary fibrosis, respectively, allowing for therapeutic delivery of an antifibrotic siRNA (88). Likewise, a library of ionizable lipids displaying the HepSC ligand anisamide yielded a top candidate that allowed for antifibrotic siRNA delivery (89). Similarly, a library of bisphosphonate-containing ionizable lipids was reported to target bone with potential applications in bone healing and regeneration (90). LNPs displaying GalNAc potently transfect hepatocytes independently of the canonical LDL receptor (LDLR)-mediated uptake mechanism, which can improve liver delivery generally but especially for patients with homozygous familial hypercholesterolemia, where little LDLR is present (91). Conjugation of mannose can potentially target antigen-presenting cells, thus enhancing self-amplifying RNA vaccine efficacy (92).

An interesting new direction is ionizable lipid-conjugated ligands with cell signaling-directed targets, as shown by a recent study where an ionizable lipid containing a TLR7/8 agonist adjuvated mRNA vaccines when used in combination with a nonconjugated lipid (93). The examples of the anisamide, bisphosphonate, and TLR7/8 agonist-containing ionizable lipids suggest substantial flexibility in ionizable lipid headgroup modifications to perform targeted biological functions while largely preserving endosomal escape ability.

Antibody conjugation has also emerged as a promising and sometimes more straightforward strategy to target specific cell types, given the extensive history of characterizing cell types by surface antigens (Fig. 2 *E, ii*). For example, anti-CD3 (94) or anti-CD5 (95) conjugated LNPs transfect T cells. In the latter case, this allowed for in vivo chimeric antigen receptor (CAR) T therapy to treat cardiac injury (95). Anti-CD117 conjugation targets hematopoietic stem cells (HSCs), the progenitor cell of the immune system (96). Notably, HSC delivery was enhanced by the use of more strongly anchored PEG-lipid to increase circulation time, a feature which may generalize to other antibody-conjugated LNPs. Importantly, in this study HSC delivery was significantly better using one monoclonal anti-CD117 antibody (2B8) rather than another (ACK2), highlighting the importance of antibody design to delivery (96).

Additionally, different antibodies or antigens may mediate delivery to and transfection of nontarget cells, and liver sequestration and transfection may still happen. These effects may or may not lead to off-target effects, depending on the application (96). Last, the additional reaction and purification steps for antibody conjugation and difficulty in precisely characterizing the efficiency and orientation of conjugation make antibody-LNP conjugates more complex than standard LNPs in both lab and industrial settings. Regardless, we believe that antibody-targeted RNA nanoparticulate delivery will continue to advance, expanding the set of tissues and cell types that can be treated using RNA medicine.

Intermediate in size between antibody and small molecule ligand conjugation, peptide conjugation is another promising strategy for targeting or improving RNA delivery (Fig. 2 *E, iii*). Recent proof of concept was provided by Herrera-Barrera and colleagues, who used phage display to identify peptides that target photoreceptor (PR) cells in the eye, a key target for treating retinal disease. When conjugated to LNPs, the top peptides led to efficient transfection of PRs as well as other retinal cells in both mice and NHPs (97). Phage display has also been used to identify a peptide targeting hepatocellular carcinoma (HCC), where peptide conjugates drove efficient mRNA delivery to HCC cells (98). Combining phage display or other peptide screening approaches with mRNA delivery systems may therefore provide a new unbiased strategy for cell type-specific targeting.

Immune Response to RNA Delivery Systems

All gene delivery vehicles administered in vivo have the potential to engage the host immune system. For mRNA vaccines against cancer and infectious diseases, the interactions with the immune system are central to their function. However, it is equally important to comprehensively assess immunological consequences in nonvaccine applications.

For cancer vaccines, optimizing antigen design to increase tumor specificity is key to immunogenicity (99), and to obtain

personalized antigens has shown clinical promise (37). The delivery system used needs to induce highly potent, systematic, and durable immune activation, which would benefit from highly targeted nanoparticles to antigen-presenting cells (100). However, for infectious diseases vaccines, immune activation must be tuned so as to provide the desired immune response without driving excessive inflammation that results in reactogenicity (101, 102).

While the field has widely adopted uridine-modified mRNA to reduce the innate recognition of the mRNA cargo (8), it remains to be systematically explored on how carrier materials, such as ionizable lipids, direct the immunogenicity and reactogenicity of mRNA vaccines. Pardi et al. reported that even without any mRNA, empty LNPs formulated with the lipid mix containing an ionizable lipid significantly adjuvanted a protein subunit vaccine (103), while a recent study found that such empty LNPs could activate human peripheral blood mononuclear cells (PBMCs) without the presence of any antigen (104).

The endosomal escape capability of ionizable lipids, which disrupts intracellular vesicular membranes, can be inflammatory in nature (105, 106). There is also accumulating evidence that LNPs can directly trigger intracellular immune sensing pathways (107). Different ionizable lipids and/or formulations were reported to trigger different pathways as researchers revisit their immunological mechanisms, and a comprehensive understanding is yet to be built on this complex territory.

The differences In immune responses to mRNA vaccines between mouse and primates (79), and even between nonhuman primates and humans, represent an additional challenge to determine optimal LNPs for human use (106). This difficulty in predicting the human immune response may be addressed in part by systems vaccinology and the study of the immune reactions of established, and well-characterized vaccines across species (108–110). In addition, human cell-based ex vivo screening platforms, such as the Modular iMmune In Vitro Construct (MIMIC) system (111), may allow the generation of multidimensional data with modules mimicking natural human immune systems to accelerate discovery of promising formulation candidates.

Gene therapy via therapeutic mRNA delivery presents perhaps the most complex immune landscape. For recessive diseases, particularly those caused by lack of a particular protein (due to nonsense, frameshift, or splicing mutations) and treated by repeated delivery of the missing gene, there is risk of an immune response against that gene due to inadequate central tolerance (112). In this case, any inflammation induced during mRNA delivery may effectively "vaccinate" the patient against the therapeutic protein. Furthermore, use of systemic corticosteroids can cause powerful side effects such as bone loss in a long-term chronic dosing context (113). While more research is needed for the mRNA case, preclinical data and limited clinical data for viral vector gene therapy for cystic fibrosis and alpha-1 antitrypsin deficiency suggest the possibility of antitransgene T cell responses (114, 115).

The immune response also complicates delivery for inflammatory diseases, as preexisting inflammation amplifies the inflammatory response to mRNA LNPs in multiple disease models (116). For example, treatment with low LNP doses (~0.1 mg/kg) in a mouse model of acute respiratory distress syndrome led to increased mortality compared to PBS treatment (117).

Better materials design can address these immunological challenges. The discovery of intrinsically inert or immunosuppressive ionizable lipids could enrich the ionizable lipid toolbox. Similarly, incorporation of dexamethasone (118) or dexamethasone prodrugs (119) into LNPs appears to have a more targeted immunosuppressive effect than systemic administration. Conversely, further adjuvanting LNPs via the STING pathway, for example, through STING agonist-derived ionizable lipids (120) or incorporation of novel manganese-based adjuvant (121), could also be useful for targeted immune activation in vaccine applications. Finally, for gene editing, where a limited number of treatments is needed, the immune response can be modulated with systemic administration of immunosuppressants (91).

Clinical Translation of RNA Delivery Systems

Currently, intramuscular vaccine delivery (the Moderna and Pfizer/BioNTech COVID-19 mRNA vaccines) and IV delivery to the liver (Onpattro, an siRNA LNP (38) are the only routes that have yielded FDA-approved RNA nanoparticle delivery (44), and these routes remain the lowest hanging fruit (Table 1). For example, Moderna's research pipeline includes clinical trials for at least 10 infectious disease vaccines and two cancer vaccines. The success of these programs will be in large part determined by choice of antigen or antigen cocktail, particularly for difficult targets such as HIV. However, improved transfection efficiency and better control over LNP-driven immunostimulation will increase the chances of success for any particular vaccine.

In the liver, Intellia Therapeutics has reported substantial LNP-based CRISPR knockout of transthyretin for patients with transthyretin amyloidosis (123). This is the first of a wave of promising LNP-driven liver gene editing therapies including Cas9-based editing of hypercholesterolemia-related diseases and hereditary angioedema (124), base editing correction of alpha-1 antitrypsin deficiency and glycogen storage disease 1a (Beam Therapeutics, undergoing IND enabling studies), and more (Table 1). Repeat-dosed liver-targeted gene therapy for metabolic diseases such as propionic acidemia (122) are also showing promise in clinical trials (Table 1). For propionic acidemia, it is unclear precisely what fraction of patients make no functional protein, as disease-causing mutations vary widely (122), but it will be valuable to test whether any patients develop an immune response to the therapeutic genes.

Delivery to other organs has advanced more slowly but there has been progress. In the respiratory tract, while the first-ever nebulized mRNA delivery gene therapy for cystic fibrosis did not advance past a phase½2 clinical study (125), two phase I nebulized trials are recruiting or ongoing (Table 1). Direct intratumoral injection of mRNA-LNPs has also advanced into clinical trials (Table 1).

While infectious disease vaccines have had the most obvious clinical success, successful cancer vaccination has proven more elusive due to the difficulty in inducing a powerful enough immune response. The ionizable lipid-free mRNA lipoplex strategy has shown impressive preliminary clinical results for pancreatic ductal adenocarcinoma, a

Table 1. Representative ongoing nanoparticle-based mRNA clinical trials

Trial ID	Phase	Route of administration	Therapy name	Cargo	Indication	Sponsor
NCT04917861	2	IM	mRNA-1893	Viral antigen	Zika virus	Moderna
NCT05397223	1	IM	mRNAs 1345, 1647, 1272, and 1010	Viral antigens	COVID/RSV/Flu/CMV	Moderna
NCT03313778	1	IM	mRNA-4157	Personalized neoantigen	Solid tumors	Moderna
NCT05398029	1	IV	VERVE-101	Base editor + sgRNA	Hypercholesterolemia and atherosclerosis	Verve Therapeutics
NCT04601051	1	IV	NTLA-2001	Cas9 + sgRNA	Transthyretin amyloidosis	Intellia Therapeutics
NCT05120830	1/2	IV	NTLA-2002	Cas9 + sgRNA	Hereditary angioedema	Intellia Therapeutics
NCT04159103	1/2	IV	mRNA-3927	PCCA, PCCB mRNA	Propionic acidemia	Moderna
NCT05659264	1	IV	mRNA-0814	Relaxin	Heart failure	Moderna
NCT04486378	2	IV	RO7198457	Personalized neoantigen	Colorectal cancer	BioNTech
NCT03946800	1	IT	MEDI1191	IL-12	Solid tumors	MedImmune/ Moderna
NCT05668741	1	Nebulized	VX-522	CFTR	CF	Vertex Pharma/ Moderna
NCT05712538	1	Nebulized	ARCT-032	CFTR	CF	Arcturus Therapeutics
NCT05737485	1	Nebulized	RCT1100	DNAi1	Primary ciliary dyskinesia	ReCode Therapeutics

IM: intramuscular administration. IV: intravenous administration. IT: intratumoral administration. RSV: respiratory syncytial virus. "Flu" refers to seasonal influenza. PCCA and PCCB: subunits of propionyl-CoA carboxylase, mutations in which cause propionic acidemia (122).

deadly cancer (37), and is in trials for colorectal cancer (RO7198457, Table 1). Lipoplex cancer mRNA vaccines are delivered IV and effectively target phagocytic antigen-presenting cells (126). Neoantigen LNP-based vaccines also show promise (Table 1).

Looking to the future of clinical mRNA delivery, lipoplexes and LNP-driven intramuscular vaccination and liver mRNA delivery continue to appear promising. Additionally, much like how the success of the COVID vaccines spurred massive investment in clinical mRNA vaccine development, the success of ongoing extrahepatic trials in any one space could lead to substantial expansion of treatments using that modality. For example, approval of existing lipoplex-based personalized cancer vaccines would be a promising signal for generalization to other types of cancer, as well as lipoplex-based tolerogenic vaccines (127). Likewise, successful nebulized mRNA delivery for cystic fibrosis may encourage clinical development of nebulized therapies for other lung diseases such as asthma (128) or idiopathic pulmonary fibrosis (129, 130).

Three additions to the current clinical trial space may also greatly expand the usefulness of RNA delivery. The first is targeted delivery vehicles. With the exception of vitamin A analogue-targeted LNPs for liver fibrosis (ND-L02-s0201, for which no trials appear to be currently ongoing), these have yet to reach the clinic. However, GalNAc conjugate LNPs may be close to clinical trials as they have reported favorable primate data (91) and are undergoing IND-enabling studies as VERVE-102. Antibody-mediated targeting of T cells could allow for the second major advance, in vivo CAR T therapy. Sidestepping the expensive, technically challenging, and time-consuming ex vivo CAR T cell generation step would make a major impact on treatment of cancer and potentially numerous other diseases including cardiac injury (95) and aging-related diseases (131). However, because mRNA delivery is transient, generating a durable CAR T response for cancer treatment is a major challenge.

Last, there has been significant progress on intranasal mRNA vaccination for respiratory infections (132). Intranasal vaccines may provide sterilizing immunity against infection via induction of mucosal immunity, which would be a dramatic step forward for prevention of seasonal and pandemic illnesses alike (133). Overall, the future of RNA nanomedicine is bright as powerful new delivery materials are brought to bear on a widening spectrum of diseases.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. This work was supported by the NIH under grant no. R01 HL162564-02. J.W. was supported by the Cystic Fibrosis Foundation under awards WITTEN19XX0 and WITTEN23F5. R.L. was supported by the NIH and the Gates Foundation. D.G.A. receives research funding from Sanofi/Translate Bio, is a Founder of oRNA Tx, and receives compensation and equity from Combined Therapeutics. R.L. is a co-founder of Moderna and serves on its board; he was on Alnylam's initial Scientific Advisory Board, and has been advisor and has received compensation and equity for Hopewell Therapeutics and Combined Therapeutics. For a list of entities with which R.L. is, or has been recently involved, compensated or uncompensated, see: https://www.dropbox.com/s/yc3xqb5s8s94v7x/Rev Langer COI.pdf?dl=0.

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