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Therapeutic RNA Delivery for COVID and Other Diseases

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RNA can alter the expression of endogenous genes and can be used to express therapeutic proteins. As a result, RNA-based therapies have recently mitigated disease in patients. Yet most potential RNA therapies cannot currently be developed, in large part because delivering therapeutic quantities of RNA drugs to diseased cells remains difficult. Here, recent studies focused on the biological hurdles that make in vivo drug delivery challenging are described. Then RNA drugs that have overcome these challenges in humans, focusing on siRNA to treat liver disease and mRNA to vaccinate against COVID, are discussed. Finally, research centered on improving drug delivery to new tissues is highlighted, including the development of high-throughput in vivo nanoparticle DNA barcoding assays capable of testing over 100 distinct nanoparticles in a single animal.

1. Delivering RNA to Diseased Cells Is an Inefficient, Multistep Process

As high-throughput genetic tools have become commonplace, our understanding of the genes that cause disease has improved. As a result, the number of potential therapeutic targets is likely to increase, especially as biological assays probe how genes[1] and noncoding elements[2] drive cellular phenotypes and contribute to disease. These biological advances are complemented by the ability to specifically manipulate gene expression using RNA therapies, which can roughly be subdivided into four categories based on their biological effects. In the first category, mRNA-based drugs can replace protein activity in order to treat diseases caused by loss-of-function mutations[3] or lead to the production of antigens that elicit an immune response[4] (**Figure 1**A). In the second, mRNA encoding nucleases can alter gene expression via RNA or DNA editing, utilizing a growing number of biochemical mechanisms that include the formation of insertions and deletions, base editing, and the purposeful insertion of specific gene sequences using a separate DNA template or reverse transcribed template[5,6] (Figure 1B). In the third, siRNA-, miRNA-, and antisense oligonucleotide (ASO)-based drugs can reduce aberrant protein activity via RNA-induced silencing complex (RISC) or RNAse H, respectively[7] (Figure 1C). Finally, disease caused by irregular splicing can be alleviated using ASOs targeting acceptor or donor sites[7] (Figure 1D).

In all four cases, the RNA drug must access the cytoplasm or nucleus of diseased cells. To enter the correct cells via systemic delivery, the RNA must be protected from proteins called nucleases that can degrade it in the blood, avoid unwanted uptake facilitated by phagocytic cells such as Kupffer cells, access the target tissue from the bloodstream, and interact with and enter the cytoplasm of the on-target cell within a complex tissue microenvironment without eliciting a strong immune response (**Figure 2**A).[8] This multistep process makes drug delivery challenging, and as a result, drug delivery is often inefficient. For example, when researchers analyzed published nanoparticle delivery datasets focused on the delivery of small-molecule drugs to tumors, they found that 0.7% of the administered nanoparticle dose was delivered to solid tumors, on average.[9] To address this issue, researchers subsequently defined the dose of nanoparticles that needs to be delivered to a single mouse to achieve efficient tumor delivery.[10] They found that by saturating delivery to liver Kupffer cells, which clear nanoparticles, they were able to bypass liver accumulation and increase nanoparticle circulation long enough to achieve 12% delivery to solid tumors after a systemic injection.[11] The need to saturate or circumvent liver-mediated clearance has also been observed when lipid nanoparticles (LNPs) carry nucleic acid-based therapeutics; scientists have shown that a significant amount of systemically administered nanoparticles ends up in hepatic cell types.[12] LNPs have been developed to bypass this challenge and specifically deliver to particular cell types in nonhepatic tissues such as lung ECs, splenic ECs, splenic T-cells, B lymphocytes, and bone marrow ECs.[13–16] In a recent example, scientists pretreated mice with a "nanoprimer" designed to saturate Kupffer cells, then subsequently injected mice with a nanoparticle carrying mRNA or siRNA (Figure 2B). Nanoprimer pretreatment increased mRNA delivery, quantified by the expression of human erythropoietin, as well as siRNA delivery, quantified by factor VII gene silencing.[17] One key question that will need to be addressed when saturating Kupffer cells, or other hepatic cell types that act as clearance cells when nonhepatic delivery is wanted (e.g., hepatocytes, endothelial cells, dendritic cells), is considered as a pretreatment is tolerability, especially given the potential role Kupffer cell activation may play in dose-limiting and systemic nanoparticle-mediated

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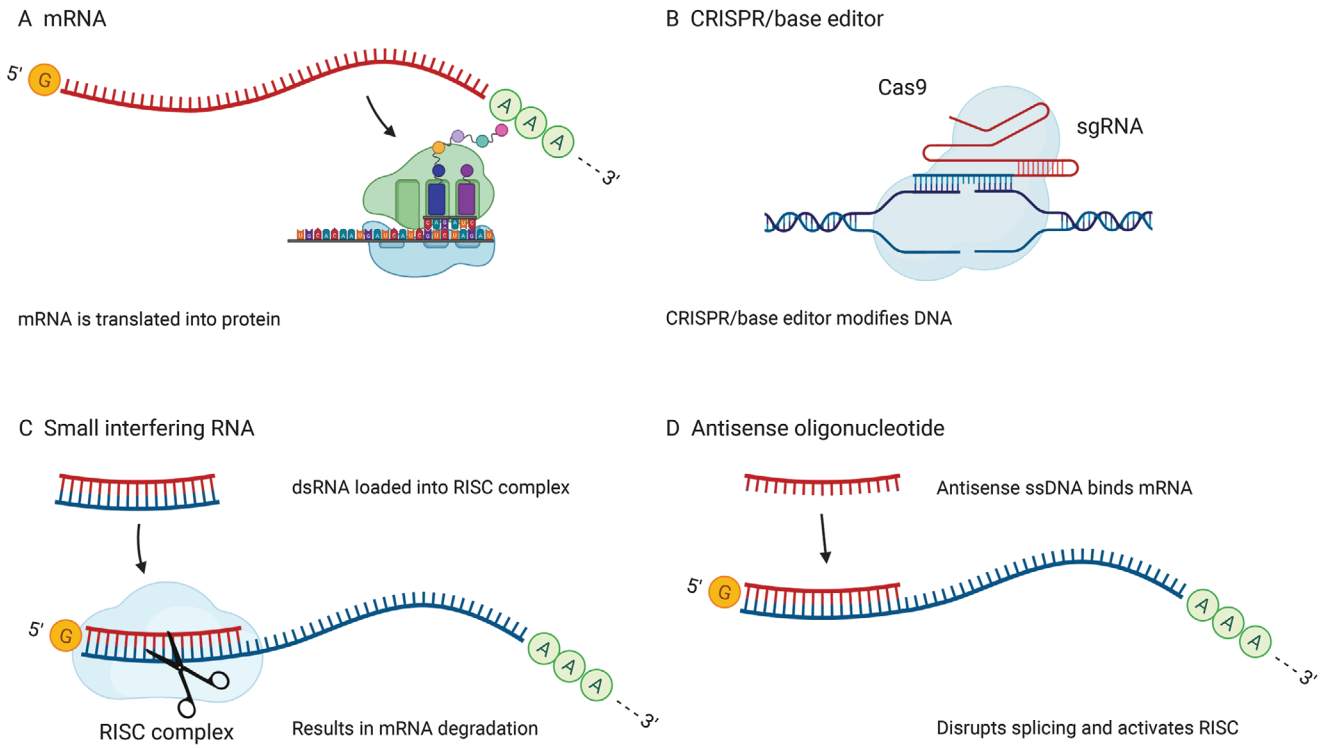

Figure 1. RNA therapies. A) mRNA delivery allows for production of missing or mutated proteins. B) CRISPR/base editors can be used to delete or edit genes to prevent aberrant protein production. C) siRNAs with the help of the RISC complex can degrade aberrant mRNA production. D) ASOs bind to complementary RNA targets, resulting in their removal with RISC or altered splicing patterns.

toxicity.[18] Specifically, authors found that platelet-activating factor, likely released by Kupffer cells, was a key driver of the immune response from nanoparticles carrying siRNA.[19] Even after a nanoparticle reaches and enters a target cell, at least 98% of the material does not enter the cytoplasm.[20]

One biological hurdle to drug delivery that is currently underappreciated is the role of endogenous cellular machinery required for the function of an RNA payload after it is released in the cytoplasm. Specifically, several lines of evidence now suggest that pathways governing endogenous mRNA translation or metabolism may influence the efficiency with which LNP-mediated mRNA delivery leads to functional protein.[21–26] In one example, researchers knocked down genes related to the mammalian target of rapamycin (mTOR) pathway in order to investigate their impact on LNP functional delivery.[21] They found that knocking down Rab4a and Rab5a, proteins necessary for early endosomal trafficking and endosomal recycling, had no impact on LNP delivery, whereas knocking down Rab7a, a protein necessary for endosomal maturation and late endosomal trafficking, significantly reduced LNP delivery. Researchers hypothesized that the decrease in Rab7a reduced mTORC1-mediated mRNA translation, thereby negatively impacting LNP-mediated mRNA delivery. To validate these results, they upregulated mTORC1 and discovered that it led to a significant increase in LNP delivery. Additional data support the hypothesis that mTOR signaling, or signaling pathways that interact with the mTOR pathway, may influence delivery.[22] Recently, scientists found that the bioactive lipid phosphatidylinositol (3,4,5)-triphosphate (PIP3) reduces LNP-mediated mRNA delivery by changing a cell's metabolic state.[22] Based on transcriptomic and metabolomic analyses, researchers concluded that reductions in LNP delivery could be mediated by two potential mechanisms: first, that PIP3 induces consumption of cellular resources, limiting resources available for the translation of exogenously delivered mRNA; second, that PIP3 induces a catabolic cell response that triggers protein degradation and decreased translation. Although the relative importance of these two mechanisms is unknown, this sharp reduction in protein translation in PIP3-altered cells suggests that the metabolic state of a cell can alter LNP delivery. A second cell signaling pathway that influences nanoparticle delivery is TLR-mediated inflammation and its subsequent effects on mRNA translation efficiency.[23] In one example, authors found that decreasing TLR activation using chemically modified mRNAs led to increased protein production.[23] In a second example, authors observed that even small increases in TLR4 signaling blocked LNP-delivered mRNA from being translated.[24] By pretreating cells with small molecules antagonizing TLR4 or its downstream mediator PKR, the authors significantly increased protein production at a given mRNA dose.[24] Given these studies, it is clear that metabolic signaling and immunological response can affect and govern LNP delivery and potency. Future studies of clinically relevant LNPs will need to assess the significance of disease states on LNP delivery in order to understand the impact that genes and pathways may have, prior to conducting clinical trials.

A third type of endogenous cell signaling that may influence the efficacy of RNA drugs has been identified using ASOs

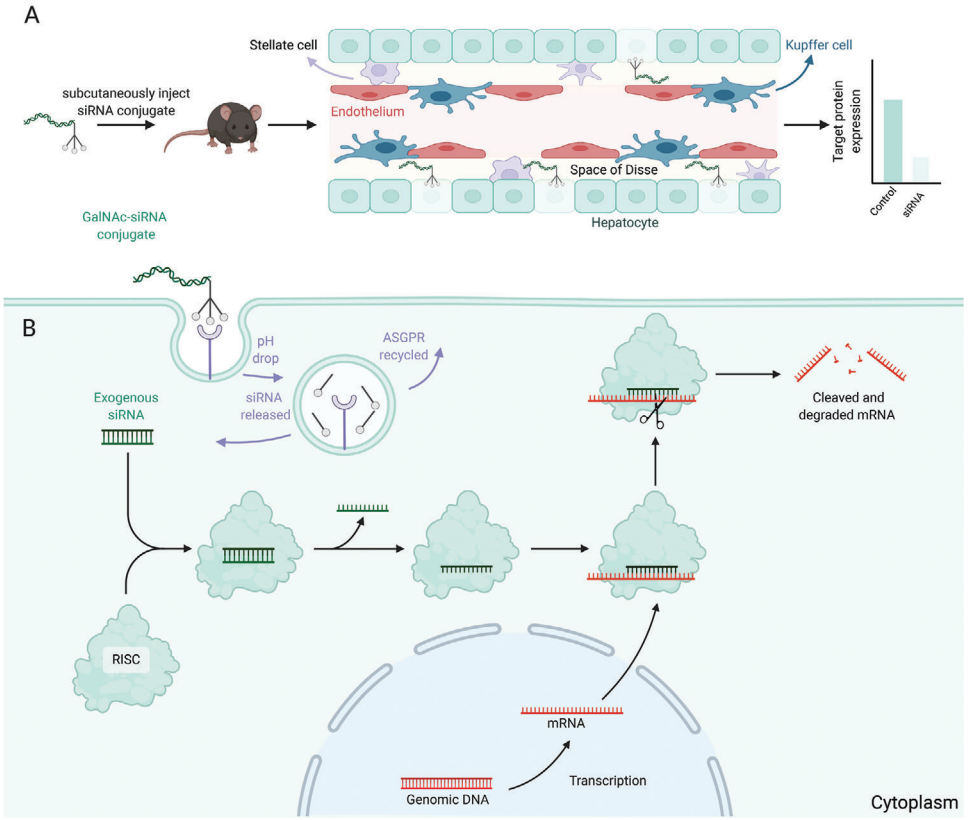

Figure 2. A) Biological barriers that prevent lipid nanoparticle delivery. Endosomal degradation, phagocytosis and immune cell clearance, receptor sequestration, and interaction with serum protein. B) Pretreatment with a nanoprimer can be used to reduce clearance of nanoparticles in the liver.

without a drug delivery system. ASOs have been found to interact with a suite of proteins within different compartments inside the cell; these interactions affect ASO efficacy.[25] In one example, authors determined that reducing the Golgi-endosome including GCC2 and M6PR resulted in less ASO release from endosomes.[26] This suggests that pharmacologically activating these genes, or inhibiting a gene that inhibits them, could improve delivery. In another example, scientists found that phosphorothioate-modified antisense oligonucleotide (PS-ASO) activity could be changed by including sugar modifications to a single nucleotide.[27] The use of a phosphorothioate backbone has been shown to block digestion by exonuclease III and some restriction enzymes.[28] Additionally, by adding a specific 2-O-methyl modification to the ASO, the interactions between the ASO and proteins were reduced, leading to decreased RNAse H1-dependent delocalization of paraspeckle proteins and subsequent nucleolar stress. By decreasing activation of cellular stress pathways, the authors reduced PS-ASO hepatotoxicity and simultaneously improved functional activity, thereby increasing the therapeutic index, a ratio that defines the amount of a therapeutic that causes a therapeutic effect relative to the amount that causes toxicity. These studies with naked ASOs led to interesting questions with direct clinical relevance. First, do the same pathways influence siRNA or mRNA drugs, and second, to what extent does the presence of a drug delivery vehicle influence these interactions?

2. siRNA and mRNA Can Be Systemically Delivered to the Liver in Humans

Despite the physical barriers that make systemic RNA delivery challenging, both siRNA- and mRNA-based drugs have been delivered to the liver in patients. In a phase 1 clinical trial led by Moderna, LNPs were formulated to carry mRNA encoding antibodies that target human chikungunya virus.[29] The LNPs were administered to 22 patients at doses of 0.1, 0.3, and 0.6 mg kg^{-1}, and detectable antibody concentrations were measured in all patients for at least 16 weeks postadministration. Of the four patients receiving the highest dose, three reported some degree of infusion-related adverse events (AEs), including a range of grade 1, grade 2, and grade 3 AEs. Grade 1 AEs are mild adverse events that require no medical intervention; grade 2 events are moderate events that require minimal, local, or noninvasive intervention; grade 3 events are severe and characterized by symptoms that often require invasive intervention and hospitalization and may be disabling, as defined by the FDA. None of the low or medium dose-receiving patients reported AEs. LNPs have also been used to deliver siRNA in patients. Specifically, Alnylam Pharmaceuticals, which develops siRNA-based therapies, received FDA approval for its LNP-mediated delivery of siRNA in hepatocytes, a drug known as patisiran (**Figure 3**). In this case, the siRNA silences mutant transthyretin (TTR) expression, which if left untreated causes amyloidosis.[30] The FDA-approved LNP utilizes

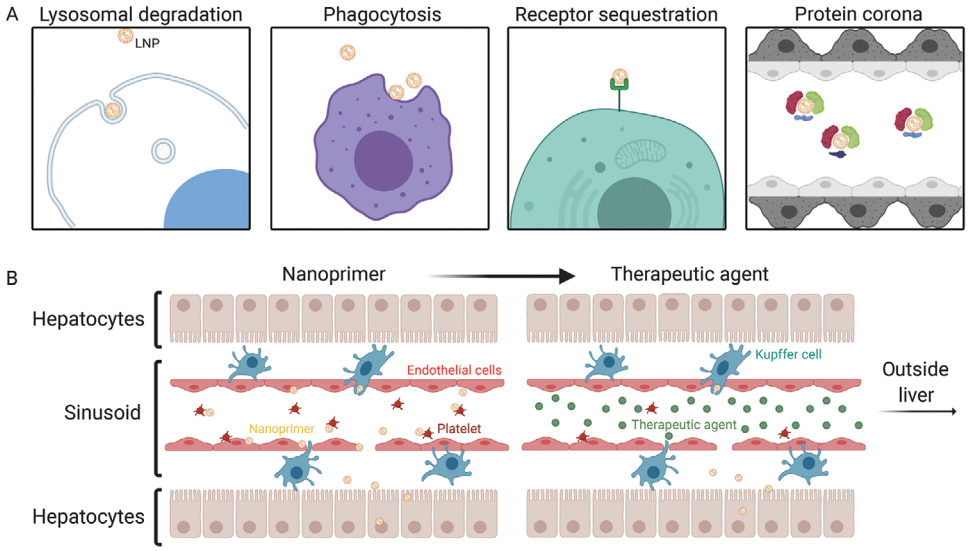

Figure 3. A) GalNAc-siRNA conjugates are subcutaneously injected and enter hepatocytes, which are accessible past the endothelial and Kupffer cell layer. Efficiency of the construct is then assessed by looking at target protein expression knockdown in mice that received the conjugate versus a saline control. B) GalNAc conjugates enter cells by binding to the asialoglycoprotein receptor (ASGPR) on hepatocytes. Once inside, the exogenous siRNA can cleave target mRNA by interacting with RISC.

DLin-MC3-DMA; this lipid is targeted to hepatocytes by interacting with the serum lipoprotein ApoE, which leads to subsequent endocytosis by cells expressing the ApoE receptor LDLR.[31] In a phase 1 clinical trial, Alnylam demonstrated that patisiran led to >80% reduction in TTR levels in patients receiving an LNP dose of 0.15–0.5 mg kg^{-1} when compared to a placebo control.[31] In the pivotal phase 3 trial, 225 patients were treated with 0.3 mg kg^{-1} LNP (148 patients) or placebo (77 patients) every three weeks. After 18 months of treatment, the neuropathy impairment score and other endpoints were used to gauge patient improvement. Once again, patisiran led to substantial (>70%) reduction in serum TTR levels for up to 81 weeks, compared to a placebo control. Whereas 97% of patients in each group reported mild or moderate AEs, including 36–40% of patients reporting serious AEs and 28–36% of patients reporting severe AEs, it is unlikely the LNP or the siRNA drug led to these, since the frequency of AEs did not increase within the treatment group as compared to a placebo control. Instead, it is more likely that the AEs were associated with the disease itself.

Alnylam has also received FDA approvals for siRNA therapies targeted to hepatocytes using GalNAc, a carbohydrate-derived conjugate that binds the rapidly recycled asialoglycoprotein receptor (ASGPR), which is specifically expressed on hepatocytes. GalNAc conjugates, which are administered subcutaneously, lead to prolonged gene silencing. For example, in the phase 3 trial for givosiran, which was subsequently approved by the FDA to treat acute intermittent porphyria, 48 patients were treated subcutaneously with 2.5 mg kg^{-1} of siRNA monthly for six months, which led to >75% reductions in mean aminolevulinic acid (ALA) and porphobilinogen (PBG) levels for up to six months.[32] Patients with acute intermittent porphyria often have accumulation of ALA and PBG, which are intermediates in the heme biosynthesis pathway.[33,34] Accumulation of these two intermediates can cause psychiatric symptoms, abdominal pain, and peripheral neuropathies; thus, increased urinary levels of ALA and PBG are also used to diagnose patients with acute intermittent porphyria.[34] By using an siRNA to reduce levels of the precursor ALA synthase (ALAS1), givosiran prevented accumulation of ALA and PBG and improved disease symptoms.[35,36] GalNAc-siRNA conjugates similarly reduced PCKS9 expression, which led to promising phase 3 clinical data.[37] More specifically, inclisiran lowered LDL cholesterol levels by >50% after 510 d when given once every six months subcutaneously. Sustained gene silencing is considered a significant advantage over statins, which require patients to take pills every day. GalNAc also served as the drug delivery system for lumasiran, which uses siRNA to silence hydroxyacid oxidase 1 (HAO1), the gene that encodes for glycolate oxidase (GO) enzyme, in order to reduce hepatic production of oxalate.[36] Scientists showed that lumasiran achieved >50% silencing of urinary oxalate levels, leading to improvements in secondary endpoints such as plasma oxalate levels and preventing kidney failure and eventual multiorgan damage from systemic oxalosis, and resulting in an FDA-approved drug treating primary hyperoxaluria type 1 (PH1).[38] Alnylam has two late-stage drugs under development for amyloidosis and hemophilia as well as multiple early-stage programs for alpha-1 liver disease, hepatitis B infection, and complement-mediated diseases, all of which utilize their enhanced stabilization chemistry (ESC)-GalNAc platform.[39] Fitusiran, which works by targeting the antithrombin gene SERPINC1, has resulted in increased thrombin levels in patients with hemophilia A or B similar to levels found in healthy patients.[40] In a phase 1 clinical trial, patients treated with fitusiran experienced up to a 90% reduction in mean antithrombin activity following a once-monthly high-dose injection and up to a 60% reduction following a once-weekly low-dose regimen.[36]

Alnylam is not the only company utilizing GalNAc-siRNA conjugates in human trials. Dicerna, Arrowhead Pharmaceuticals, and Silence Therapeutics also have ongoing clinical trials[5,41–43] (**Table 1**). For example, nedosiran, made by Dicerna, has been used in clinical trials to treat primary hyperoxaluria types 1, 2,

Table 1. Select list of current mRNA and RNAi therapies in clinical trials.

Sponsor	RNAi therapy: disease	Mechanism of action	Administration route	Clinical trial phase: NCT #	Comments
Select[a] siRNA clinical trials					
Alnylam Pharmaceuticals Genzyme (Sanofi)	Fitusiran (ALN-AT3SC): Hemophilia A/ hemophilia B	Antithrombin III inhibitors, hemostasis stimulants	Subcutaneous	Hemophilia A—Phase II/III: NCT03974113 Hemophilia B—Phase II/III: NCT03974113	One patient death in phase II for hemophilia A; phase II/III recruiting and expected to be complete in Oct 2021.
Alnylam Pharmaceuticals The Medicines Company	Inclisiran (ALN-PCSSC): Hypercholesterolaemia, atherosclerotic cardiovascular disease, renal impairment	PCSK9 protein inhibitors	Subcutaneous	Phase I, II, III; NCT03060577, NCT03159416, NCT02963311, NCT03705234	Up to 50% reduction in low-density lipoprotein (LDL) cholesterol at 180 d. Alnylam intends to market inclisiran in year 2020.
Alnylam Pharmaceuticals	Givosiran (ALN-AS1): Acute intermittent porphyria	5-Aminolevulinate synthetase inhibitors	Subcutaneous	Phase I, I/II, III; NCT02949830, NCT03338816, NCT02240784, NCT03547297	Monthly injection (2.5 mg kg^{-1}) for 6 months resulted in a 70% decrease of porphyria attacks.
Alnylam Pharmaceuticals	Lumasiran (A16ALN-GO1): Primary hyperoxaluria	Glycolate oxidase expression inhibitors	Subcutaneous	Phase I/II, II, III: NCT02706886, NCT03350451, NCT03681184	Dosed monthly at 3 mg kg^{-1} for 3 months followed by quarterly maintenance doses; normalization of urinary oxalate levels observed after 6 months.
Arrowhead Pharmaceuticals	ARO-AAT: α1-antitrypsin deficiency liver disease	α1-antitrypsin inhibitors	Subcutaneous	Phase I: NCT03362242	Up to 93% AAT reduction at 6 weeks after single dose; no severe AEs up to 300 mg kg^{-1}. Enrollment in the first sequential cohort in its phase II trial complete.
Arrowhead Pharmaceuticals (Janssen)	ARO-HBV: Hepatitis B	Antivirals, hepatoprotectants	Subcutaneous	Phase I, II: NCT03365947	100% of patients achieved >1 log10 reduction in HBsAg; well tolerated up to 400mg.
Arrowhead Pharmaceuticals (Amgen)	AMG 890: Cardiovascular diseases	Reduce production of apolipoprotein A	Subcutaneous	Phase II: NCT04270760	Phase II recruiting in progress.
Arrowhead Pharmaceuticals	ARO-APOC3: Hypertriglyceridemia	Reduce production of apolipoprotein C-III	Subcutaneous	Phase I: NCT03783377	Well tolerated; patients achieved high levels of pharmacologic activity against the target.
Arrowhead Pharmaceuticals	ARO-ANG3: Dyslipidemia	Reduce production of angiopoietin-like protein 3	Subcutaneous	Phase I: NCT03747224	Multiple doses achieved high levels of APOC3 and ANGPTL3 protein knockdown in phase I.
Arrowhead Pharmaceuticals	ARO-HSD: Liver diseases	Reduce production of hydroxysteroid dehydrogenase HSD17B13	Subcutaneous	Phase I: NCT04202354	Phase I recruiting in progress.
Arrowhead Pharmaceuticals	ARO-ENaC: Cystic fibrosis	Reduce production of the epithelial sodium channel alpha subunit (αENaC) in the airways of the lung	Nebulization	Phase II: NCT04375514	Phase I/II recruiting in progress.
Arrowhead Pharmaceuticals	ARO-HIF2: Renal cell carcinoma	inhibit the production of HIF-2α	Intravenous	Phase I: NCT04169711	Phase I recruiting in progress.
Dicerna Pharmaceuticals	Nedosiran (DCR-PHXC): Primary hyperoxaluria	Oxalate modulators	Subcutaneous	Phase I: NCT03392896	Well tolerated; most patients reach normal circulating oxalate.
Dicerna Pharmaceuticals	DCR-A1AT: α1-antitrypsin deficiency	α1-antitrypsin inhibitors	Subcutaneous	Phase I: NCT04174118	Phase I recruitment in progress. Administration of a single or multiple doses will be explored.

(*Continued*)

Table 1. (Continued).

Sponsor	RNAi therapy: disease	Mechanism of action	Administration route	Clinical trial phase: NCT #	Comments
Silence Therapeutics	SLN360: Cardiovascular diseases	Reduce production of Lp(a)	Subcutaneous	Phase I: NCT04606602	Phase I recruitment in progress.
Silence Therapeutics	SLN124: ß-thalassemia Myelodysplastic syndrome	Lower serum iron levels, modulate tissue iron distribution	Intramuscular	Phase I: NCT04176653	Phase I recruitment in progress.
Select[a] mRNA clinical trial					
Moderna Therapeutics	mRNA-1273: COVID-19 infections	Immunostimulants	Intramuscular	Phase III: NCT04470427	Two doses of the vaccine via IM injection in the upper arm ≈28 d apart. Emergency use authorization in Dec 2020 by the FDA.
Moderna Therapeutics	mRNA-1944: Chikungunya virus Infection	mRNA encoding antibodies	Intravenous	Phase I: NCT03829384	3 doses tested (0.1, 0.3, and 0.6 mg kg^{-1}). 3 patients receiving the highest dose showed AEs, including 1 patient showing grade 3 AEs.
BioNTech/Pfizer	BNT162b1: COVID-19 infections	Immunostimulants	Intramuscular	Phase III: NCT04368728	Emergency use authorization in Dec 2020 by the FDA

[a] These clinical trials are described in the text. In the interest of space, additional clinical trials that are not described in the text were omitted from the table.

and 3. In a long-term, open-label extension trial, nedosiran led to long-term reductions in urinary oxalate levels to the normal range found in healthy patients. Dicerna reported multiple AEs but only two serious AEs, which were unrelated to the therapy and a common disease-related occurrence.[41] In collaboration with Roche, Dicerna is also conducting a phase 1 clinical trial for the treatment of noncirrhotic chronic hepatitis B infection. The lead candidate, RG6346, targets hepatitis B mRNA for long-term viral clearance. Dicerna is also recruiting patients for a phase 1 trial for the treatment of alpha-1 antitrypsin deficiency using DCR-A1AT, a GalNAc-siRNA conjugate therapy.

Arrowhead Pharmaceuticals is currently undergoing a phase 2 clinical trial with Takeda for the treatment of alpha-1 antitrypsin-associated liver disease after a preliminary phase 1 trial showed robust reduction in serum alpha-1 antitrypsin (AAT) levels following injections of ARO-AAT, their lead candidate.[40] Interim phase 1I results show >95% reduction in hepatic AAT levels as well as improvements in other secondary outcomes measured.[44] Arrowhead is also undergoing phase 1I trials for the treatment of hepatitis B and cardiovascular disease, the lead candidates of which are licensed to Janssen and Amgen, respectively. In addition, Arrowhead has multiple phase 1 clinical trials near completion for hypertriglyceridemia, dyslipidemia, and liver disease, as well as two ongoing phase 1 clinical trials for cystic fibrosis and renal cell carcinoma. Recent phase 1/II clinical data for cardiovascular disease candidate ARO-APOC3, which targets apolipoprotein C-III, shows reductions in APOC3 levels and secondary reductions in triglycerides, low-density lipoprotein cholesterol, and apolipoprotein B, and an increase in high-density lipoprotein cholesterol. These lipid profile changes are consistent with requirements for combating cardiovascular disease. Similarly, phase 1/II data for ARO-ANG3, which targets angiopoietin-like protein 3 in order to treat dyslipidemia, has shown similar changes in patient lipid profiles after dosing once every four to six months. Silencing of angiopoietin-like protein 3 lowers serum LDL and triglyceride levels—making ARO-ANG3 a good candidate for cardiovascular disease as well.[42]

Finally, Silence Therapeutics has initiated or is in the process of initiating phase 1 clinical trials for cardiovascular disease (lead candidate SLN360), ß-thalassemia (lead candidate SLN124), and myelodysplastic syndrome (lead candidate SLN124). SLN360 is an siRNA-based therapeutic that targets lipoprotein(a), a protein whose levels are high in patients with cardiovascular disease, and Silence Therapeutics has stated they will begin dosing healthy volunteers with SLN360 by the end of 2020. Meanwhile, a double-blind, placebo-controlled phase 1 trial has been initiated for the GalNAc-siRNA conjugate candidate SLN124, and the FDA has granted SLN124 orphan drug designation.[43] These clinical datasets have given scientists a chance to thoroughly evaluate the safety of GalNAc-siRNA conjugates. In many of the ongoing clinical trials mentioned above, mild and severe AEs were reported. However, these AEs were similar to AEs in the placebo groups, indicating that they may be unrelated to the treatment itself. Given that many hundreds of patients have been treated with GalNAc-siRNA drugs, and that GalNAc-siRNA conjugates have been consistently safe, it is likely that GalNAc-siRNA conjugates will be used to treat even more hepatic diseases in the future.

3. LNPs Have Delivered mRNA Encoding SARS-CoV-2 Antigens in Humans

Partially driven by the evidence that LNPs can be approved for human therapies, these nanoparticles have recently been used to deliver mRNA encoding SARS-CoV-2 antigens in order to develop

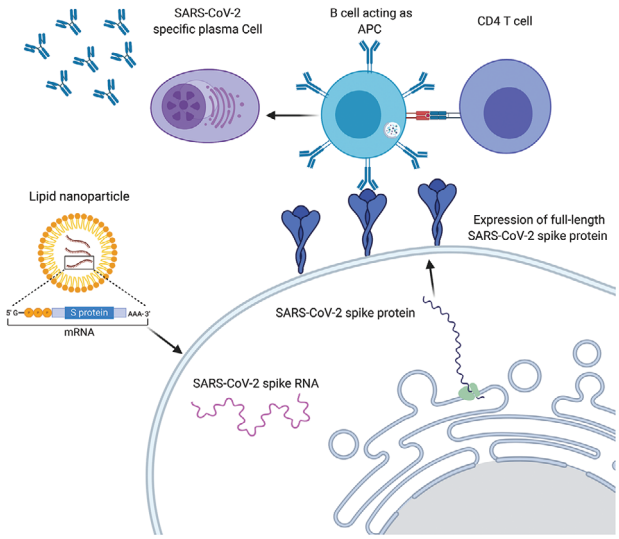

Figure 4. 20–250 µg of the formulated LNP is administered using intramuscular injection. Upon delivery of the mRNA, full-length SARS-CoV-2 spike proteins are produced via translation and translocated onto the cell. B cells with receptors that recognize the SARS-CoV-2 spike protein are stimulated and become partially activated. Following T cell activation, these specific B cells expand and differentiate into plasma cells releasing copious amounts of antibody targeting SARS-CoV-2 virions.

COVID-19 vaccines. Both the Moderna mRNA-1273 and BioNTech/Pfizer BNT162b1 vaccines have generated promising phase 1 and phase 2 readouts, and are now progressing through 30,000-person phase 3 clinical trials in order to determine whether these drugs can be rapidly approved for eventual distribution to hundreds of millions or even billions of people.[45] Although there are important differences between the vaccines, described below, both vaccines have the overarching goal of training the immune system to recognize and neutralize specific components of the SARS-CoV-2 virus. These LNP-based SARS-CoV-2 vaccines contain RNA for the glycosylated spike proteins found on the surface of all coronaviruses. These exterior regions of the virus are accessible to neutralizing antibodies and are used for both adsorption and entry of the virion.[46] Using convalescent patient sera and B cell receptor sequencing, the region found to be the most effective at developing broadly neutralizing antibodies is the S2 region, in particular the HR1 domain.[47] Because the S2 region is primarily used for virion-cell fusion, it is likely that neutralizing antibodies prevent new fusion events as well as canonical agglutination and opsonization.[48]

Using the LNPs loaded with HR1-RNA allows for expression of the SARS-CoV-2 protein in host cells. The expression of a non-human protein results in a T cell response though HLA-ABC processing of the HR1 peptides. This T cell response allows for full activation of the humoral immune response, in particular activation of B cells and further differentiation into antibody-producing plasma cells.[49] In addition, both vaccines require two shots (the first shot termed a "prime" and the second shot termed a "boost") three to four weeks apart. Using this prime-boost approach, these vaccines may generate long-lived immunity against SARS-CoV-2 (**Figure 4**). This prime-and-boost approach has been utilized in other vaccine candidates, in particular subunit vaccines such as the hepatitis B or human papilloma virus vaccines.[50] By developing this strong humoral and cell-mediated memory response, it is thought long-lived immunity to SARS-CoV-2 will develop as long as significant mutations in HR1 do not arise.

The Moderna LNP formulation likely consists of a proprietary ionizable lipid (SM-102) along with cholesterol, PEG2000 DMG, and DSPC at a 50:38.5:1.5:10 ratio, respectively.[29] The components that likely make up this LNP are commonly used in the field of drug delivery. Cholesterol, DSPC, and PEG2000 have been used in previous preclinical studies as well as approved FDA delivery vehicles.[51] In an early example, researchers systemically delivered siRNA to the liver in nonhuman primates (NHPs) using an LNP.[52] This LNP, which had a hydrodynamic diameter between 70 and 90 nm and led to protein knockdown at a dose of 1.0 mg kg^{-1}, contained the lipid DLinDMA, cholesterol, PEG-C-DMA, and DSPC, combined at a 40:48:2:10 molar ratio.[52] Future studies screened ionizable lipids in vitro in order to understand the impact of the lipomer–RNA ratio, PEG chain length, and particle size on an LNP's pharmacokinetics and biodistribution.[53] In one example, the lipid C12-200 delivered siRNA to the liver in mice and NHPs at low doses and was formulated with cholesterol, PEG2000 DMG, and DSPC at a formulation ratio of 50:38.5:1.5:10.[54] In a second example, researchers first formulated the lipid MC3 with cholesterol, PEG2000, and DSPC at a ratio of 40:40:10:10. They then optimized this formulation ratio for siRNA-mediated silencing in NHPs such that the lipid was formulated with cholesterol, PEG-C-DMA, and DPPC at a ratio of 57.1:34.3:1.4:1.7.[55] Finally, as a third example, authors identified the lipid cKK-E12 as a potent material for siRNA and mRNA delivery,[56,57] typically formulating this LNP with cholesterol, PEG2000 DMG, and DSPC at a ratio of 50:38.5:1.5:10 for siRNA delivery and with cholesterol, PEG2000, and DOPE at a ratio of 35:46.5:2.5:16 for mRNA delivery. Thus, the molar ratio that may be used by Moderna has been reported to work well with other commonly used ionizable lipids such as C12-200, cKK-E12, and MC3,[54,56,58] making it a valid choice as a vaccine candidate.

Studies have also highlighted the importance and impact of PEG structure as well as PEG molar ratios on LNP delivery.[59] Scientists have shown that PEG can reduce interactions with opsonizing proteins and subsequent immune cells by creating a hydrophilic barrier around the LNP.[60] However, it has also been shown that too much PEG in an LNP can inhibit the adsorption of protein coronas that promote delivery to target cells.[61,62] Additionally, PEG has been used as a conjugate to improve the delivery of peptides, antibodies, and siRNAs.[62,63] Alternatives to PEG such as PLGA have also been explored for siRNA conjugates.[64]

The Moderna LNP was selected based on data from an LNP vaccine candidate containing Zika mRNA,[65] highlighting the true platform nature of this technology; Zika mRNA was quickly replaced by the CoV-2 mRNA, creating a potential new therapeutic candidate in a matter of weeks. The Moderna LNP formulation carries mRNA encoding the S2 region of the SARS-Cov-2 spike protein. Notably, the sequence was modified with two proline substitutions at the top of the central helix in order to stabilize the S protein in a prefusion conformation, thereby increasing immunogenicity.[66] Upon successful mRNA delivery, full-length SARS-CoV-2 spike proteins are produced via translation and are translocated onto the cell surface. As of the publication of this review, Moderna has completed phase 1 and 2 clinical trials and

reported successful topline results from a phase 3. The phase 1 open-label clinical trial was conducted to determine overall safety as well as the proper dose in 120 adult participants. Four doses were chosen based on nonhuman primate data: 10, 25, 100, and 250 µg. LNPs were administered in a 0.5 cc dose via an intramuscular (IM) injection at day 1 followed by a booster on day 29. Participants will be followed for 12 months after the first injection with visits occurring 1, 2, and 4 weeks post first vaccination and 3, 6, and 12 months post second vaccination. The majority of adverse events were mild and most frequently included fatigue, chills, headache, myalgia, and injection site pain with most serious AEs associated with the second booster at the highest dose (250 µg). The mean S-2P antibody titer was 7–10 times higher in patients who received the medium dose (100 µg) as compared to the lowest dose (25 µg), indicating that 100 µg would be the best candidate for further clinical trials. A significant CD4 T cell cytokine response also developed, indicating full immune activation and subsequent immunological memory. The phase 1 clinical trial showed that the 100 µg dose, administered twice, would be an effective and safe dose for the phase 2 clinical trial.[67] These encouraging results showed that, for the first time, an LNP could be used to deliver a vaccine payload with a significant immunological response.

The phase 2 placebo-controlled clinical trial was fully enrolled in early July and is scheduled to be fully completed in March 2021. In this study, 300 adult participants (split into two groups ages 18–54 and 55+) were randomized and administered either 50 µg, 100 µg, or a placebo (PBS). The objectives of this clinical trial are to determine effectiveness at developing an S-2P antibody response as compared to the placebo control as well as determining if there are any additional adverse events that were missed during the phase 1 clinical trial. The phase 3 clinical trial is now completely enrolled with 30 000 adult participants. The 100 µg dose was chosen based on its ability to develop an increased S-2P titer with mild adverse events. This randomized, double-blind study will be used to determine how protective the S-2P antibody response is in preventing infection with SARS-CoV-2; in late 2020, Moderna reported topline results from the phase 3 clinical trial suggesting that the vaccine reduced infection rates by 94.5% compared with placebo.

The BioNTech LNP formulation was not developed by BioNTech scientists, but rather was in-licensed from Acuitas, a company that focuses on LNP design. Similar to previously reported LNP formulations, the LNP consists of four components: a proprietary lipid, a cholesterol, a PEG-lipid, and a helper lipid, and may be similar to previously reported LNP formulations used by Acuitas collaborators for IM delivery of mRNA.[68] The overall mechanism of immune activation and subsequent protection from SARS-CoV-2 is similar to the Moderna vaccine apart from the region of the SARS-CoV-2 spike protein encoded for. Two candidates were developed for use as a SARS-CoV-2 vaccine: BNT162b1, which encodes a secreted trimerized SARS-CoV-2 receptor-binding domain, and BNT162b2, which encodes a membrane-anchored SARS-CoV-2 full-length spike stabilized in a perfusion conformation. The BNT162b1 vaccine candidate was developed in hopes that an antibody response to the receptor-binding domain of SARS-CoV-2 would prevent de novo virion binding to cellular receptors, thus eliciting greater protection. The BNT162b2 candidate was developed to produce a wide gamut of SARS-CoV-2 antibodies, allowing for a strong humoral immune response similar to what is developed during active infection.[69]

Both candidates were evaluated in the phase 1 clinical trial using two IM injections administered three weeks apart with either 10, 20, 30, or 100 µg. The purpose of this phase 1 clinical trial was to determine which vaccine candidate was safe and effective and at what dose. A total of 195 adult participants were randomized and split into two groups, ages 18–55 and 65–85. Both candidates resulted in significant production of SARS-CoV-2 antibodies, similar to or greater than the titers seen in convalescent patients. Midway through the trial it was determined that BNT162b1 resulted in more serious systemic adverse events in older adults, most likely because of the secretion of the SARS-CoV-2 SP-1 protein into the bloodstream. Following this result BNT162b2 was determined to be the best candidate and was advanced into the phase 2/3 clinical trials.[70] Currently, the phase 2/3 clinical trial is recruiting participants for a large-scale double-blind efficacy trial with initial results showing a SARS-CoV-2 prevention efficacy of 90%, and full-scale results should be available by mid-2021. The efficacy and safety profiles of the Moderna and BioNTech vaccines suggest that mRNA vaccines delivered by LNPs may be effective weapons against COVID. Both of these vaccine candidates show strong efficacy rates that are on par with or better than some of the best vaccines we have available, such as MMR (97%-88%), HPV (88%), and HBV (80–90%).[50,71]

4. Preclinical RNA Delivery to the Lung, Spleen, and Immune System Is Emerging

The clinical results described above demonstrate that RNA therapies can benefit patients, and that effective drug delivery is required for clinical results. Given this, academic and industry labs have allocated significant resources to improving drug delivery. For example, from 2006 to 2014, the dose required to deliver siRNA to hepatocytes decreased more than 10,000-fold.[52,56] These advances, which led to clinically relevant hepatocyte delivery,[31] were enabled by two key facts. The first is well-known: unique physiology and structure make the liver easier to target with drug delivery systems than most, but not all, other organs. The liver exhibits discontinuous basement membranes in hepatic sinusoids, which makes hepatocytes and other hepatic cell types physically accessible from the bloodstream.[72] This discontinuous basement membrane results in 7.5-fold more nanomaterial interactions with hepatic cells, compared to peripheral cells.[12] Similarly, slow blood flow in liver sinusoids increases nanoparticle resonance time within the liver sinusoids, thereby increasing the odds that nanoparticles interact with, and therefore are taken up by, cells within the liver microenvironment.[73] The second fact is that the nanomedicine field has a simple, inexpensive way to quantify siRNA delivery to hepatocytes. More specifically, a kit quantifying factor VII (FVII) serum protein is regularly used to quantify how well nanoparticles deliver siRNA targeting FVII. Since FVII is only expressed by hepatocytes, the hepatocyte-targeting efficiency of an LNP can be easily determined by quantifying FVII levels in serum. Critically, this assay quantifies how well the siRNA functioned inside the cell (i.e., the siRNA-mediated silencing of FVII protein), rather than quantifying just biodistribution (i.e., where the nanoparticle went). FVII

assays have been used to study hundreds of chemically distinct nanoparticles in vivo, leading to the discovery of an LNP that delivered siRNA to mice at a dose of 0.01 mg kg^{-1}, and subsequently patisiran.[74]

Historically, it has been more challenging to evaluate cell type-specific delivery to other cell types in vivo. This may partially explain why other cells and tissues that share important physiological traits that promote delivery to the liver have not had the same degree of clinical success to date. For example, endothelial cells are physically accessible to nanoparticles injected into the blood; the bone marrow contains discontinuous endothelium, which could promote nanoparticle extravasation from the blood; and the spleen has a specialized structure that likely dramatically increases nanoparticle resonance time within the organ. Yet a growing number of publications have shown that effective non-liver delivery is possible in mice and nonhuman primates. In one example, screening epoxide-modified lipid-polymer hybrids at different lipid:RNA ratios in vitro resulted in the identification of an LNP containing a new lipid-bas_{ed} compound, termed 7C1, that potently delivers siRNA to heart ($ED_{50} \approx 0.05$ mg kg^{-1}) and lung ($ED_{50} \approx 0.02$ mg kg^{-1}) endothelial cells in mice after a single systemic injection.[75] This same LNP was validated in NHPs, where researchers saw up to 80% protein silencing in lung endothelial cells after a 1 mg kg^{-1} systemic administration.[76] Other researchers have delivered RNA to the lung by altering the phospholipid molar ratio. Increasing the phospholipid molar ratio of LNPs formulated with the hepatocyte-targeting ionizable lipid cKK-E12 led to potent and specific mRNA delivery to the lungs with minimal off-target delivery detected in the liver and spleen.[77] More recently, selective organ targeting (SORT) was used to deliver mRNA to nonliver tissues by including a supplemental SORT lipid.[78] Researchers used SORT to demonstrate that increasing the molar ratio of charged phospholipids in LNP formulations shifted LNP tropism away from the liver. Specifically, the use of negatively charged SORT molecules (e.g., 18PA) shifted delivery to the spleen, while positively charged SORT molecules (e.g., DOTAP) shifted delivery to the lungs. Finally, it was also demonstrated that changing the overall charge of an LNP, by varying the molar ratio of DOPE or DOTMA, resulted in a shift of delivery toward the lungs (when cationic) or the spleen (when neutral).[79]

RNA has also been delivered to the lung by locally administering nanoparticles via intranasal or nebulized administrations in order to tackle delivery in disease models of cancer and cystic fibrosis.[80–82] In one example, authors found that an LNP previously optimized for liver delivery could deliver mRNA encoding cystic fibrosis transmembrane conductance regulator (CFTR) to epithelial cells after intranasal administration in $CFTR^{-/-}$ mouse models of cystic fibrosis.[81] In another, authors screened a series of functional polyester nanoparticles and identified nanoparticles that resulted in preferential RNA delivery to lung cancer cells compared to normal cells.[82] Similarly, Translate Bio, an mRNA company, has evaluated inhalation-based mRNA drugs for the treatment of cystic fibrosis. In a phase 1 clinical study, CF patients showed improvement after they were treated with MRT5005, an mRNA drug contained within a nanoparticle that encoded for CFTR. Patients were given one of three mRNA doses (8, 16, or 24 mg) or a placebo control. Of the 12 patients in the study, seven were already taking an FDA-approved CFTR modulator. All patients in the medium- and high-dose groups saw significant improvement in percent predicted forced expiratory volume in one second (ppFEV1), one of the primary measures of lung function. There were no severe AEs but multiple mild treatment-emergent AEs reported in each treatment group, including the placebo; despite this, patient symptoms tended to resolve after 24 h.[83]

Nanoparticles have also been used to target the spleen as well as immune cells.[84–86] Researchers found that biodegradable variants of the potent ionizable lipid cKK-E12 led to splenic immune cell delivery, albeit with off-target delivery to the liver.[84] Moreover, a targeting platform known as Anchored Secondary scFv Enabling Targeting (ASSET) was developed to coat LNPs with monoclonal antibodies in order to facilitate cell targeting.[87] One key aspect to ASSET was the ability to easily create antibody-lipid conjugates that could be anchored into the hydrophobic regions of LNPs, and the ability for ASSET to direct liver-trophic LNPs to other cell types was exploited for applications in inflammatory bowel disease.[85] LNPs with novel ionizable lipids have also been designed to deliver nucleic acids to leukocytes, a traditionally difficult-to-transfect cell type.[88] Authors formulated siRNA-based LNPs with novel linker moieties including hydrazine, hydroxylamine, and ethanolamine. Specifically, 14 different lipid structures were formulated to contain cholesterol, DSPC, and PEG-lipid at the fixed molar ratio 50:38.5:10:1.5 and then screened in vitro. Authors then formulated the most potent in vitro LNPs and modified them so that they contained a surface-based anti-β_7-mAb or an isotype control and confirmed their potency in vivo.[88] LNPs surface modified with the anti-β_7-mAb carrying siRNA against CD45 achieved ≈30–50% silencing of the CD45 protein in CD4+ and CD8+ cells in the spleen and lymph nodes. In another example of delivering mRNA to immune cells, researchers demonstrated that LNPs containing vitamin C could be used to deliver mRNA to macrophages in vitro for the treatment of multidrug-resistant bacterial sepsis.[89] Vitamin C was included so that LNPs specifically accumulated in macrophage lysosomes, where they released an antimicrobial peptide and cathepsin B. Adoptive transfer of these macrophages into an immunocompromised mouse model of sepsis led to the elimination of multidrug-resistant bacteria and complete animal recovery.[89] Delivery of mRNA to Kupffer cells, specialized macrophages in the liver microenvironment, has also been achieved in vivo without the use of targeting ligands. More specifically, an unbiased high-throughput in vivo DNA barcoding was used to identify LNPs that preferentially deliver mRNA to Kupffer cells, relative to other cell types in the liver.[24,90]

5. Identifying Nanoparticles with Tropism to New Cells Using High-Throughput In Vivo Assays

These studies suggest that clinical RNA delivery to the lung, spleen, or immune system may emerge. However, delivery to the heart, brain, kidney, pancreas, muscle, and other tissues may be more difficult and may require testing increasingly diverse nanoparticle chemistries. Even if nanoparticle size, shape, and charge are ignored, it is possible to envision 10^{10} or more chemically distinct nanoparticles[91] created using standard synthetic routes like Michael addition-, epoxide-, peptide-, esterification-, and thiol-based chemistries.[15,80,92] This leads to a key question:

what is the best way to evaluate a very large nanoparticle chemical space? In this instance, an LNP chemical space refers to all of the possible chemical compounds that can be used to formulate an LNP. One potential answer is to markedly improve the ease with which delivery is measured in vivo by creating an assay that is i) high throughput (i.e., >100 nanoparticles at once), ii) quantifies functional (i.e., mRNA being translated into active protein, or siRNA silencing protein) delivery, and iii) does so directly in vivo, to any desired cell type. The value of a high-throughput in vivo screen is supported by recent evidence that in vitro nanoparticle delivery does not predict systemic in vivo nanoparticle delivery.[93–95]

To address the need for more efficient discovery of potent LNPs, new assays that enable multiplexed evaluation of nanoparticle delivery in animals have been developed. These systems use a unique molecular tag to individually tag nanoparticles; the nanoparticles are then pooled together and administered.[93,95,96] After waiting for drug delivery to occur, the signals are detected and the delivery efficiency of each individual nanoparticle is deconvoluted using its unique molecular tag. Several molecules can serve as multiplexed molecular tags, including peptides and fluorescent nanodots.[96,97] In one example, Negron et al. used nanoparticles containing ZsGreen plasmids, labeled with Cy5, to monitor delivery in spheroid cultures of a glioma cell line. Using this approach, the authors identified nanoparticles that worked successfully in vivo.[95] In a second example, the Lindfors group used radiolabeled mRNA-containing nanoparticles of different sizes to determine their efficacy at delivering and releasing mRNA in an iPSC-derived liver model.[98]

One alternative to these molecular tags is the use of DNA. DNA-based tags, often referred to as DNA barcodes, are inherently stable, can be modified chemically to increase their drug-like properties,[99] and are similar to the nucleic acid drugs typically formulated into nanoparticles. Moreover, DNA barcodes allow for many nanoparticles to be easily tagged at once, since the number of combinations with a DNA sequence N nucleotides long is 4^N. For example, an eight-nucleotide barcode can be used to distinguish up to 65 536 distinct nanoparticles, far more nanoparticles than can be made in a single experiment at this time. In addition, DNA barcodes provide a sensitive, robust, and specific signal—they can easily be amplified using PCR, and then sequenced using next-generation sequencing (NGS), an approach that is affordable and fast. For example, a sequencer the size of a desktop computer can now be purchased for less than $50 000 and can generate over 20 million datapoints per experiment within a few hours.

Currently, predominant nucleic acid barcoding technologies utilize a small DNA oligo. In one early system, each barcode was a DNA oligo that was differentiated by either its size or unique PCR primer sites found on each end of the barcode; successful drug delivery was analyzed and quantified using gel electrophoresis and qPCR readouts.[100] Using this system, chemotherapies were loaded into an LNP library and tested in an in vivo tumor model using a single administration. Researchers isolated dead cells from tumor tissue and amplified the DNA barcodes to determine which chemotherapies were effective. By contrast, scientists developed a distinct DNA barcoding strategy that relies on an NGS readout. This system uses a DNA oligo composed of a universal primer region, a unique molecular identifier (UMI), and a carefully designed 8 nt barcode region.[101] More specifically, each barcode should be unique at three of the eight barcode nucleotides, to allow for enough diversity to reduce sequencing errors. The DNA barcode is then incorporated at a 1:10 ratio with functional RNAs into an LNP (**Figure 5**A). Using DNA barcodes in combination with NGS allows for hundreds of LNPs to be administered to a single animal, resulting in a robust and efficient high-throughput screening system (Figure 5B). This methodology has successfully been used to develop high-throughput in vivo screening systems for both siRNA and mRNA delivery. Furthermore, incorporation of a payload (e.g., mRNA, siRNA) and DNA barcode allows for quantification of both functional delivery and biodistribution (Figure 5C). For example, we utilized Cre mRNA in a Lox-Stop-Lox-CAG-tdTomato mouse model, which only expresses tdTomato when Cre protein is expressed and translocates to the nucleus to excise the stop signal.[14] More specifically, an LNP containing Cre mRNA must be taken up by the target cell, released from the endosome, and translated for a tdTomato signal to be detected. Because this results in a genotypic change within the cell, the signal is long-lived, allowing us to expand our timepoints for in-depth biochemical analyses. Using fluorescence-activated cell sorting (FACS), tdTomato-expressing cells are sorted from multiple organ systems. Sorting on the tdTomato signal makes it easy to distinguish between cells with LNPs that are bound to the surface of the cell or stuck in endosomes (tdTomato negative) as compared to LNPs that have been fully processed and released into the cytoplasm (tdTomato positive) (Figure 5C). The ability to distinguish between biodistribution and functional delivery is key in any nanoparticle-screening assay since the majority of nanoparticles are not released from endosomes.[20,102] Similarly, being able to determine which LNPs are functionally delivered increases the possibility of finding correlations between LNP structures created by novel biomaterials and their potential impact on endosomal escape. More recently, a new mRNA barcoding system was reported.[103] In this system, luciferase mRNA is synthesized in vitro so that it contains unique barcode sequences downstream of the T7 promoter as well as the same universal primer sites used in previous DNA barcoding papers.[104]

6. Conclusions and Future Perspectives

Emerging datasets demonstrate that RNA targeted to cells via manufacturable, safe, and effective delivery systems can treat disease in humans. This clinical success underscores the need for at least two significant efforts in the field. The first effort is to improve our understanding of the genes that influence the safety and efficacy of drug delivery systems in vivo. For example, it remains unclear whether subsets of a given immune cell type (e.g., subsets of Kupffer cells) respond to drug delivery systems the same way. Given that cell subsets have been shown to influence immune response to other stimuli,[105] it is conceivable that a small subset of immune cells act as "super responders" that drive systemic toxicity. Similarly, mRNA-based vaccines may be improved upon in the coming years by identifying the subsets of immune cells that, when delivered to, lead to the safest, yet most durable immune response.

A second near-term focus of the drug delivery field could center on improving the efficiency with which drug delivery systems

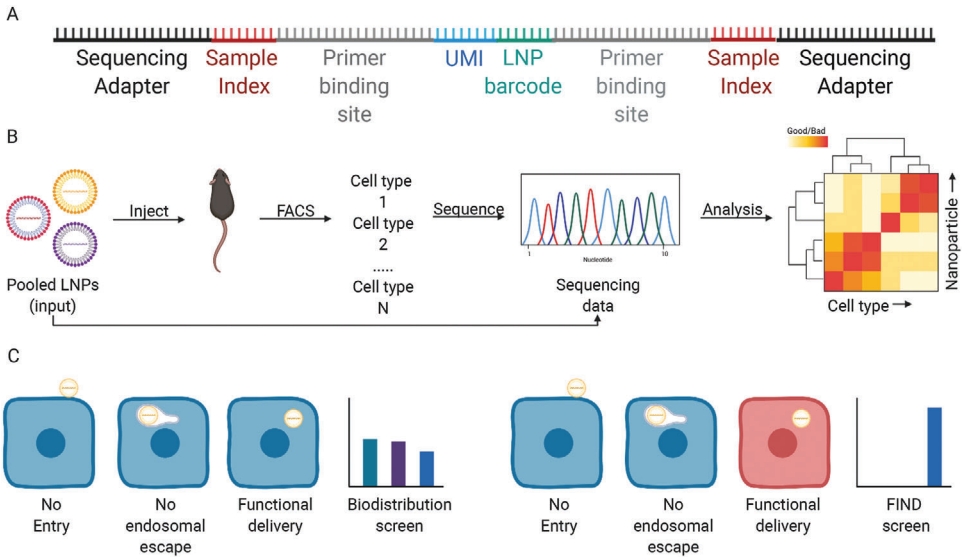

Figure 5. High-throughput LNP barcoding can be used to find potent delivery vehicles. A) DNA barcodes contain universal primer sites, a 7 nt randomized region, and an 8 nt barcode region, allowing us to generate hundreds of distinct DNA barcodes. B) Joint Rapid DNA Analysis of Nanoparticles (JORDAN) uses DNA barcodes and NGS to analyze the biodistribution of thousands of particles in vivo. C) Screening LNPs using JORDAN does not differentiate between LNPs at the cell surface, LNPs trapped in an endosomal compartment, and LNPs that facilitate functional delivery (i.e., mRNA translated into functional protein). Fast Identification of Nanoparticle Delivery (FIND) can be used in order to test specifically for LNP functional delivery. FIND relies on the identification of cells that undergo Cre-mediated gene editing and, therefore, only LNPs that have been functionally delivered.

are discovered. Historically, nanoparticles have been screened in cell culture, in large part because synthesizing thousands of nanoparticles was feasible but studying them in vivo was not. The ability to quantify delivery to new tissues via high-throughput multiplexed experiments now makes it possible to study thousands of drug delivery systems directly in vivo.[14,16,93] By testing hundreds of nanoparticles at once in vivo, scientists have been able to find LNPs that functionally deliver to novel cell types. This development is important for two reasons. First, it allows researchers to analyze and understand LNP on-target functional delivery for any combination of desired cell types that can be isolated using standard techniques such as FACS. Specifically, functional delivery of an siRNA-based delivery vehicle is defined as delivery that leads to protein silencing, whereas functional delivery of an mRNA-based vehicle is defined as delivery that leads to production of a new, functional protein. Second, LNP on-target functional delivery can be studied alongside LNP biodistribution, thereby allowing scientists to identify cells where LNPs enter the cell but do not lead to functional payload delivery. This is especially important when considering delivery to off-target cells such as phagocytic Kupffer cells. Thus, understanding the on-target to off-target ratio of a drug delivery vehicle is crucial when developing therapeutics.

A second critical design criterion for successful delivery vehicles is their tolerability and efficacy in large-animal models such as NHPs. cKK-E12, currently licensed for clinical development, has been shown to have an ED_{50} as low as 0.002 mg kg^{-1} for the delivery of siRNA in mice.[56] A lower ED_{50} leads to a higher therapeutic index, a quantitative measurement that describes the relative safety of a drug. However, this therapeutic index typically decreases as a therapeutic is tested in larger-animal models, in part because the dose required to achieve the same potency goes down and the immune response in a large animal tends to be more severe than in mice.[106,107] Specifically, it may be that LNPs typically well tolerated in mice are not well tolerated in NHPs. As an example, early studies that attempted to translate DNA vaccines from mice to NHPs showed that immunogenicity in a small-animal model did not necessarily translate to a large-animal model, leading to decreased vaccine efficacy.[108] Therefore, the way an LNP behaves in terms of functional delivery and immune response in a small-animal model, such as a mouse, might be very different from how it functionally delivers in a larger-animal model such as an NHP or in a clinical trial. Given this potential disparity, it may be necessary to identify novel methods to test LNPs that are more indicative of the cellular and physiological environment present in an NHP or a human. It is unknown whether drug delivery in smaller animals predicts drug delivery in NHPs, which are considered the gold standard in preclinical assays. We find it likely that species-to-species predictivity will vary with cell type. Although additional work is needed, if the drug delivery field is able to understand the genes that influence delivery and streamline the preclinical selection of drug delivery systems, we find it likely that RNA therapies may be applied to a growing number of diseases.

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Conflict of Interest

J.E.D. is a co-founder of Guide Therapeutics.

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[1] O. Shalem, N. E. Sanjana, E. Hartenian, X. Shi, D. A. Scott, T. S. Mikkelsen, D. Heckl, B. L. Ebert, D. E. Root, J. G. Doench, F. Zhang, *Science* **2014**, *343*, 84.
[2] J. Joung, J. M. Engreitz, S. Konermann, O. O. Abudayyeh, V. K. Verdine, F. Aguet, J. S. Gootenberg, N. E. Sanjana, J. B. Wright, C. P. Fulco, Y. Y. Tseng, C. H. Yoon, J. S. Boehm, E. S. Lander, F. Zhang, *Nature* **2017**, *548*, 343.
[3] U. Sahin, K. Kariko, O. Tureci, *Nat. Rev. Drug Discovery* **2014**, *13*, 759.
[4] N. Pardi, M. J. Hogan, F. W. Porter, D. Weissman, *Nat. Rev. Drug Discovery* **2018**, *17*, 261.
[5] A. V. Anzalone, L. W. Koblan, D. R. Liu, *Nat. Biotechnol.* **2020**, *38*, 824.
[6] A. V. Wright, J. K. Nuñez, J. A. Doudna, *Cell* **2016**, *164*, 29.
[7] A. A. Levin, *N. Engl. J. Med.* **2019**, *380*, 57.
[8] a) E. Blanco, H. Shen, M. Ferrari, *Nat. Biotechnol.* **2015**, *33*, 941; b) C. Wan, T. M. Allen, P. R. Cullis, *Drug Delivery Transl. Res.* **2014**, *4*, 74.
[9] S. Wilhelm, A. J. Tavares, Q. Dai, S. Ohta, J. Audet, H. F. Dvorak, W. C. W. Chan, *Nat. Rev. Mater.* **2016**, *1*, 16014.
[10] B. Ouyang, W. Poon, Y. N. Zhang, Z. P. Lin, B. R. Kingston, A. J. Tavares, Y. Zhang, J. Chen, M. S. Valic, A. M. Syed, P. MacMillan, J. Couture-Senécal, G. Zheng, W. C. W. Chan, *Nat. Mater.* **2020**, 1362.
[11] B. Ouyang, W. Poon, Y. N. Zhang, Z. P. Lin, B. R. Kingston, A. J. Tavares, Y. Zhang, J. Chen, M. S. Valic, A. M. Syed, P. MacMillan, J. Couture-Senécal, G. Zheng, W. C. W. Chan, *Nat. Mater.* **2020**, *19*, 1362.
[12] a) K. M. Tsoi, S. A. MacParland, X. Z. Ma, V. N. Spetzler, J. Echeverri, B. Ouyang, S. M. Fadel, E. A. Sykes, N. Goldaracena, J. M. Kaths, J. B. Conneely, B. A. Alman, M. Selzner, M. A. Ostrowski, O. A. Adeyi, A. Zilman, I. D. McGilvray, W. C. Chan, *Nat. Mater.* **2016**, *15*, 1212; b) A. J. Tavares, W. Poon, Y. N. Zhang, Q. Dai, R. Besla, D. Ding, B. Ouyang, A. Li, J. Chen, G. Zheng, C. Robbins, W. C. W. Chan, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E10871.
[13] a) J. E. Dahlman, C. Barnes, O. Khan, A. Thiriot, S. Jhunjunwala, T. E. Shaw, Y. Xing, H. B. Sager, G. Sahay, L. Speciner, A. Bader, R. L. Bogorad, H. Yin, T. Racie, Y. Dong, S. Jiang, D. Seedorf, A. Dave, K. S. Sandu, M. J. Webber, T. Novobrantseva, V. M. Ruda, A. K. R. Lytton-Jean, C. G. Levins, B. Kalish, D. K. Mudge, M. Perez, L. Abezgauz, P. Dutta, L. Smith, K. Charisse, M. W. Kieran, K. Fitzgerald, M. Nahrendorf, D. Danino, R. M. Tuder, U. H. von Andrian, A. Akinc, A. Schroeder, D. Panigrahy, V. Kotelianski, R. Langer, D. G. Anderson, *Nat. Nanotechnol.* **2014**, *9*, 648; b) O. S. Fenton, K. J. Kauffman, J. C. Kaczmarek, R. L. McClellan, S. Jhunjhunwala, M. W. Tibbitt, M. D. Zeng, E. A. Appel, J. R. Dorkin, F. F. Mir, J. H. Yang, M. A. Oberli, M. W. Heartlein, F. DeRosa, R. Langer, D. G. Anderson, *Adv. Mater.* **2017**, *29*, 1606944.
[14] C. D. Sago, M. P. Lokugamage, K. Paunovska, D. A. Vanover, C. M. Monaco, N. N. Shah, M. Gamboa Castro, S. E. Anderson, T. G. Rudoltz, G. N. Lando, P. Munnilal Tiwari, J. L. Kirschman, N. Willett, Y. C. Jang, P. J. Santangelo, A. V. Bryksin, J. E. Dahlman, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E9944.
[15] M. P. Lokugamage, C. D. Sago, Z. Gan, B. R. Krupczak, J. E. Dahlman, *Adv. Mater.* **2019**, *31*, 1902251.
[16] C. D. Sago, M. P. Lokugamage, F. Z. Islam, B. R. Krupczak, M. Sato, J. E. Dahlman, *J. Am. Chem. Soc.* **2018**, *140*, 17095.
[17] N. R. M. Saunders, M. S. Paolini, O. S. Fenton, L. Poul, J. Devalliere, F. Mpambani, A. Darmon, M. Bergère, O. Jibault, M. Germain, R. Langer, *Nano Lett.* **2020**, *20*, 4264.
[18] M. A. Jackson, S. S. Patel, F. Yu, M. A. Cottam, E. B. Glass, E. N. Hoogenboezem, R. B. Fletcher, B. R. Dollinger, P. Patil, D. D. Liu, I. B. Kelly, S. K. Bedingfield, A. R. King, R. E. Miles, A. M. Hasty, T. D. Giorgio, C. L. Duvall, *Biomaterials* **2021**, *268*, 120528.
[19] M. A. Jackson, S. S. Patel, F. Yu, M. A. Cottam, E. B. Glass, E. N. Hoogenboezem, R. B. Fletcher, B. R. Dollinger, P. Patil, D. D. Liu, I. B. Kelly, S. K. Bedingfield, A. R. King, R. E. Miles, A. M. Hasty, T. D. Giorgio, C. L. Duvall, *Biomaterials* **2020**, *268*, 120528.
[20] J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier, M. Zerial, *Nat. Biotechnol.* **2013**, *31*, 638.
[21] S. Patel, N. Ashwanikumar, E. Robinson, A. DuRoss, C. Sun, K. E. Murphy-Benenato, C. Mihai, O. Almarsson, G. Sahay, *Nano Lett.* **2017**, *17*, 5711.
[22] K. Paunovska, A. Da Silva Sanchez, M. T. Foster, D. Loughrey, E. L. Blanchard, F. Z. Islam, Z. Gan, A. Mantalaris, P. J. Santangelo, J. E. Dahlman, *Sci. Adv.* **2020**, *6*, eaba5672.
[23] K. Karikó, H. Muramatsu, F. A. Welsh, J. Ludwig, H. Kato, S. Akira, D. Weissman, *Mol. Ther.* **2008**, *16*, 1833.
[24] M. P. Lokugamage, Z. Gan, C. Zurla, J. Levin, F. Z. Islam, S. Kalathoor, M. Sato, C. D. Sago, P. J. Santangelo, J. E. Dahlman, *Adv. Mater.* **2019**, *32*, 1904905.
[25] S. T. Crooke, T. A. Vickers, X. H. Liang, *Nucleic Acids Res.* **2020**, *48*, 5235.
[26] X. H. Liang, H. Sun, C. W. Hsu, J. G. Nichols, T. A. Vickers, C. L. De Hoyos, S. T. Crooke, *Nucleic Acids Res.* **2020**, *48*, 1372.
[27] W. Shen, C. L. De Hoyos, M. T. Migawa, T. A. Vickers, H. Sun, A. Low, T. A. Bell 3rd, M. Rahdar, S. Mukhopadhyay, C. E. Hart, M. Bell, S. Riney, S. F. Murray, S. Greenlee, R. M. Crooke, X. H. Liang, P. P. Seth, S. T. Crooke, *Nat. Biotechnol.* **2019**, *37*, 640.
[28] a) F. Eckstein, *Nucleic Acid Ther.* **2014**, *24*, 374; b) S. D. Putney, S. J. Benkovic, P. R. Schimmel, *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 7350.
[29] N. Kose, J. M. Fox, G. Sapparapu, R. Bombardi, R. N. Tennekoon, A. D. de Silva, S. M. Elbashir, M. A. Theisen, E. Humphris-Narayanan, G. Ciaramella, S. Himansu, M. S. Diamond, J. E. Crowe Jr., *Sci. Immunol.* **2019**, *4*, eaaw6647.
[30] D. Adams, A. Gonzalez-Duarte, W. D. O'Riordan, C. C. Yang, M. Ueda, A. V. Kristen, I. Tournev, H. H. Schmidt, T. Coelho, J. L. Berk, K. P. Lin, G. Vita, S. Attarian, V. Planté-Bordeneuve, M. M. Mezei, J. M. Campistol, J. Buades, T. H. Brannagan 3rd, B. J. Kim, J. Oh, Y. Parman, Y. Sekijima, P. N. Hawkins, S. D. Solomon, M. Polydefkis, P. J. Dyck, P. J. Gandhi, S. Goyal, J. Chen, A. L. Strahs, S. V. Nochur, M. T. Sweetser, P. P. Garg, A. K. Vaishnaw, J. A. Gollob, O. B. Suhr, *N. Engl. J. Med.* **2018**, *379*, 11.
[31] A. Akinc, M. A. Maier, M. Manoharan, K. Fitzgerald, M. Jayaraman, S. Barros, S. Ansell, X. Du, M. J. Hope, T. D. Madden, B. L. Mui, S. C. Semple, Y. K. Tam, M. Ciufolini, D. Witzigmann, J. A. Kulkarni, R. van der Meel, P. R. Cullis, *Nat. Nanotechnol.* **2019**, *14*, 1084.
[32] M. Balwani, E. Sardh, P. Ventura, P. A. Peiró, D. C. Rees, U. Stölzel, D. M. Bissell, H. L. Bonkovsky, J. Windyga, K. E. Anderson, C. Parker, S. M. Silver, S. B. Keel, J. D. Wang, P. E. Stein, P. Harper, D. Vassiliou, B. Wang, J. Phillips, A. Ivanova, J. G. Langendonk, R. Kauppinen, E. Minder, Y. Horie, C. Penz, J. Chen, S. Liu, J. J. Ko, M. T. Sweetser, P. Garg, A. Vaishnaw, J. B. Kim, A. R. Simon, L. Gouya, *N. Engl. J. Med.* **2020**, *382*, 2289.
[33] a) R. S. Ajioka, J. D. Phillips, J. P. Kushner, *Biochim. Biophys. Acta* **2006**, *1763*, 723; b) J. T. Marsden, D. C. Rees, *J. Clin. Pathol.* **2014**, *67*, 60.

[34] K. E. Anderson, J. R. Bloomer, H. L. Bonkovsky, J. P. Kushner, C. A. Pierach, N. R. Pimstone, R. J. Desnick, *Ann. Intern. Med.* **2005**, *142*, 439.
[35] a) S. Agarwal, A. R. Simon, V. Goel, B. A. Habtemariam, V. A. Clausen, J. B. Kim, G. J. Robbie, *Clin. Pharmacol. Ther.* **2020**, *108*, 63; b) R. Wang, G. Liu, C. Wang, *BMC Bioin.* **2019**, *20*, 471.
[36] E. Sardh, P. Harper, M. Balwani, P. Stein, D. Rees, D. M. Bissell, R. Desnick, C. Parker, J. Phillips, H. L. Bonkovsky, D. Vassiliou, C. Penz, A. Chan-Daniels, Q. He, W. Querbes, K. Fitzgerald, J. B. Kim, P. Garg, A. Vaishnaw, A. R. Simon, K. E. Anderson, *N. Engl. J. Med.* **2019**, *380*, 549.
[37] K. K. Ray, R. S. Wright, D. Kallend, W. Koenig, L. A. Leiter, F. J. Raal, J. A. Bisch, T. Richardson, M. Jaros, P. L. J. Wijngaard, J. J. P. Kastelein, *N. Engl. J. Med.* **2020**, *382*, 1507.
[38] S. Garrelfs, Y. Frishberg, S. Hulton, M. Koren, W. O'Riordan, P. Cochat, G. Deschenes, H. Shasha-Lavsky, J. Saland, W. Van't Hoff, D. G. Fuster, D. Magen, S. Moochhala, G. Schalk, E. Simkova, J. Groothoff, D. Sas, K. Meliambro, J. Lu, P. Garg, J. Gansner, T. McGregor, J. Lieske, *Nephrol., Dial., Transplant.* **2020**, *35*, gfaa146.LB002.
[39] A. D. Springer, S. F. Dowdy, *Nucleic Acid Ther.* **2018**, *28*, 109.
[40] K. J. Pasi, S. Rangarajan, P. Georgiev, T. Mant, M. D. Creagh, T. Lissitchkov, D. Bevan, S. Austin, C. R. Hay, I. Hegemann, R. Kazmi, P. Chowdary, L. Gercheva-Kyuchukova, V. Mamonov, M. Timofeeva, C. H. Soh, P. Garg, A. Vaishnaw, A. Akinc, B. Sørensen, M. V. Ragni, *N. Engl. J. Med.* **2017**, *377*, 819.
[41] B. Hoppe, M. Coenen, G. Schalk, P. Cochat, G. Lipkin, J. Lieske, . . . R. Rosskamp, **2020**. PHYOX3: A Long-Term, Open-Label Extension Trial of Nedosiran in Patients with Primary Hyperoxaluria Type 1, 2, or 3. Poster presented at the American Society of Nephrology, Kidney Week. https://investors.dicerna.com/index.php/static-files/683eb0a3-532b-4df6-9127-7b740457b119.
[42] Arrowhead Presents Positive New Phase 1/2 Clinical Data on Cardiometabolic Candidates ARO-APOC3 and ARO-ANG3 at European Society of Cardiology Congress 2020. **2020**. [Press release] https://www.businesswire.com/news/home/20200831005208/en/.
[43] Silence Therapeutics Provides Research and Development Update. **2020**. [Press release] https://www.silence-therapeutics.com/media/2079/200908-silence-business-update_final.pdf.
[44] Arrowhead ARO-AAT Phase 2 Interim Results in Patients with Alpha-1 Liver Disease Demonstrate Improvements in Key Parameters after Six Months of Treatment. **2020**. [Press release] https://www.businesswire.com/news/home/20200916005224/en/.
[45] L. DeFrancesco, *Nat. Biotechnol.* **2020**, *38*, 1132.
[46] B. S. Graham, *Immunol. Rev.* **2013**, *255*, 230.
[47] Y. Huang, C. Yang, X. F. Xu, W. Xu, S. W. Liu, *Acta Pharmacol. Sin.* **2020**, *41*, 1141.
[48] F. Krammer, *Nature* **2020**, *586*, 516.
[49] B. Pulendran, R. Ahmed, *Nat. Immunol.* **2011**, *12*, 509.
[50] a) N. Muñoz, R. Manalastas Jr., P. Pitisuttithum, D. Tresukosol, J. Monsonego, K. Ault, C. Clavel, J. Luna, E. Myers, S. Hood, O. Bautista, J. Bryan, F. J. Taddeo, M. T. Esser, S. Vuocolo, R. M. Haupt, E. Barr, A. Saah, *Lancet* **2009**, *373*, 1949; b) W. Szmuness, C. E. Stevens, E. A. Zang, E. J. Harley, A. Kellner, *Hepatology* **1981**, *1*, 377.
[51] M. Rizk, Ş. Tüzmen, *Pharmgenomics Pers. Med.* **2017**, *10*, 267.
[52] T. S. Zimmermann, A. C. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M. N. Fedoruk, J. Harborth, J. A. Heyes, L. B. Jeffs, M. John, A. D. Judge, K. Lam, K. McClintock, L. V. Nechev, L. R. Palmer, T. Racie, I. Röhl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A. J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H. P. Vornlocher, I. MacLachlan, *Nature* **2006**, *441*, 111.
[53] a) B. L. Mui, Y. K. Tam, M. Jayaraman, S. M. Ansell, X. Du, Y. Y. Tam, P. J. Lin, S. Chen, J. K. Narayanannair, K. G. Rajeev, M. Manoharan, A. Akinc, M. A. Maier, P. Cullis, T. D. Madden, M. J. Hope, *Mol. Ther.–Nucleic Acids* **2013**, *2*, e139; b) A. Akinc, M. Goldberg, J. Qin, J. R. Dorkin, C. Gamba-Vitalo, M. Maier, K. N. Jayaprakash, M. Jayaraman, K. G. Rajeev, M. Manoharan, V. Koteliansky, I. Röhl, E. S. Leshchiner, R. Langer, D. G. Anderson, *Mol. Ther.* **2009**, *17*, 872.
[54] K. T. Love, K. P. Mahon, C. G. Levins, K. A. Whitehead, W. Querbes, J. R. Dorkin, J. Qin, W. Cantley, L. L. Qin, T. Racie, M. Frank-Kamenetsky, K. N. Yip, R. Alvarez, D. W. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1864.
[55] J. B. Miller, P. Kos, V. Tieu, K. Zhou, D. J. Siegwart, *ACS Appl. Mater. Interfaces* **2018**, *10*, 2302.
[56] Y. Dong, K. T. Love, J. R. Dorkin, S. Sirirungruang, Y. Zhang, D. Chen, R. L. Bogorad, H. Yin, Y. Chen, A. J. Vegas, C. A. Alabi, G. Sahay, K. T. Olejnik, W. Wang, A. Schroeder, A. K. Lytton-Jean, D. J. Siegwart, A. Akinc, C. Barnes, S. A. Barros, M. Carioto, K. Fitzgerald, J. Hettinger, V. Kumar, T. I. Novobrantseva, J. Qin, W. Querbes, V. Koteliansky, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3955.
[57] a) M. P. Lokugamage, Z. Gan, C. Zurla, J. Levin, F. Z. Islam, S. Kalathoor, M. Sato, C. D. Sago, P. J. Santangelo, J. E. Dahlman, *Adv. Mater.* **2020**, *32*, 1904905; b) O. S. Fenton, K. J. Kauffman, R. L. McClellan, E. A. Appel, J. R. Dorkin, M. W. Tibbitt, M. W. Heartlein, F. DeRosa, R. Langer, D. G. Anderson, *Adv. Mater.* **2016**, *28*, 2939.
[58] M. Jayaraman, S. M. Ansell, B. L. Mui, Y. K. Tam, J. Chen, X. Du, D. Butler, L. Eltepu, S. Matsuda, J. K. Narayanannair, K. G. Rajeev, I. M. Hafez, A. Akinc, M. A. Maier, M. A. Tracy, P. R. Cullis, T. D. Madden, M. Manoharan, M. J. Hope, *Angew. Chem., Int. Ed. Engl.* **2012**, *51*, 8529.
[59] V. Kumar, J. Qin, Y. Jiang, R. G. Duncan, B. Brigham, S. Fishman, J. K. Nair, A. Akinc, S. A. Barros, P. V. Kasperkovitz, *Mol. Ther.–Nucleic Acids* **2014**, *3*, e210.
[60] J. S. Suk, Q. Xu, N. Kim, J. Hanes, L. M. Ensign, *Adv. Drug Delivery Rev.* **2016**, *99*, 28.
[61] S. Schöttler, G. Becker, S. Winzen, T. Steinbach, K. Mohr, K. Landfester, V. Mailänder, F. R. Wurm, *Nat. Nanotechnol.* **2016**, *11*, 372.
[62] R. Michel, S. Pasche, M. Textor, D. G. Castner, *Langmuir* **2005**, *21*, 12327.
[63] a) A. P. Chapman, *Adv. Drug Delivery Rev.* **2002**, *54*, 531; b) S. H. Kim, J. H. Jeong, S. H. Lee, S. W. Kim, T. G. Park, *J. Controlled Release* **2006**, *116*, 123; c) M. J. Roberts, M. D. Bentley, J. M. Harris, *Adv. Drug Delivery Rev.* **2002**, *54*, 459; d) Y. Dong, D. J. Siegwart, D. G. Anderson, *Adv. Drug Delivery Rev.* **2019**, *144*, 133.
[64] M. Oishi, Y. Nagasaki, K. Itaka, N. Nishiyama, K. Kataoka, *J. Am. Chem. Soc.* **2005**, *127*, 1624.
[65] J. M. Richner, S. Himansu, K. A. Dowd, S. L. Butler, V. Salazar, J. M. Fox, J. G. Julander, W. W. Tang, S. Shresta, T. C. Pierson, G. Ciaramella, M. S. Diamond, *Cell* **2017**, *168*, 1114.
[66] K. S. Corbett, D. K. Edwards, S. R. Leist, O. M. Abiona, S. Boyoglu-Barnum, R. A. Gillespie, S. Himansu, A. Schäfer, C. T. Ziwawo, A. T. DiPiazza, K. H. Dinnon, S. M. Elbashir, C. A. Shaw, A. Woods, E. J. Fritch, D. R. Martinez, K. W. Bock, M. Minai, B. M. Nagata, G. B. Hutchinson, K. Wu, C. Henry, K. Bahl, D. Garcia-Dominguez, L. Ma, I. Renzi, W. P. Kong, S. D. Schmidt, L. Wang, Y. Zhang, E. Phung, L. A. Chang, R. J. Loomis, N. E. Altaras, E. Narayanan, M. Metkar, V. Presnyak, C. Liu, M. K. Louder, W. Shi, K. Leung, E. S. Yang, A. West, K. L. Gully, L. J. Stevens, N. Wang, D. Wrapp, N. A. Doria-Rose, G. Stewart-Jones, H. Bennett, G. S. Alvarado, M. C. Nason, T. J. Ruckwardt, J. S. McLellan, M. R. Denison, J. D. Chappell, I. N. Moore, K. M. Morabito, J. R. Mascola, R. S. Baric, A. Carfi, B. S. Graham, *Nature* **2020**, *586*, 567.
[67] L. A. Jackson, E. J. Anderson, N. G. Rouphael, P. C. Roberts, M. Makhene, R. N. Coler, M. P. McCullough, J. D. Chappell, M. R. Denison, L. J. Stevens, A. J. Pruijssers, A. McDermott, B. Flach, N. A. Doria-Rose, K. S. Corbett, K. M. Morabito, S. O'Dell, S. D. Schmidt, P. A. Swanson 2nd, M. Padilla, J. R. Mascola, K. M. Neuzil, H. Bennett, W. Sun, E. Peters, M. Makowski, J. Albert, K. Cross, W.

Buchanan, R. Pikaart-Tautges, J. E. Ledgerwood, B. S. Graham, J. H. Beigel, *N. Engl. J. Med.* **2020**, *382*, 1920.

[68] P. F. McKay, K. Hu, A. K. Blakney, K. Samnuan, J. C. Brown, R. Penn, J. Zhou, C. R. Bouton, P. Rogers, K. Polra, P. J. C. Lin, C. Barbosa, Y. K. Tam, W. S. Barclay, R. J. Shattock, *Nat. Commun.* **2020**, *11*, 3523.

[69] U. Sahin, A. Muik, E. Derhovanessian, I. Vogler, L. M. Kranz, M. Vormehr, A. Baum, K. Pascal, J. Quandt, D. Maurus, S. Brachtendorf, V. Lörks, J. Sikorski, R. Hilker, D. Becker, A. K. Eller, J. Grützner, C. Boesler, C. Rosenbaum, M. C. Kühnle, U. Luxemburger, A. Kemmer-Brück, D. Langer, M. Bexon, S. Bolte, K. Karikó, T. Palanche, B. Fischer, A. Schultz, P. Y. Shi, C. Fontes-Garfias, J. L. Perez, K. A. Swanson, J. Loschko, I. L. Scully, M. Cutler, W. Kalina, C. A. Kyratsous, D. Cooper, P. R. Dormitzer, K. U. Jansen, Ö. Türeci, *Nature* **2020**, *586*, 594.

[70] E. E. Walsh, R. W. Frenck, A. R. Falsey, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, K. Neuzil, M. J. Mulligan, R. Bailey, K. A. Swanson, P. Li, K. Koury, W. Kalina, D. Cooper, C. Fontes-Garfias, P.-Y. Shi, Ö. Türeci, K. R. Tompkins, K. E. Lyke, V. Raabe, P. R. Dormitzer, K. U. Jansen, U. Şahin, W. C. Gruber, *N. Engl. J. Med.* **2020**, *383*, 2439.

[71] T. Vesikari, E. L. Ala-Laurila, A. Heikkinen, A. Terho, E. D'Hondt, F. E. André, *Am. J. Dis. Child.* **1984**, *138*, 843.

[72] H. Sarin, **2010**, *2*, 14.

[73] K. M. Tsoi, S. A. Macparland, X.-Z. Ma, V. N. Spetzler, J. Echeverri, B. Ouyang, S. M. Fadel, E. A. Sykes, N. Goldaracena, J. M. Kaths, J. B. Conneely, B. A. Alman, M. Selzner, M. A. Ostrowski, O. A. Adeyi, A. Zilman, I. D. McGilvray, W. C. W. Chan, *Nat. Mater.* **2016**, *15*, 1212.

[74] a) S. C. Semple, A. Akinc, J. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Sah, D. Stebbing, E. J. Crosley, E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K. F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M. Srinivasulu, M. J. Weinstein, Q. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T. Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A. Tracy, A. de Fougerolles, I. MacLachlan, P. R. Cullis, T. D. Madden, M. J. Hope, *Nat. Biotechnol.* **2010**, *28*, 172; b) S. Chen, Y. Y. Tam, P. J. Lin, A. K. Leung, Y. K. Tam, P. R. Cullis, *J. Controlled Release* **2014**, *196*, 106.

[75] a) J. E. Dahlman, C. Barnes, O. F. Khan, A. Thiriot, S. Jhunjunwala, T. E. Shaw, Y. Xing, H. B. Sager, G. Sahay, L. Speciner, A. Bader, R. L. Bogorad, H. Yin, T. Racie, Y. Dong, S. Jiang, D. Seedorf, A. Dave, K. Singh Sandhu, M. J. Webber, T. Novobrantseva, V. M. Ruda, A. K. R. Lytton-Jean, C. G. Levins, B. Kalish, D. K. Mudge, M. Perez, L. Abezgauz, P. Dutta, L. Smith, K. Charisse, M. W. Kieran, K. Fitzgerald, M. Nahrendorf, D. Danino, R. M. Tuder, U. H. Von Andrian, A. Akinc, D. Panigrahy, A. Schroeder, V. Koteliansky, R. Langer, D. G. Anderson, *Nat. Nanotechnol.* **2014**, *9*, 648; b) H. B. Sager, P. Dutta, J. E. Dahlman, M. Hulsmans, G. Courties, Y. Sun, T. Heidt, C. Vinegoni, A. Borodovsky, K. Fitzgerald, G. R. Wojtkiewicz, Y. Iwamoto, B. Tricot, O. F. Khan, K. J. Kauffman, Y. Xing, T. E. Shaw, P. Libby, R. Langer, R. Weissleder, F. K. Swirski, D. G. Anderson, M. Nahrendorf, *Sci. Transl. Med.* **2016**, *8*, 342ra80; c) H. B. Sager, M. Hulsmans, K. J. Lavine, M. B. Moreira, T. Heidt, G. Courties, Y. Sun, Y. Iwamoto, B. Tricot, O. F. Khan, J. E. Dahlman, A. Borodovsky, K. Fitzgerald, D. G. Anderson, R. Weissleder, P. Libby, F. K. Swirski, M. Nahrendorf, *Circ. Res.* **2016**, *119*, 853; d) S. Yun, M. Budatha, J. E. Dahlman, B. G. Coon, R. T. Cameron, R. Langer, D. G. Anderson, G. Baillie, M. A. Schwartz, *Nat. Cell Biol.* **2016**, *18*, 1043; e) K. White, Y. Lu, S. Annis, A. E. Hale, B. N. Chau, J. E. Dahlman, C. Hemann, A. R. Opotowsky, S. O. Vargas, I. Rosas, M. A. Perrella, J. C. Osorio, K. J. Haley, B. B. Graham, R. Kumar, R. Saggar, R. Saggar, W. D. Wallace, D. J. Ross, O. F. Khan, A. Bader, B. R. Gochuico, M. Matar, K. Polach, N. M. Johannessen, H. M. Prosser, D. G. Anderson, R. Langer, J. L. Zweier, L. A. Bindoff, D. Systrom, A. B. Waxman, R. C. Jin, S. Y. Chan, *EMBO Mol. Med.* **2015**, *7*, 695.

[76] O. F. Khan, P. S. Kowalski, J. C. Doloff, J. K. Tsosie, V. Bakthavatchalu, C. B. Winn, J. Haupt, M. Jamiel, R. Langer, D. G. Anderson, *Sci. Adv.* **2018**, *4*, eaar8409.

[77] K. J. Kauffman, M. A. Oberli, J. R. Dorkin, J. E. Hurtado, J. C. Kaczmarek, S. Bhadani, J. Wyckoff, R. Langer, A. Jaklenec, D. G. Anderson, *Mol. Ther.–Nucleic Acids* **2018**, *10*, 55.

[78] Q. Cheng, T. Wei, L. Farbiak, L. T. Johnson, S. A. Dilliard, D. J. Siegwart, *Nat. Nanotechnol.* **2020**, *15*, 313.

[79] L. M. Kranz, M. Diken, H. Haas, S. Kreiter, C. Loquai, K. C. Reuter, M. Meng, D. Fritz, F. Vascotto, H. Hefesha, C. Grunwitz, M. Vormehr, Y. Husemann, A. Selmi, A. N. Kuhn, J. Buck, E. Derhovanessian, R. Rae, S. Attig, J. Diekmann, R. A. Jabulowsky, S. Heesch, J. Hassel, P. Langguth, S. Grabbe, C. Huber, O. Tureci, U. Sahin, *Nature* **2016**, *534*, 396.

[80] K. Zhou, L. H. Nguyen, J. B. Miller, Y. Yan, P. Kos, H. Xiong, L. Li, J. Hao, J. T. Minnig, H. Zhu, D. J. Siegwart, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 520.

[81] E. Robinson, K. D. MacDonald, K. Slaughter, M. McKinney, S. Patel, C. Sun, G. Sahay, *Mol. Ther.* **2018**, *26*, 2034.

[82] Y. Yan, L. Liu, H. Xiong, J. B. Miller, K. Zhou, P. Kos, K. E. Huffman, S. Elkassih, J. W. Norman, R. Carstens, J. Kim, J. D. Minna, D. J. Siegwart, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E5702.

[83] Translate Bio Announces Interim Results from Phase 1/2 Clinical Trial of MRT5005 in Patients with Cystic Fibrosis. **2019**. [Press release] http://www.globenewswire.com/news-release/2019/07/31/1894563/0/en/Translate-Bio-Announces-Interim-Results-from-Phase-1-2-Clinical-Trial-of-MRT5005-in-Patients-with-Cystic-Fibrosis.html.

[84] O. S. Fenton, K. J. Kauffman, J. C. Kaczmarek, R. L. McClellan, S. Jhunjhunwala, M. W. Tibbitt, M. D. Zeng, E. A. Appel, J. R. Dorkin, F. F. Mir, J. H. Yang, M. A. Oberli, M. W. Heartlein, F. DeRosa, R. Langer, D. G. Anderson, *Adv. Mater.* **2017**, *29*, 1606944.

[85] D. Rosenblum, N. Joshi, W. Tao, J. M. Karp, D. Peer, *Nat. Commun.* **2018**, *9*, 1410.

[86] T. P. J. Wyatt, J. Fouchard, A. Lisica, N. Khalilgharibi, B. Baum, P. Recho, A. J. Kabla, G. T. Charras, *Nat. Mater.* **2020**, *19*, 109.

[87] R. Kedmi, N. Veiga, S. Ramishetti, M. Goldsmith, D. Rosenblum, N. Dammes, I. Hazan-Halevy, L. Nahary, S. Leviatan-Ben-Arye, M. Harlev, M. Behlke, I. Benhar, J. Lieberman, D. Peer, *Nat. Nanotechnol.* **2018**, *13*, 214.

[88] S. Ramishetti, I. Hazan-Halevy, R. Palakuri, S. Chatterjee, S. Naidu Gonna, N. Dammes, I. Freilich, L. Kolik Shmuel, D. Danino, D. Peer, *Adv. Mater.* **2020**, *32*, 1906128.

[89] X. Hou, X. Zhang, W. Zhao, C. Zeng, B. Deng, D. W. McComb, S. Du, C. Zhang, W. Li, Y. Dong, *Nat. Nanotechnol.* **2020**, *15*, 41.

[90] K. Paunovska, A. J. Da Silva Sanchez, C. D. Sago, Z. Gan, M. P. Lokugamage, F. Z. Islam, S. Kalathoor, B. R. Krupczak, J. E. Dahlman, *Adv. Mater.* **2019**, *31*, 1807748.

[91] M. P. Lokugamage, C. D. Sago, J. E. Dahlman, *Curr. Opin. Biomed. Eng.* **2018**, *7*, 1.

[92] a) A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D. N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. De Fougerolles, J. R. Dorkin, K. Narayanannair Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K. G. Rajeev, D. W. Y. Sah, J. Soutschek, I. Toudjarska, H.-P. Vornlocher, T. S. Zimmermann, R. Langer, D. G. Anderson, *Nat. Biotechnol.* **2008**, *26*, 561; b) K. T. Love, K. P. Mahon, C. G. Levins, K. A. Whitehead, W. Querbes, J. R. Dorkin, J. Qin, W. Cantley, L. L. Qin, T. Racie, M. Frank-Kamenetsky, K. N. Yip, R. Alvarez, D. W. Y. Sah, A. De Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1864; c) Y. Dong, K. T. Love, J. R. Dorkin, S. Sirirungruang, Y. Zhang, D. Chen, R. L. Bogorad, H. Yin, Y. Chen, A. J. Vegas, C. A. Alabi, G. Sahay, K. T. Olejnik, W. Wang, A.

Schroeder, A. K. R. Lytton-Jean, D. J. Siegwart, A. Akinc, C. Barnes, S. A. Barros, M. Carioto, K. Fitzgerald, J. Hettinger, V. Kumar, T. I. Novobrantseva, J. Qin, W. Querbes, V. Koteliansky, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3955.

[93] K. Paunovska, C. D. Sago, C. M. Monaco, W. H. Hudson, M. G. Castro, T. G. Rudoltz, S. Kalathoor, D. A. Vanover, P. J. Santangelo, R. Ahmed, A. V. Bryksin, J. E. Dahlman, *Nano Lett.* **2018**, *18*, 2148.

[94] C. D. Sago, M. P. Lokugamage, K. Paunovska, D. A. Vanover, C. M. Monaco, N. N. Shah, M. Gamboa Castro, S. E. Anderson, T. G. Rudoltz, G. N. Lando, P. Mummilal Tiwari, J. L. Kirschman, N. Willett, Y. C. Jang, P. J. Santangelo, A. V. Bryksin, J. E. Dahlman, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, e9944.

[95] K. Negron, N. Khalasawi, B. Lu, C.-Y. Ho, J. Lee, S. Shenoy, H.-Q. Mao, T.-H. Wang, J. Hanes, J. S. Suk, *J. Controlled Release* **2019**, *303*, 1.

[96] K. Toome, A.-M. A. Willmore, Paiste P., A. Tobi, K. N. Sugahara, K. Kirsimäe, E. Ruoslahti, G. B. Braun, T. Teesalu, *Nanoscale* **2017**, *9*, 10094.

[97] a) D. X. Medina, K. T. Householder, R. Ceton, T. Kovalik, J. Heffernan, R. V. Shankar, R. P. Bowser, R. J. Wechsler-Reya, R. W. Sirianni, *J. Controlled Release* **2017**, *253*, 171; b) Y. S. Yang, P. U. Atukorale, K. D. Moynihan, A. Bekdemir, K. Rakhra, L. Tang, F. Stellacci, D. J. Irvine, *Nat. Commun.* **2017**, *8*, 14069.

[98] M. Yanez Arteta, T. Kjellman, S. Bartesaghi, S. Wallin, X. Wu, A. J. Kvist, A. Dabkowska, N. Székely, A. Radulescu, J. Bergenholtz, L. Lindfors, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E3351.

[99] C. D. Sago, S. Kalathoor, J. P. Fitzgerald, G. N. Lando, N. Djeddar, A. V. Bryksin, J. E. Dahlman, *J. Mater. Chem. B* **2018**, *6*, 7197.

[100] Z. Yaari, D. Da Silva, A. Zinger, E. Goldman, A. Kajal, R. Tshuva, E. Barak, N. Dahan, D. Hershkovitz, M. Goldfeder, J. S. Roitman, A. Schroeder, *Nat. Commun.* **2016**, *7*, 13325.

[101] J. E. Dahlman, K. J. Kauffman, Y. Xing, T. E. Shaw, F. F. Mir, C. C. Dlott, R. Langer, D. G. Anderson, E. T. Wang, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 2060.

[102] A. Wittrup, A. Ai, X. Liu, P. Hamar, R. Trifonova, K. Charisse, M. Manoharan, T. Kirchhausen, J. Lieberman, *Nat. Biotechnol.* **2015**, *33*, 870.

[103] P. P. G. Guimaraes, R. Zhang, R. Spektor, M. Tan, A. Chung, M. M. Billingsley, R. El-Mayta, R. S. Riley, L. Wang, J. M. Wilson, M. J. Mitchell, *J. Controlled Release* **2019**, *316*, 404.

[104] C. D. Sago, M. P. Lokugamage, G. N. Lando, N. Djeddar, N. N. Shah, C. Syed, A. V. Bryksin, J. E. Dahlman, *Nano Letters* **2018**, *18*, 7590.

[105] A. K. Shalek, R. Satija, J. Shuga, J. J. Trombetta, D. Gennert, D. Lu, P. Chen, R. S. Gertner, J. T. Gaublomme, N. Yosef, S. Schwartz, B. Fowler, S. Weaver, J. Wang, X. Wang, R. Ding, R. Raychowdhury, N. Friedman, N. Hacohen, H. Park, A. P. May, A. Regev, *Nature* **2014**, *510*, 363.

[106] E. Khera, G. M. Thurber, *BioDrugs* **2018**, *32*, 465.

[107] M. A. Maier, M. Jayaraman, S. Matsuda, J. Liu, S. Barros, W. Querbes, Y. K. Tam, S. M. Ansell, V. Kumar, J. Qin, X. Zhang, Q. Wang, S. Panesar, R. Hutabarat, M. Carioto, J. Hettinger, P. Kandasamy, D. Butler, K. G. Rajeev, B. Pang, K. Charisse, K. Fitzgerald, B. L. Mui, X. Du, P. Cullis, T. D. Madden, M. J. Hope, M. Manoharan, A. Akinc, *Mol. Ther.* **2013**, *21*, 1570.

[108] F. K. Stevenson, C. H. Ottensmeier, J. Rice, *Curr. Opin. Immunol.* **2010**, *22*, 264.

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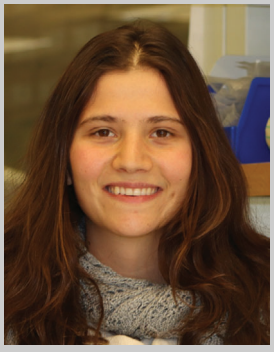

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