Lipids and Lipid Derivatives for RNA Delivery

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ABSTRACT: RNA-based therapeutics have shown great promise in treating a broad spectrum of diseases through various mechanisms including knockdown of pathological genes, expression of therapeutic proteins, and programmed gene editing. Due to the inherent instability and negative-charges of RNA molecules, RNA-based therapeutics can make the most use of delivery systems to overcome biological barriers and to release the RNA payload into the cytosol. Among different types of delivery systems, lipid-based RNA delivery systems, particularly lipid nanoparticles (LNPs), have been extensively studied due to their unique properties, such as simple chemical synthesis of lipid components, scalable manufacturing processes of LNPs, and wide packaging capability. LNPs represent the most widely used delivery systems for RNA-based therapeutics, as evidenced by the clinical approvals of three LNP-RNA formulations, patisiran, BNT162b2, and mRNA-1273. This review covers recent advances of lipids, lipid derivatives, and lipid-derived macromolecules used in RNA delivery over the past several decades. We focus mainly on their chemical structures, synthetic routes, characterization, formulation methods, and structure—activity relationships. We also briefly describe the current status of representative preclinical studies and clinical trials and highlight future opportunities and challenges.

CONTENTS
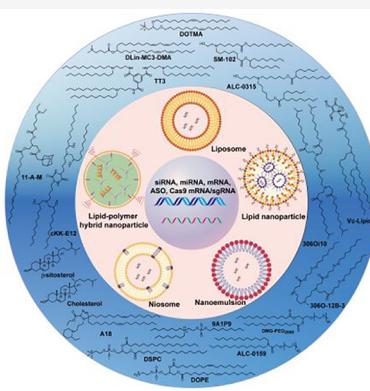
1. INTRODUCTION

1.1. RNA-Based Therapeutics

RNA-based therapeutics have gained extensive interest in treating diverse diseases including those associated with "undruggable" targets.[1–3] In the past decade, a series of RNA-based therapies have been approved for therapeutic applications in different types of diseases such as macular degeneration, spinal muscular atrophy, hypercholesterolemia, and TTR-mediated amyloidosis.[4–6] Table 1 lists representative examples

Table 1. Representative RNA-Based Therapeutics Approved for Clinical Use

RNA-based therapeutic products	Approval year	Therapeutic Indication
ASO		
Mipomersen	2013	Familial hypercholesterolemia
Eteplirsen	2016	Duchenne muscular dystrophy
Nusinersen	2016	Spinal muscular atrophy
Inotersen	2018	Hereditary transthyretin amyloidosis
Golodirsen	2019	Duchenne muscular dystrophy
Volanesorsen	2019	Familial chylomicronaemia syndrome
siRNA		
Patisiran	2018	Hereditary transthyretin amyloidosis
Givosiran	2019	Acute hepatic porphyria
Lumasiran	2020	Primary Hyperoxaluria Type 1
Inclisiran	2020	Hypercholesterolemia or mixed dyslipidemia
mRNA		
BNT162b2	2020	COVID-19 vaccine
mRNA-1273	2020	COVID-19 vaccine

of RNA-based therapeutics for clinical use. Most recently, two formulations of lipid nanoparticles encapsulating mRNA, BNT162b2 and mRNA-1273, have obtained emergency use authorizations (EUA) from the FDA and EMA as SARS-CoV-2 vaccines for the prevention of coronavirus disease 2019 (COVID-19).[7–9] RNA molecules mainly include antisense oligonucleotides (ASOs), small interfering RNA (siRNA), microRNA (miRNA), and mRNA (mRNA).

Short antisense oligonucleotides (ASOs) consist of single antisense stranded DNAs or RNAs with a sequence length of 8–50 nucleotides that can specifically bind to their target mRNA through complementary base pairing, leading to the degradation of mRNA by endogenous cellular RNase H[10,11] or a functional blockade of mRNAs through steric effects.[12,13] Chemical modification of ASOs, such as phosphorothioate ASOs, can increase the stability of ASOs and facilitate their interactions with targeted cells.[14,15]

In 1998, Fire et al. discovered the RNA interference (RNAi) pathway,[16] which involves the formation of an RNA-induced silencing complex (RISC) in cell cytosol and subsequent decay of the target mRNA. These important findings led to the emergence of RNAi as a new type of RNA-based therapeutics.[17] Small interfering RNA (siRNA) and microRNA (miRNA) are two major types of RNA molecules for RNA interference. siRNA, one of the most important classes of RNAi therapeutics, is typically a double-stranded RNA (dsRNA) molecule that consists of less than 30 base pairs. siRNA-based therapeutics have been investigated as potential therapies for diseases caused by abnormal expression or mutation such as cancers,[18–21] viral infections,[22,23] and genetic disorders.[24] Following Onpattro

(patisiran), the first approved RNAi therapeutic,[25–28] three other RNAi therapeutics have been approved for clinical application, including givosiran,[29,30] inclisiran,[31,32] and lumasiran.[33,34] Meanwhile, miRNA, usually an endogenous small noncoding RNA (ncRNA), negatively controls the expression of the target mRNA.[35,36] Researchers have discovered numerous miRNA for the treatment of cancer[37,38] and fibrosis.[39] For instance, miR-34a was studied for the treatment of lung cancer.[40]

Messenger RNA (mRNA) carries genetic information transcribed from the genomic DNA in the nucleus to the sites of protein synthesis in the cytoplasm.[41,42] The sequences of mRNA play important roles in coding a specific protein and modulating the post-translational modifications. Besides, mRNA has a relatively short half-life, which induces transient protein expression. Given these properties, mRNA has become a new class of therapeutics,[43–45] which have shown considerable promise in vaccine development,[46–57] allergy tolerization,[58] and the treatment of a broad spectrum of diseases, including sepsis,[59] hemophilia B,[60,61] HIV,[62] myocardial infarction,[63] and several types of cancer.[64,65] Theoretically, engineered mRNA can act as a vaccine platform to produce any emerging immunogen. Additionally, mRNA has been used for gene editing and genomic engineering.[66,67] In recent years, gene editing systems have been a biotechnological breakthrough, providing a strategy for the treatment of various diseases. Specifically, the CRISPR/Cas system[68] uses programmable DNA nucleases to permanently and precisely manipulate the genome.[69] The codelivery of Cas9 mRNA and single-guide RNA (sgRNA) against a certain genomic target via base pairing between the sgRNA and the target DNA has been examined for gene editing in numerous genes.[70–74] Additionally, RNA aptamer,[75–78] RNA decoys,[79] ribozymes,[80,81] and circular RNA (cirRNA)[82–85] have also been explored for biological and therapeutic applications, which have been well-summarized in other reviews.

1.2. Biological Barriers to RNA Delivery

Despite the great potential of RNA-based therapeutics for treating a variety of diseases, many barriers must be circumvented for the successful delivery of these therapeutic RNAs into targeted cell types. Biological obstacles to the effective delivery of RNAs include extracellular and intracellular barriers (Figure 1). The extracellular matrix (ECM) is the first barrier that protects the integrity of the cells from foreign agents, which can inhibit the transport of the RNA molecules from the extracellular environment to the target cells. Cell membrane and endosomal trapping are two major obstacles as intracellular barriers.[86–88] As shown in Figure 1, once RNA-loading nanoparticles are administered into the bloodstream, they need to protect RNAs from rapid degradation by serum ribonucleases (RNase).[89,90] Meanwhile, nanoparticles must evade phagocytosis, cross the vascular endothelial cells, and traverse the extracellular space to reach the target cells.[91] Typical nanoparticles, with small particle size, can penetrate the vascular endothelial pores to pass the extracellular environment. Cellular uptake of RNA-loading nanoparticles into the cytoplasm involves many different pathways, such as clathrin-mediated endocytosis (CME),[92,93] caveolae-mediated endocytosis (CvME), and macropinocytosis.[94,95] Then, the nanoparticles must escape from the endosome before the lysosome formation which would result in the enzymatic degradation of the nanoparticles.[96–98] It was reported that only 1–2% of lipid nanoparticles (LNPs) can escape the endosomes.[99] Two main
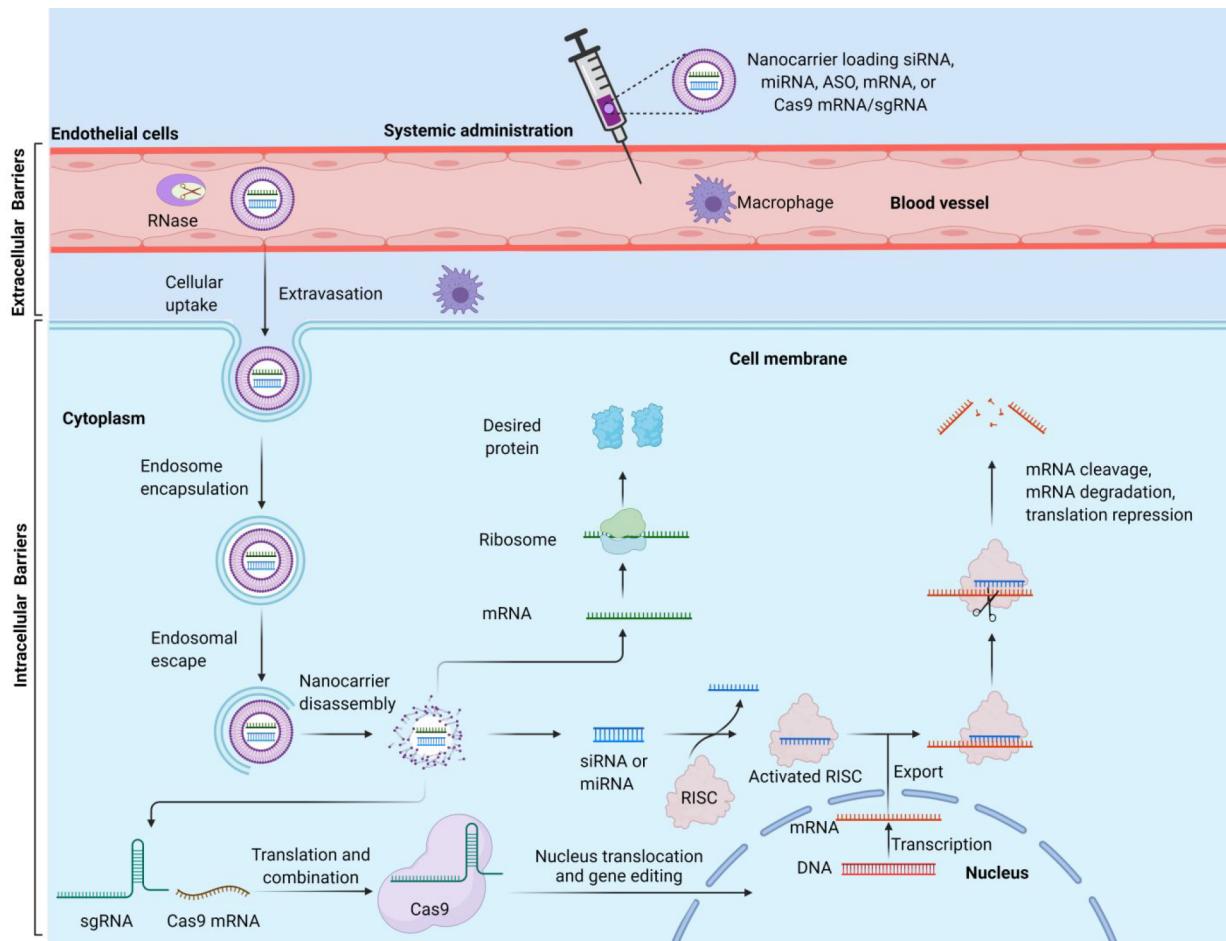
Figure 1. Schematic illustration of the extracellular and intracellular barriers to effective systemic delivery of RNAs and the mechanism of RNA-based therapeutics. Figure was created with BioRender.com.

mechanisms have been proposed for the process of nano-particles endosomal escape including the proton sponge effect and lipid flipping by fusogenic properties during nonlamellar phase transitions.[100] During the process of endosomal maturation, the endosomal environment changes from neutral to slightly acidic (pH ∼ 6.3 in early endosomes; pH ∼ 5.5 in late endosomes; and pH ∼ 4.7 in lysosomes),[101,102] which makes the ionizable components of the nanoparticles become protonated. Protonation of the ionizable components destabilizes the anionic vesicular membrane and facilitates nanoparticle disassembly, leading to the release of RNA to the cytosol, where RNA elicits its functions.[97,103] Upon acidification, lipid nanoparticles that contain protonated ionizable lipids or cationic lipids may adopt an inverted hexagonal (H_{II}) phase and rapidly fuse with anionic endosomal membranes, resulting in the endosomal escape of nanoparticles.[104] Incorporation of helper lipids, such as 1,2-dioleoyl-*sn*-glycerol-3-phosphatidylethanol-amine (DOPE), can enhance endosomal fusion as well as endosomal escape of lipid nanoparticles, by undergoing a conformational change upon protonation and promoting an inverted hexagonal (H_{II}) phase change.[105−108]

1.3. Techniques for RNA Delivery

Effective delivery of RNA molecules into cells is a crucial step for successful RNA-based therapeutics. An ideal RNA delivery technique should have high delivery efficiency, low toxicity, as well as high cell specificity. Currently, techniques for RNA delivery can be divided into three types: physical methods, biological carriers, and synthetic approaches Table 2.

1.3.1. Physical Methods. The physical methods for RNA delivery generally provide external forces, magnetic field or electrical field to the cells of interest, including microinjection, electroporation, sonoporation, magnetofection, photoporation, hydrodynamic delivery, and microfluidic squeezing.[109,110]

Table 2. RNA Delivery Techniques

RNA Delivery Techniques		refs
Physical Methods	Microinjection	111−113
	Electroporation	114−116
	Sonoporation	117−119
	Photoporation	120, 121
	Magnetofection	122, 123
	Hydroporation	124
	Microfluidic squeezing	125−129
Biological Carriers	Extracellular vesicles (EVs)	146−167
	Cell/cell membrane-based vectors	135,168−171
Synthetic Approaches	Lipid-based nanocarriers	175−177
	Polymer-based delivery systems	206−217
	Inorganic nanoparticles	221−232
	Nucleic acid nanostructures	236, 238, 243, 246−253
	Chemically conjugated RNAs	261−278

Table 3. Representative Clinical Trials of Lipid-Based RNA Therapies

Name	Indication	RNA Payload	Delivery System	Delivery Route	Sponsoring Institution	Phase	Clinical Trial Identifier
siRNA-based therapeutics							
ALN-TTR02 (Patisiran)	TTR-mediated Amyloidosis	siRNA against TTR	LNP (MC3/CHOL/DSPC/PEG$_{2000}$-C-DMG)	IV	Alnylam Pharmaceuticals	Approved	NCT03862807
ALN-VSP02	Solid tumor	siRNA targeting VEGF and KSP	LNP	IV	Alnylam Pharmaceuticals NCT00882180	I	NCT01158079
TKM-080301	Tumor	siRNA targeting PLK1	LNP	IV	Tekmira Pharmaceuticals	I	NCT01262235 NCT01437007 NCT02191878
ALN-PCS02	Hypercholesterolemia	siRNA against PCSK9	LNP	IV	Alnylam Pharmaceuticals	I	NCT01437059
siRNA-EphA2-DOPC	Advanced malignant solid neoplasm	siRNA targeting EphA2	Liposome (DOPC, Tween 20)	IP	M.D. Anderson Cancer Center	I	NCT01591356
Atu027	Pancreatic ductal carcinoma	siRNA targeting PKN3	Liposome (AtuFECT01/PEG-DSPE/CHOL)	IV	Silence Therapeutics GmbH	I/II	NCT01808638 NCT00938574
ND-L02-s0201	Hepatic fibrosis, Idiopathic pulmonary fibrosis	siRNA against HSP47	LNP (DC-6-14/Chol/DOPE/Vitamin A)	IV	Bristol-Myers Squibb	II	NCT03538301
ARB-001467	Hepatitis B	siRNA against HBV gene	LNP	IV	Arbutus Biopharma	II	NCT02631096
DCR-MYC	Hepatocellular carcinoma	siRNA targeting MYC	LNP	IV	Dicerna Pharmaceuticals, Inc.	I/II	NCT02314052
DCR-HBVS	Hepatitis B	siRNA against HBV gene	LNP	IV	Dicerna Pharmaceuticals, Inc.	I	NCT03772249
mRNA-based vaccines against infection							
mRNA-1273	COVID-19	mRNA encoding SARS-CoV-2 spike protein	LNP (SM-102/CHOL/DSPC/DMG-PEG$_{2000}$)	IM	Moderna Therapeutics	Emergency Use Authorization (EUA)	NCT04470427
BNT162b2	COVID-19	mRNA encoding SARS-CoV-2 spike protein	LNP (ALC-0315/CHOL/DSPC/ALC-0159)	IM	BioNTech/Pfizer	Emergency Use Authorization (EUA)	NCT04537949
ARCoV	COVID-19	mRNA encoding RBD of SARS-CoV-2	LNP	IM	Abogen Biosciences	I	ChiCTR2000003411
ARCT-021	COVID-19	mRNA encoding SARS-CoV-2 spike protein	LNP (ATX/DSPC/CHOL/DMG-PEG$_{2000}$)	IM	Arcturus Therapeutics	II	NCT04728347
ChulaCov19	COVID-19	mRNA encoding SARS-CoV-2-specific antigen	LNP	IM	Chulalongkorn University	I	NCT04566276
CVnCoV	COVID-19	mRNA encoding SARS-CoV-2 spike protein	LNP	IM	CureVac	II/III	NCT04652102
CV7202	Rabies	mRNA encoding Rabies virus G glycoprotein	LNP	IM	CureVac	I	NCT03713086
mRNA-1440	Influenza H10N8	mRNA encoding Hemagglutinin	LNP	ID or IM	Moderna Therapeutics	I	NCT03076385
mRNA-1851	Influenza H7N9	mRNA encoding Hemagglutinin	LNP	ID or IM	Moderna Therapeutics	I	NCT03345043
mRNA-1653	hMPV/PIV3	mRNA encoding fusion protein of hMPV and PIV3	LNP	ID	Moderna Therapeutics	I	NCT03392389
mRNA-1325	Zika	mRNA encoding prM-E glycoproteins	LNP	IM	Moderna Therapeutics	I	NCT03014089
mRNA-1893	Zika	mRNA encoding prM-E glycoproteins	LNP	IM	Moderna Therapeutics	I	NCT04064905
mRNA-1647 and mRNA-1443	Cytomegalovirus	mRNA encoding Pentameric complex and B glycoprotein	LNP	ID	Moderna Therapeutics	I	NCT03392389
mRNA-1388	Chikungunya	mRNA encoding Chikungunya virus antigens	LNP	IM	Moderna Therapeutics	I	NCT03325075
mRNA-1944	Chikungunya	mRNA encoding Chikungunya virus antigens	LNP	IV	Moderna Therapeutics	I	NCT03829384

Table 3. continued

Name	Indication	RNA Payload	Delivery System	Delivery Route	Sponsoring Institution	Phase	Clinical Trial Identifier
mRNA-based vaccines against infection							
GSK 692342	Tuberculosis	mRNA encoding immunogenic fusion protein (M72) of tuberculosis	LNP	IM.	GlaxoSmithKline	II	NCT01669096
mRNA-based cancer immunotherapy							
BNT111	Melanoma	mRNA encoding TAAs	Liposome (DOTMA/DOPE)	IV	BioNTech RNA Pharmaceuticals GmbH	I	NCT04382898
BNT112	Prostate cancer	mRNA encoding TAAs	Liposome (DOTMA/DOPE)	IV	BioNTech RNA Pharmaceuticals GmbH	I	NCT03418480
BNT113	HPV16-positive cancers	mRNA encoding TAAs	Liposome (DOTMA/DOPE)	IV	BioNTech RNA Pharmaceuticals GmbH	I	NCT04534205
BNT114	Triple negative breast cancer	mRNA encoding TAAs	Liposome (DOTMA/DOPE)	IV	BioNTech RNA Pharmaceuticals GmbH	I	NCT02410733
BNT115	Ovarian cancer	mRNA encoding TAAs	Liposome (DOTMA/DOPE)	IM	BioNTech RNA Pharmaceuticals GmbH	I	NCT04163094
RO7198457 (BNT122)	Locally advanced and metastatic tumors	mRNA encoding TAAs	Liposome	IV	Genentech, Inc.	II	NCT03815058
mRNA-2752	Solid tumors and lymphomas	mRNA encoding OX40L, IL-23, and IL-36γ	LNP	IT	Moderna Therapeutics	I	NCT03739931
mRNA-2416	Solid tumors lymphomas and ovarian cancer	mRNA encoding OX40L	LNP	IT	Moderna Therapeutics	I	NCT03323398
mRNA-4157	Bladder carcinoma, Melanoma	mRNA encoding TAAs	LNP	IM	Moderna Therapeutics	II	NCT03897881
mRNA-4650	Gastrointestinal cancer	mRNA encoding TAAs	LNP	IM	National Cancer Institute (NCI)	I/II	NCT03480152
mRNA-5671/V941	Nonsmall cell lung cancer, colorectal cancer, pancreatic adenocarcinoma	mRNA encoding KRAS antigens	LNP	IM	Merck Sharp & Dohme Corp.	I	NCT03948763
HARE-40	HPV positive cancers	mRNA encoding HPV oncoproteins E6 and E7	LNP	ID	University of Southampton	I/II	NCT03418480
SAR441000 (BNT131)	Solid tumors	mRNA encoding L-12sc, IL-15sushi, IFNα and GM-CSF	LNP	IT	Sanofi	I	NCT03871348
W_ova1	Ovarian cancer	mRNA encoding TAAs	Liposome	IV	University Medical Center Groningen	I	NCT04163094
MEDI1191	Solid tumors	mRNA encoding IL-12	LNP	IT	MedImmune LLC.	I	NCT03946800
mRNA-based therapeutics for gene disorders							
mRNA-3704	Isolated Methylmalonic Acidemia	mRNA encoding Methylmalonyl-CoA mutase	LNP	IV	Moderna Therapeutics	I/II	NCT03810690
mRNA-3927	Propionic acidemia	mRNA encoding Propionyl-CoA carboxylase	LNP	IV	Moderna Therapeutics	I/II	NCT04159103
MRT5201	Ornithine transcarbamylase deficiency	mRNA encoding Ornithine transcarbamylase	LNP	IV	Translate Bio, Inc.	I/II	NCT03767270
MRT5005	Human Cystic fibrosis	mRNA encoding CFTR	LNP	INH	Translate Bio, Inc.	I/II	NCT03375047
NTLA-2001	Transthyretin amyloidosis with polyneuropathy	CRISPR/Cas9 gene editing system	LNP	IV	Intellia Therapeutics	I	NCT04601051
Other RNA-based therapeutics							
MTL-CEBPA	Hepatocellular carcinoma	CEBPA-51 saRNA targeting CEBPA	Liposome (POPC/DOPE/MoChol/CHEMS)	IV	MiNA Therapeutics	I	NCT02716012
BP1001	AML, ALL, MDS, and CML	ASO targeting Grb2 mRNA	Liposome (DOPC/Tween 20)	IV	Bio-Path Holdings, Inc.	II	NCT02781883
LErafAON-ETU	Advanced cancer	ASO targeting C-raf	LNP	IV	INSYS Therapeutics	I	NCT00100672

Microinjection involves direct injection of RNAs into the cytosol using a glass micropipette.[111−113] Electroporation employs an electric field, transiently increasing cell membrane permeability, to import RNAs from extracellular compartments into cells.[114−116] Sonoporation leads to a perforated cell membrane using ultrasound waves.[117−119] Photoporation applies a focused laser beam to produce a submicron hole in cell membranes, which is most commonly used to treat single cells.[120,121] Magnetofection involves attaching RNA with cationic magnetic nanoparticles. These nanoparticles are concentrated into target cells under a magnetic field.[122,123] Hydroporation is the hydrodynamic capillary effect that can create pores in the cell membrane to allow entry of RNAs.[124] Microfluidic squeezing is a microfluidic membrane deformation technique for delivering macromolecules in the surrounding medium into cells by forming transient pores in the plasma membrane.[125−129] This technique shows low effects on the normal functions of cells and is broadly applicable for the cytosolic delivery of various macromolecules (e.g., RNA, carbon nanotubes, proteins, quantum dots) to different types of cells.[130]

1.3.2. Biological Carriers. Biological carriers are delivery vehicles obtained from living organisms including extracellular vesicles (EVs) and cell/cell membrane-based vehicles, such as exosomes-based vehicles, red blood cells extracellular vesicles (RBCEVs), platelet membrane-coating vehicles, red blood cell (RBC) membrane coating nanoparticles, cancer cell membrane-coated nanoparticles, and macrophage-based vehicles.[131−136] These carriers can protect RNA cargos from the degradation by RNase and early clearance by the immune system.[137,138]

Extracellular vesicles (EVs) are important mediators involved in intercellular communications, which are cell-derived membranous nanosized particles with a lipid bilayer membrane.[139−142] Based on their size, surface markers, and mode of biogenesis, EVs are classified into three classes: exosomes (40−120 nm), microvesicles (100−500 nm), and apoptotic bodies (800−5000 nm).[143−145] EVs have been applied as an RNA delivery system due to their characteristics such as high biocompatibility, long circulation time, and low toxicity.[146−148] Exosomes are considered as "nature's delivery system", as it has been shown that exosomes naturally transport DNAs and RNAs between cells, inducing genetic modifications in both biological and pathogenic processes.[149−151] Accumulating interests have been focused on harnessing exosomes as vehicles for siRNA[152−155] and miRNA[156−159] delivery to induce gene silencing.[145,160−162] mRNA-loading exosomes have shown tumor-suppressor function in orthotopic phosphatase and tensin homologue (PTEN)-deficient glioma mouse models.[163] EVs released from mature red blood cells (RBCEVs), for example, have been used as an RNA delivery system for miRNA inhibiting and CRISPR/Cas9 genome editing in xenograft mouse models.[164] RBCs are selected to produce EVs for RNA delivery because mature RBCs lack both mitochondrial and nuclear DNA,[165] so the risk of horizontal gene transfer is avoided. In previous studies, RBCEVs were loaded with ASOs, Cas9 mRNA, and sgRNAs or plasmids, respectively, and delivered these agents to target cells in both solid and liquid tumors.[164] For example, RBCEVs encapsulating miRNA-125b ASO significantly silenced miRNA-125b and reduced infiltrated cancer cells in acute myeloid leukemia (AML) MOLM13 engrafted mice. Cas9 mRNA and sgRNAs can be codelivered to MOLM13 cells using RBCEVs, inducing genome editing effects.[164] Platelet-derived microparticles (PMPs) are extracellular vesicles, 0.1−1 μm in diameter, that are involved in the

enhancement of angiogenesis, invasion, and metastasis of tumors.[166] PMPs have been shown to infiltrate solid tumors and deliver platelet-derived miRNA to tumor cells both *in vivo* and *in vitro*, resulting in gene silencing in tumor cells with broad tumor type specificity.[167] Macrophages are appealing carriers for solid tumor targeting RNA delivery due to their inherent capacities to home to tumors at significant numbers throughout tumor progression.[168,169] Moreover, macrophages can easily load and secrete nanoparticles into the surrounding microenvironment. Wayne et al. developed a macrophage-based targeted siRNA delivery system that delivered calcium integrin binding protein-1 (CIB1)-siRNA to MDA-MB-468 human breast cancer cells, leading to reduced expression of CIB1 and KI67 and decreased tumor growth.[170] Zhang et al. prepared platelet membrane-camouflaged PLGA/DOTAP nanoparticles to deliver anti-Pcsk9 siRNA efficiently, resulting in ∼28% reduction in the level of plasma LDL-C.[171]

1.3.3. Synthetic Approaches. Synthetic approaches have constructed numerous types of natural or synthetic materials and formulations for delivering RNAs into cells, including lipid-based nanocarriers, polymer-based systems, inorganic nanoparticles, nucleic acid nanostructures, chemically modified RNAs, and many others.[172−174]

Among the various synthetic approaches for RNA-based therapeutics, lipid-based nanocarriers have been recognized as one of the most promising RNA delivery systems.[175−177] These nanocarriers can be prepared in various forms such as cationic liposomes, ionizable lipid nanoparticles (LNPs), lipid−polymer hybrid nanoparticles (LPHNPs), lipid calcium phosphate (LCP) nanoparticles, niosomes, cationic nanoemulsions (CNEs), and neutral lipid nanoemulsions (NLEs).[178−183] Examples of clinical trials of lipid-based RNA therapies are summarized in Table 3. In the 1960s, Bangham et al. reported that biocompatible lipid/phospholipid spontaneously formed closed phospholipid bilayer structures in an excess of water, which was termed as liposome.[184,185] Liposomes contain an aqueous compartment that is surrounded by one or more phospholipid bilayers, which can serve as unique vehicles for the entrapment of hydrophilic drugs (e.g., Doxil)[186] and DNA.[187,188] Cationic liposomes are also among the earliest synthetic approaches used for RNA delivery.[189−191] The positive charges of the cationic lipid-based liposomes can improve the RNA encapsulation efficiency as the result of electrostatic interactions between the negatively charged phosphate backbone of RNA molecules and the positively charged head groups of cationic lipids. Generally, the nitrogen/phosphate ratio (N/P) is modulated so that the liposome has a net positive charge, thus neutralizing RNA molecules and avoiding aggregation of liposomes. Besides, the excess positive charge facilitates the binding of liposomes to the negatively charged cell membranes.[192] PEGylated cationic liposomes were developed to increase the circulation stability of liposomes, thus improving RNA delivery efficiency *in vivo*.[192,193]

Later on, researchers synthesized ionizable lipids with apparent pK_a values less than 7, which exhibit positive charges and interact with RNA molecules when protonated under acidic conditions, while they are neutral in physiologic conditions (pH = 7.4). Apart from ionizable lipids, PEG lipids and helper lipids are fundamental lipid components in the LNP formulations, such as DMG-PEG$_{2000}$, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and cholesterol.[194,195] Microfluidic mixing of solutions of lipid components in organic solvent with aqueous solutions is a readily scalable and precisely controlled technique
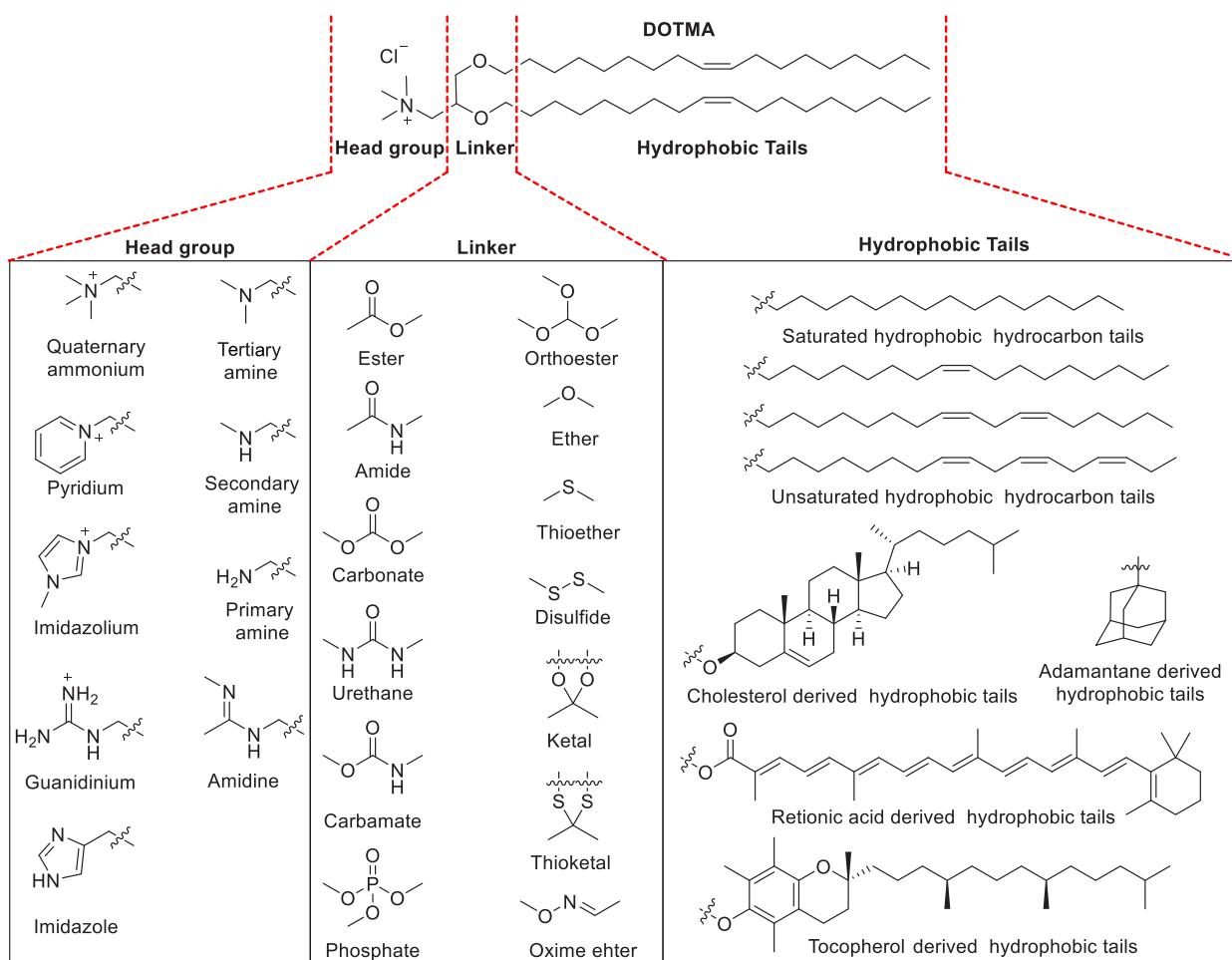
Figure 2. Chemical structures of the cationic lipid DOTMA and three segments of lipids.

for the preparation of LNPs.[196−198] Different from cationic liposomes, typical LNPs only have a single phospholipid outer layer encapsulating the electron-dense core, where the ionizable lipids aggregate into inverted micelles around the entrapped RNA molecules.[199−201] Additionally, under acidic conditions of the endosomes, these ionizable lipids are protonated and can bind to the negatively charged endosomal membranes, inducing endosome disruption and resulting in enhanced endosomal escape.[202] These characteristics make lipid nanoparticles important materials for RNA delivery. In terms of clinical research, lipid nanoparticles (LNPs) are the most advanced synthetic approaches for RNA therapies for treating a range of diseases up to date.[203] The approvals of patisiran,[204,205] BNT162b2,[8,9] and mRNA-1273[7,8] in the clinical application are milestones in the development of LNP-based RNA delivery.

In addition to lipid nanoparticles, both naturally derived and synthetic polymers have been utilized for RNA delivery, such as poly(ethyleneimine) (PEI),[206,207] poly-L-lysine (PLL),[208−210] poly(β-amino ester) (PBAE),[211−213] chitosan,[214−216] and polysaccharide.[217] By tuning the physiochemical characteristics of polymers, efficient RNA delivery can be achieved in cell and animal models.[218−220] A broad range of inorganic nanoparticles have also been explored as carriers for the controlled and targeted RNA delivery, such as gold nanoparticles,[221−223] silica nanoparticles,[224−226] calcium phosphate nanoparticles,[227−229] and iron oxides nanoparticles.[230−232] DNA and RNA strands are versatile building blocks for creating functional nucleic acid

nanostructures with structural programmability, spatial addressability, molecular recognition capability, and biocompatibility.[233−236] Over the past several decades, nucleic acid-based nanotechnology has made great achievements in various applications.[237−246] For example, DNA and RNA nanostructures have been used in the delivery of ASOs and siRNA.[236,238,243,246−253] Additionally, researchers have developed numerous chemical strategies of RNA conjugation, which can improve the RNA-binding affinity, thermostability, circulation time, and pharmacokinetic properties of RNA.[254−260] A chemically conjugated RNA is a direct covalent conjugation of an RNA molecule and various moieties that promotes intracellular uptake, targets the drug to specific cells/tissues, or reduces clearance from the circulation. These moieties include lipids (e.g., cholesterol,[261] α-Tocopherol[262,263]), peptides (e.g., cell-penetrating peptide[264,265]), aptamers,[266−268] antibodies,[269−271] and receptor ligand.[272−276] The conjugation of siRNA and N-acetylgalactosamine (GalNAc) increases the cellular internalization in the liver through interactions of the GalNAc with the asialoglycoprotein receptor (ASGPR) on the surface of hepatocytes.[259,277,278] This hepatocyte-specific delivery platform has led to the clinical use of givosiran,[29,30] inclisiran,[32] and lumasiran.[33,34]

In this review article, we focus on the chemical perspectives of lipids including a variety of lipid derivatives and lipid-derived macromolecules used in lipid-based RNA delivery systems over the past three decades. We summarize the advances of lipids,
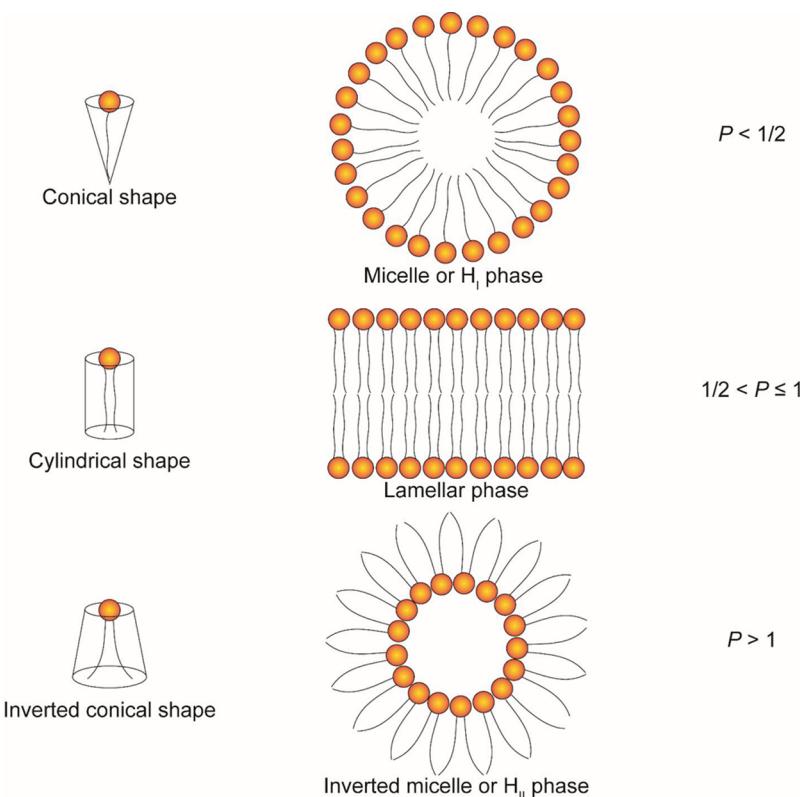
Figure 3. Schematic illustration of the shape structure concept of lipids.

lipid derivatives, and lipopolymers regarding their chemical structures, synthetic routes, characterizations, and structure–activity relationships. We also briefly introduce the status of representative preclinical and clinical studies and highlight future opportunities and challenges.

## 2. CATIONIC, IONIZABLE, AND ZWITTERIONIC LIPIDS

Cationic or ionizable lipids are of great importance for the delivery of RNAs because their positively charged head groups under the formulation environment can interact with the negatively charged phosphate backbone of the RNA cargos.[279,280] In 1978, Dimitriadis reported the delivery of rabbit globin mRNA into mouse lymphocytes *ex vivo* using phosphatidylserine-based unilamellar liposomes.[189] In 1987, Felgner et al. synthesized the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA, Figure 2) and used it for *in vitro* gene delivery.[281] The encapsulation efficiency of DOTMA-based liposomes to pDNA is about 100%, and their pDNA delivery efficiency is 5–100 times higher than that of calcium phosphate or diethylaminoethyl-dextran.[281] In 1989, Malone et al. developed DOTMA-based liposomes (Lipofection) for *in vitro* delivery of luciferase mRNA into NIH 3T3 mouse cells.[191]

Structurally, synthetic lipids usually contain three parts: (i) cationic or ionizable head groups, (ii) linker groups, and (iii) hydrophobic tails (Figure 2).[282,283] The head groups exhibiting positive charge(s) can interact with the negatively charged RNA backbone through electrostatic attractions; in this way complexes containing condensed RNA are formed. The lipids and lipid derivatives can be classified into various categories based on the characteristics of their head groups: (i) cationic lipids, (ii) ionizable lipids, and (iii) zwitterionic lipids.[282−285] The structure of the hydrophobic tails of lipids can affect their

pK_a, lipophilicity, transition temperature, and potency for RNA delivery.[105,286] A cholesterol derivative or a hydrocarbon chain or even a tocopherol derivative can act as a hydrophobic component of lipids. The hydrocarbon tails are generally between 8 to 18 carbon units in length with various unsaturation degrees (e.g., oleoyl group, linoleoyl group), and symmetry is not necessary for them.[280] Incorporation of unsaturated fatty acid as lipid tails has resulted in higher delivery efficiency in certain formulations, possibly owing to their low transition temperature and their influence on increasing membrane fluidity.[287] An ideal linker group should be biodegradable and preserve strong circulation stability to survive in a biological environment. The commonly employed linker groups include ethers and esters, phosphate or phosphonate linker, glycerol-type moiety, or peptides. Carbamate and amide are also frequently used as linker, as both of them are chemically stable and biodegradable. Ester and ether are alternative linkers, which are chemically stable. The linker groups can be designed to be tunable; thus, they are stable enough for storage and have higher circulation stability but can be degraded rapidly at the target sites to facilitate the release of the RNA payload.

Geometry is an important characteristic of amphiphile lipids with regard to their application as RNA carriers. Amphiphile lipids form aggregates above a certain concentration in an aqueous environment, adopting various structures, including the micellar phase, hexagonal (H_I) phase, lamellar phase, inverted micellar phase, and inverted hexagonal (H_{II}) phase (Figure 3). These different types of structures can be predicted by the packing parameter P of the lipid, which is defined as the ratio of the amphiphile lipid volume (V) to its head group area (a), and the critical tail length (l_c) ($P = V/al_c$).[288−290] When P is less than 1/2, the conical-shaped amphiphile lipids assemble into micelles or a hexagonal (H_I) phase. When $1/2 < P \leq 1$, cylindrical-shaped
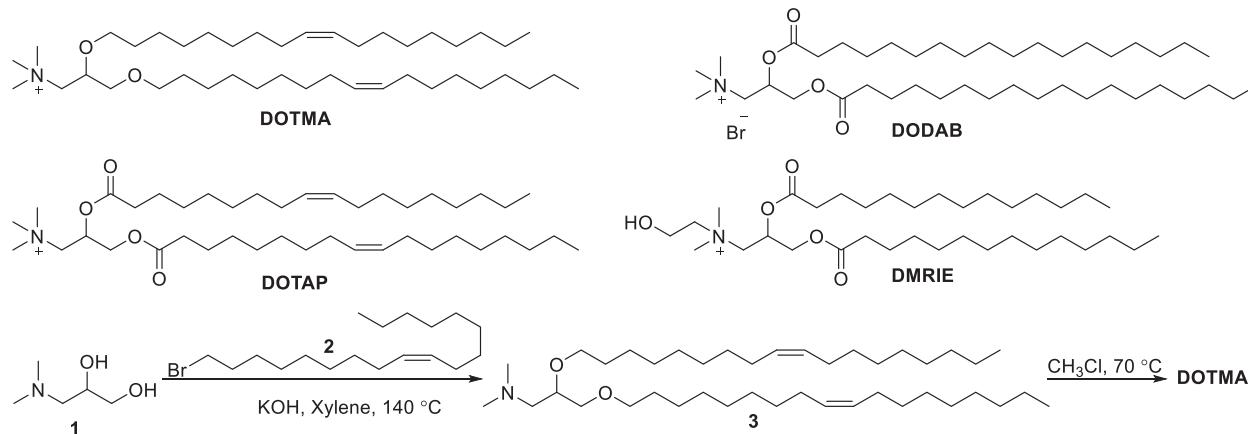
Figure 4. Chemical structures and synthesis of DOTMA and its analogs.

amphiphile lipids with a curvature close to 0 adopt the stable lamellar phase. The inclusion of lipids with a cylindrical shape, such as DSPC, increases the stability and circulation time of lipid-based nanoparticles.[291] When $P > 1$, the structures formed by the inversed conical-shaped amphiphile lipids tend to adopt inverted micelles or inverted hexagonal (H_{II}) phases. Thus, when $P > 1$, the inverted conical-shaped lipids (e.g., DOPE) can destabilize the endosomal membrane and allow the endosomal escape and release of the RNA payload into the cytosol of the target cells.[286,292,293] This section describes a large number of lipid derivatives with their chemical structures, synthetic routes, and RNA delivery properties.

2.1. Cationic Lipids

Cationic lipids refer to lipids with head groups bearing permanent positive charges. They have been well-explored for nucleic acids (DNA and RNA) delivery as components of liposomes and lipoplexes, due to their capability of encapsulating nucleic acids. According to the chemical structures of their head groups, they are grouped into four types in this part: quaternary ammonium lipids, guanidinium lipids, pyridinium lipids, and imidazolium lipids. Cationic lipids are permanently positively charged and are chemically stable even in the environment of strong oxidants and acids. However, the positive charges might lead to potential cytotoxicity, e.g., hemolytic and undesired immunostimulation.[294,295] Cytotoxicity of cationic lipids may be related to the generation of reactive oxygen species (ROS) and the increase of cellular calcium levels.[296,297] Besides, the positive charge of cationic lipids could result in their rapid plasma clearance and short circulation time.[294,295] It is noteworthy that cationic lipids with delocalized positive charges, such as pyridinium, imidazolium, and guanidinium, showed lower cytotoxicity as compared to quaternary ammonium lipids.[298−301] In certain cases, cationic lipids may act as vaccine adjuvants by taking advantage of their inflammatory effects.[302]

2.1.1. Quaternary Ammonium Lipids. Ever since the 1980s, numerous cationic lipids with quaternary ammonium head groups have been developed for the delivery of DNA and RNA, such as DOTAP, DMRIE, and DODAB, which were reported to be effective for RNA delivery (Figure 4).[303] As the purification of positively charged compounds is relatively challenging, the cationic head groups are preferentially formed via quaternization of the corresponding tertiary amines in the final step of the synthesis.[304−306] The synthesis of DOTMA, for example, began with the combination of amino alcohol **1** with

oleyl bromide **2** via ether bond formation; then quaternization of the resulting amine **3** with chloroform under reflux condition gave DOTMA.[281] In 2016, Kranz et al. developed a DOTMA/DOPE LNPs-mRNA vaccine that specifically targeted dendritic cells (DCs) *in vivo* by changing the surface charge from positive to slightly positive or neutral. The optimized vaccine induced specific immune responses following intravenous administration.[307] This DOTMA/DOPE LNPs formulation has also been used to deliver mRNA containing 1-methylpseudouridine (m1Ψ) instead of uracil for precision therapy of autoimmune diseases in mice.[308] In 2020, Reinhard et al. engineered T cells by utilizing DOTMA-based LNPs to deliver mRNA encoding a single-chain variable fragment (scFv) that could specifically recognize the overexpressed cancer cell surface protein claudin 6 (CLDN6). The resulting CAR-T cells led to improved regression in mouse models of intractable tumors, such as CT26 colon carcinoma mouse models.[309] In a recent phase 1 clinical trial of an mRNA vaccine for melanoma (melanoma FixVac), DOTAP-based LNPs were formulated to encapsulate mRNA encoding four tumor associated antigens (TAAs) that show high prevalence in melanoma; mRNA molecules were delivered into immature DCs in lymphoid tissues, driving TAA presentation on both MHC I and MHC II molecules after intravenous administration. Patients showed TLR activation, increased body temperature, elevated cytokines level in plasma, and specific response against at least one TAA after vaccination.[310] In 2017, Cheng et al. used LNPs formulated with DOTAP/Cholesterol/eggPC/Tween 80 at a molar ratio of 25:20:50:5 to deliver G3139, an antisense oligonucleotide, into A549 lung cancer cells, resulting in 40% knockdown of bcl-2 mRNA and approximately 83% reduction of the bcl-2 protein level, respectively.[311] In 2019, DOTAP LNPs encapsulating h*ARG1* mRNA were used to treat arginase deficiency in inherited metabolic liver disorder, achieving 54% of normal hepatic arginase 24 h after administration in mice.[312] Cationic liposomes can act as immunomodulators that stimulate the innate immune response in some cases.[313] For example, DOTAP-based cationic liposomes have been used as a vaccine adjuvant.[302] The immunostimulatory effects of the components of cationic liposomes were related to the length and saturation degree of hydrophobic tails. Lipids possessing unsaturated tails or short saturated tails may be stronger immunomodulators than lipids with long saturated tails.[313] Cationic liposomes formulated with DOTAP/DSPC/cholesterol were shown to be capable of activating Toll-like receptor 4 (TLR4), inducing a
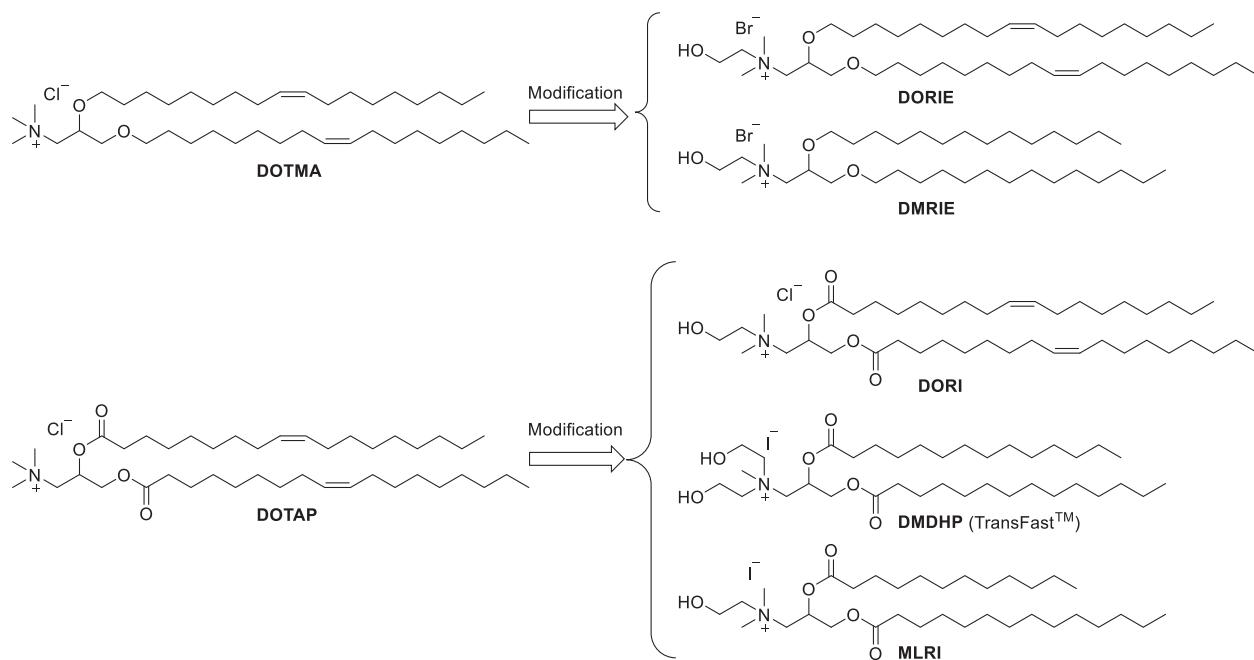
Figure 5. Chemical modifications of DOTMA and DOTAP by introducing hydroxyethyl groups
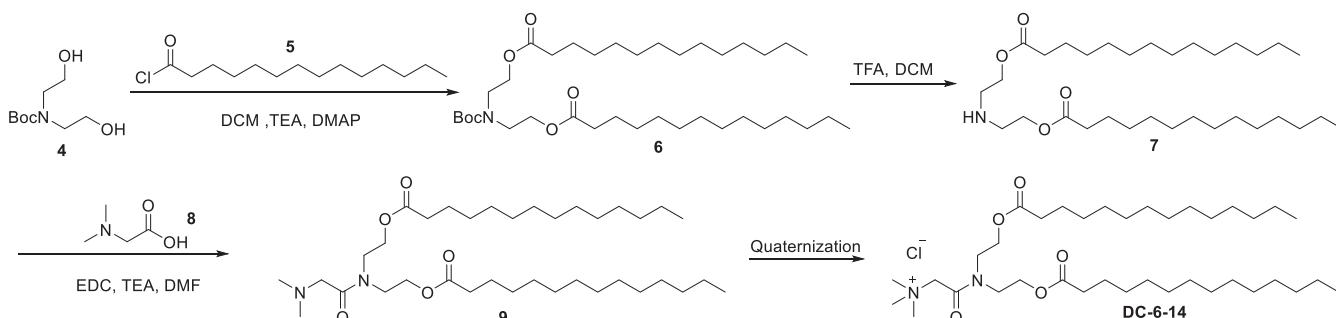
Figure 6. Chemical structure and synthetic route of DC-6-14.

greater pro-inflammatory response with enhanced Th1 cytokines expression in mice compared with ionic liposomes formulated with DSPG/HSPC/cholesterol.[314] DOTAP-based cationic nanoemulsions (CNEs) have also been used as vectors for mRNA delivery.[315−320] For instance, CNEs formulated with DOTAP/ sorbitan trioleate/polysorbate 80/squalene were reported to deliver an mRNA vaccine against several viral and bacterial infections in nonhuman primates with two doses of 75 μg.[317] In a follow-up study, CNEs encapsulating the HIV Type 1 envelope protein mRNA were shown to be well-tolerated and immunogenic in nonhuman primates.[318] Additionally, DOTAP was incorporated to prepare lipid−polymer hybrid nanoparticles (LPHNPs), which are composed of a biodegradable RNA-loaded polymer core surrounded by lipid/PEG-lipid layers.[321,322] LPHNPs combine the unique strengths of liposomes and polymeric nanoparticles but exclude some of their limitations such as short circulation time and structural disintegration.[323] Gao et al. formulated LPHNPs with DOTAP/ DOPE/cholesterol (25:43:25) and poly(amidoamine)/siRNA for siRNA delivery. The resulting LPHNPs effectively delivered T7-modified anti-EGFR siRNA to an MCF-7 tumor xenograft murine model and inhibited tumor growth.[324]

A hydroxyalkyl chain incorporated in the head group was capable of providing hydrogen bonding to neighboring head groups, thus decreasing the head group hydration and improving the encapsulation of nucleic acids via hydrogen bonding with the lipid. In the previous studies lipids used in DNA delivery, DORIE and DORI, were obtained by replacing one of the methyl groups in the head groups of DOTMA and DOTAP with a hydroxyethyl group, respectively, and both of them exhibited greater DNA delivery activity than DOTMA or DOTAP (Figure 5).[325,326] DMDHP and MLRI were synthesized as analogs of DOTAP for mRNA delivery.[327,328] MLRI, an asymmetric analog of DORI, contains a myristoyl group and a lauroyl group as the hydrophobic tails. Results indicated that mRNA/cationic lipid lipoplexes formulated with MLRI or DMDHP could protect mRNA from degradation by RNases in human cerebrospinal fluid (hCSF) for at least 4 h.[327]

In 1999, Kikuchi et al. developed a series of quaternary ammonium lipids and identified DC-16-4 as the lead cationic lipid for delivering pDNA to human peritoneal disseminated tumors both *in vitro* and *in vivo*.[329] As shown in Figure 6, the hydrophobic tails were installed via acylation of diol **4** with myristoyl chloride **5**, and the head group was installed via an amide bond formation followed by quaternization of the tertiary amine group. In 2008, Sato et al. reported the delivery of gp46 siRNA using vitamin A-coupled DC-6-14 LNPs in rats, resulting in effective treatment of liver fibrosis and prolonged survival
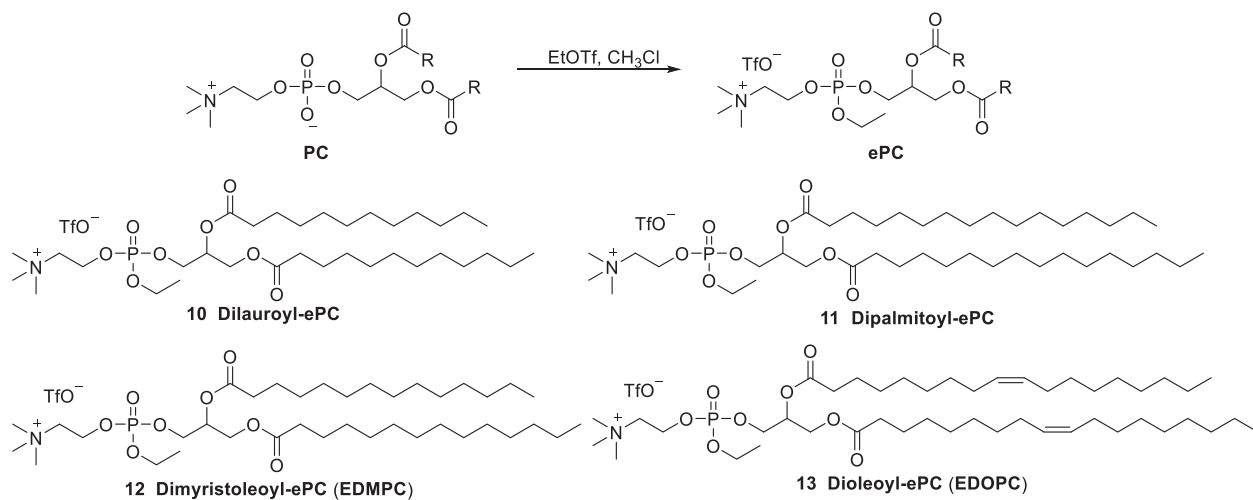
Figure 7. Chemical structures and general synthetic route of ethylphosphatidylcholines (ePCs).
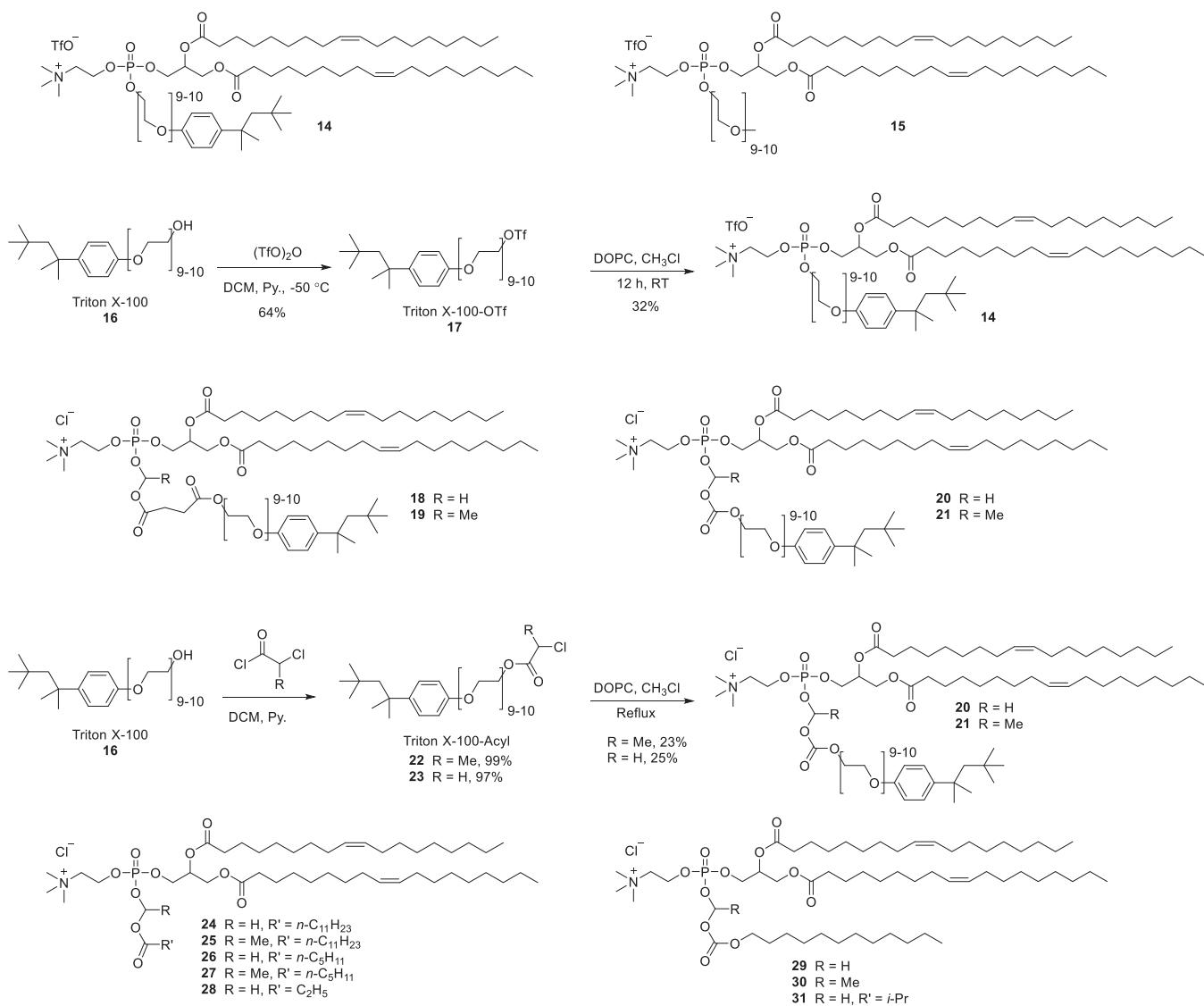

Figure 8. Representative chemical structures and synthetic routes of DOPC-based lipids.

time by targeting hepatic stellate cells.[330] ND-L02-s0201 is another vitamin A-coupled DC-6-14 LNP encapsulating siRNA targeting heat shock protein 47 (HSP47), which is involved in

the fibrosis of the liver. Results of the phase I clinical study of ND-L02-s0201 showed that intravenous administration of ND-
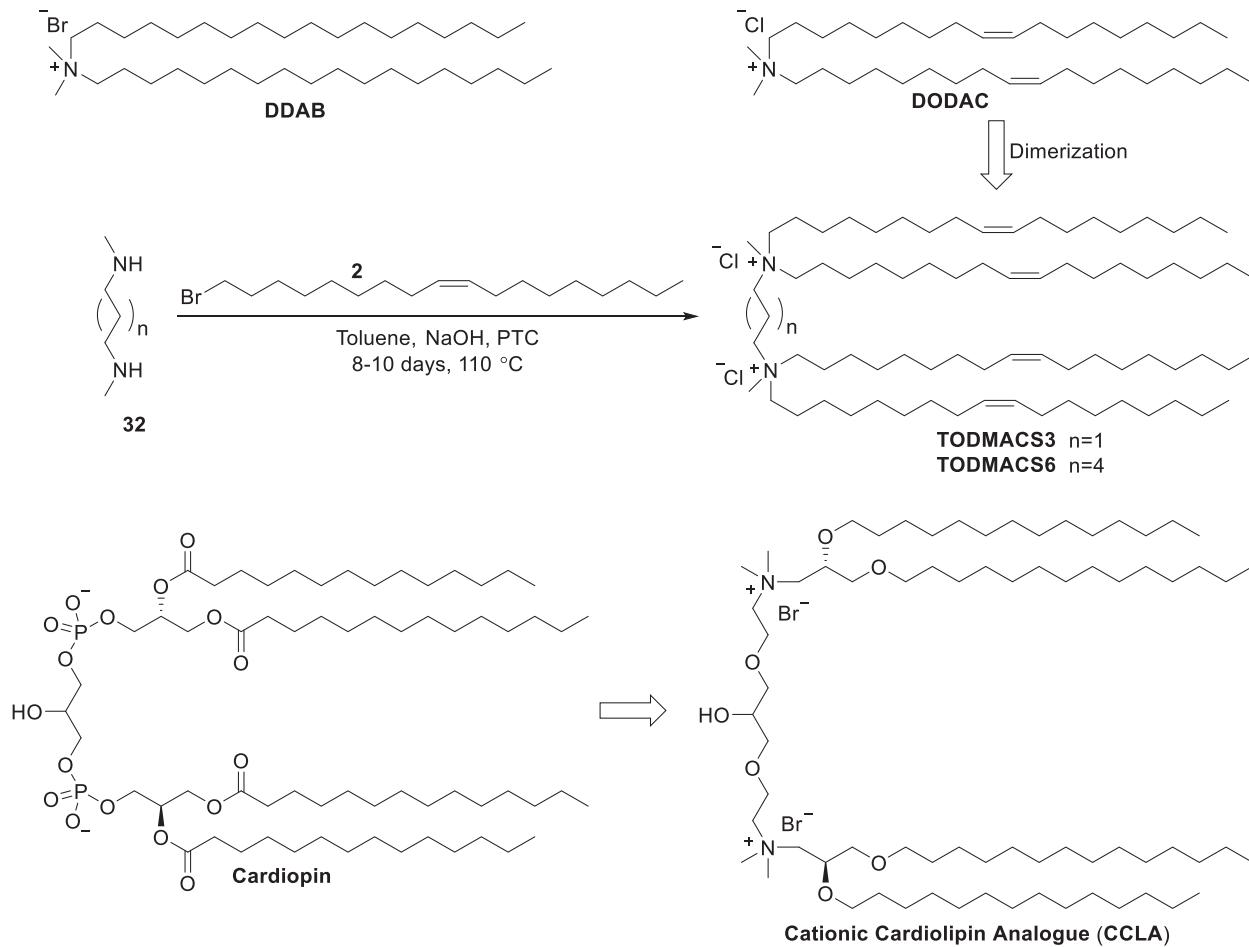
Figure 9. Synthesis of gemini diquaternary ammoniums TODMACS3, TODMACS 6, and CCLA.

L02-s0201 at a siRNA dose of 90 mg for 3 weeks was well-tolerated in healthy adults.[331]

In 1997, Gorman et al. reported that dimyristoyl-*sn*-glycero-3-ethylphosphocholine (EDMPC, **12**), a phosphotriester derived from phosphocholine, was able to mediate efficient gene delivery.[332] Then, Macdonald et al. found that phosphotriesters were slowly metabolized by intracellular phospholipases in endosomes and lysosomes and showed low cytotoxicity.[333] In 1999, Macdonald et al. developed a series of alkyl phosphatidylcholine triesters, the cationic ethylphosphatidylcholines (ePCs), by introducing a third alkyloxy group, through substitution reaction between the phosphoric acid and ethyl trifluoromethylsulfonates (Figure 7).[334] This method represents a straightforward way to convert zwitterionic phospholipids to cationic phospholipids via a simple substitution reaction. This transformation not only eliminates the negative-charge of phosphatidylcholines but also reduces their hydrogen bond accepting potential. This class of cationic lipids has shown effective delivery of *p*DNA both *in vitro* and *in vivo* for anticystic fibrosis and antitumor gene therapies.[335−338] Dimyristoleoyl-ePC (EDMPC) **12** was identified as the most efficient ePC that could be used for delivering GFP siRNA into breast cancer cells.[339] The structure−activity relationship of the ePCs showed that the high siRNA delivery efficiency of dimyristoleoyl-ePC (EDMPC) **12** stems from its high fusogenicity and ability to induce inverted hexagonal (H$_{II}$) phases.[339] In 2017, a lipopolyplex mRNA vaccine was prepared by trapping the mRNA/PBAE core in a bilayer lipid shell containing EDOPC/DOPE/

DSPE-PEG. This mRNA vaccine showed adjuvant activity by stimulating the expression of INF-β and IL-12 in dendritic cells. Subcutaneous administration of this mRNA vaccine resulted in a reduction of tumor nodules by 90% in mice with lung metastatic B16-OVA tumors.[340] In 2019, Zhang et al. prepared LNPs with dipalmitoyl-ePC **11** and cholesterol at a molar ratio of 70:30 for delivering mRNA.[341] Results indicated that these LNPs could deliver mRNA encoding an anti-RAS antibody into a range of human cancer cells.[341]

The membrane-disruptive properties of detergent (e.g., Triton X-100 (TX100)) are considered for improving nucleic acids delivery. Pierrat et al. synthesized a panel of cationic phospholipid−detergent conjugates by covalently attaching a detergent molecule (such as Triton X-100 (TX100)) to DOPC (Figure 8).[342] Conjugate **14** was able to deliver luciferase siRNA into mammalian cell cytosol without helper lipids, but its application was limited by its high toxicity. To address the toxicity issue, DOPC and Triton X-100 were conjugated through linker groups showing various chemical and biological stabilities (Figure 8).[342] Results showed that conjugates **20** and **21** obtained by replacing the phosphoester bond of conjugate **14** with a phospho(alkyl)enecarbonate group showed no loss of siRNA delivery activity, whereas the cytotoxicities of conjugates **20** and **21** were significantly decreased. The conjugates incorporating the succinate moiety (conjugates **18** and **19**) showed even lower toxicity along with a reduced siRNA delivery efficiency. The low siRNA delivery efficiency of conjugates **18** and **19** may be attributed to their more labile chemical
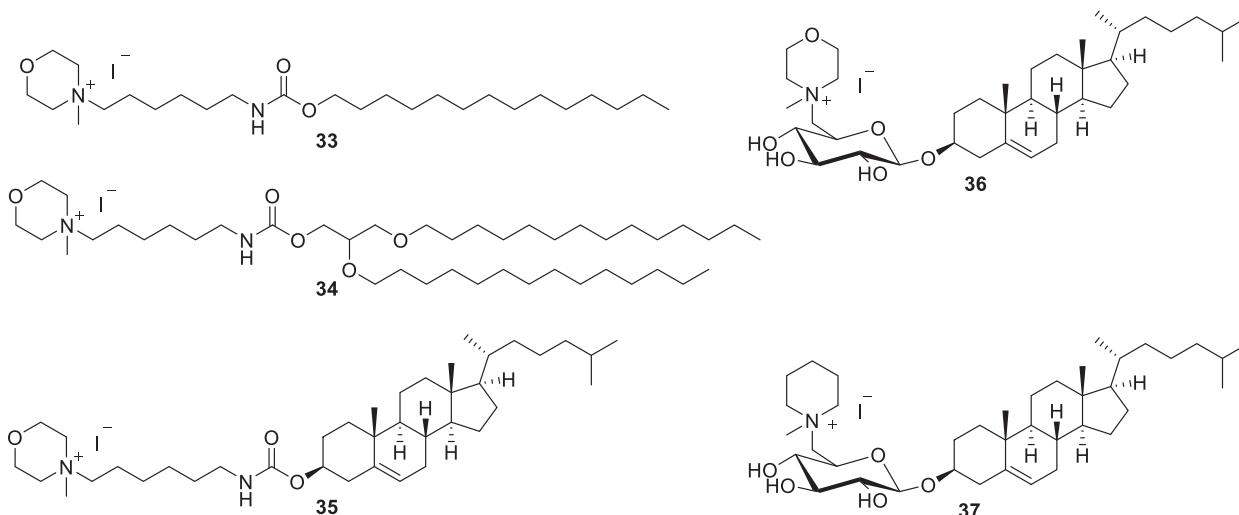
**Figure 10.** Chemical structures of piperidinium and morpholinium lipids.

structures. Another such series of conjugates were synthesized by coupling DOPC with low molecular weight alcohol or carboxylic acids.[343] Lipids **30** and **31** efficiently delivered luciferase siRNA into U87 cells, provoking up to 80% of luciferase gene knockdown, whereas the other lipids (**24**−**28**) were inactive.[343]

Didodecyldimethylammonium bromide (DDAB) (Figure 9) was previously used to prepare nanoparticles as gene delivery vectors, such as cationic liposomes,[344] niosomes,[345] and LNPs.[346] In 2011, lipid nanoparticles prepared with DDAB/MOG/siRNA showed effective siRNA delivery and induced gene silencing *in vitro*.[347] DDAB-based liposomes have also been used in delivering siRNA to lung metastasized tumor following systemic injection[348] and siRNA delivery targeting dendritic cells.[349] The quaternary ammonium lipids can be further modified by introducing a second quaternary ammonium group, giving cationic gemini lipids (Figure 9).[350,351] In 2001, Rosenzweig et al. developed a class of diquaternary ammonium lipids for DNA delivery via quaternization of their corresponding tetramethyldiamines with alkyl halides.[352] A class of dimers of DODAC were synthesized by the Cullis group, among which TODMACS6 exhibited the best delivery ability. Results suggested that the second quaternary ammonium group may strengthen the interactions with DNA, and the delivery efficiency could be tuned by the length of the linker between the two head groups.[353] Cardiolipin is a class of natural phospholipids that exists mainly in the heart and skeletal muscles.[354] In 2005, Kasireddy et al. synthesized a series of gemini quaternary ammonium cardiolipin analogs (CCLAs) by replacing the two negatively charged phosphate groups of cardiolipin with two quaternary ammonium groups (Figure 9).[355] The CCLA-based liposomes, formulated with CCLA/DOPA at a ratio of 1:2, delivered c-raf siRNA efficiently both *in vitro* and *in vivo*, inducing up to 62% of tumor growth repression in mice.[356] A CCLA-based liposome encapsulating anti-Raf-1 siRNA, designated as NeoPhectin-AT, was shown to repress Raf-1 gene expression and concomitantly downregulate cyclin D1 gene expression.[357]

Previous reports suggested that the incorporation of carbohydrate in cationic lipids increases the stability of DNA-loading cationic liposomes, decreases cytotoxicity, and enhances DNA delivery efficiency.[306,358] Besides, cholesterol is biologically compatible and able to stabilize membranes and form

stable liposomes. Maslov et al. synthesized a library of cholesterol-based cationic lipids with morpholinium, pyridinium, or imidazolium as their head groups (Figure 10). They also synthesized other cholesterol-based cationic lipids incorporating a carbohydrate residue with piperidinium, morpholinium, pyridinium, or imidazolium as the head groups.[359,360] LNPs formulated with lipid **37**/DOPE showed effective EGFP siRNA delivery *in vitro* and provided pronounced downregulation of EGFP expression in BHK cells.[348]

Nucleolipids are amphiphilic compounds that possess head groups that can recognize nucleic acids and hydrophobic tails.[361−364] Ceballos et al. developed a series of nucleoside lipids containing different bases attached at the anomeric position, a quaternary ammonium head group at the 5′, and two hydrophobic tails at the 2′ and 3′ positions. As for nucleolipids **38**−**43** (Figure 11), two oleoyls were installed to the 2′ and 3′ positions and 3-nitropyrrole, 5-nitroindole, or 4-nitroimidazole was attached at the anomeric carbon atom with different stereochemistry.[365−367] Nucleolipids **44** and **45** are cationic lipids with hydrophobic domains connected to the nucleosides via a ketal linker (**44**) or an orthoester linker (**45**), respectively.[367,368] The synthesis of nucleolipid **38** started with the selective protection of the hydroxy groups of D-ribose **46**, giving protected sugar **47**.[369] Next, the protected sugar **47** was stereoselectively chlorinated with hexamethylphosphorus triamide (HMPT) and CCl$_4$[370] followed by attachment of 3-nitropyrrole.[371] Removal of the TBS protecting group gave alcohol **49**, which was tosylated, desilylated, and coupled with oleic acids to afford compound **50**. The final quaternization reaction with trimethylamine afforded the nucleolipids **38**. In the case of nucleolipid **45**, the starting material 2′-deoxythymidine **53** was methanesulfonylated with methanesulfonyl chloride and the resulting compound underwent a coupling reaction with trihexadecyl orthoformate **52** promoted by pyridinium *p*-toluenesulfonate (PPTS).[372] siRNA delivery by nucleolipid **38** LNPs resulted in protein knockdown in several cell lines, such as hamster ovarian cells, mouse fibroblast cells, and human liver cells. In the following studies based on lipids **40**−**42**, results showed that both the stereochemistry at the anomeric carbon and the properties of the bases affected the formation of nucleolipids−siRNA complexes. Nucleolipid **45** LNPs were shown to be able to deliver the human RecQ helicase (RECQL4) siRNA into tumor cells. The cleavage of the
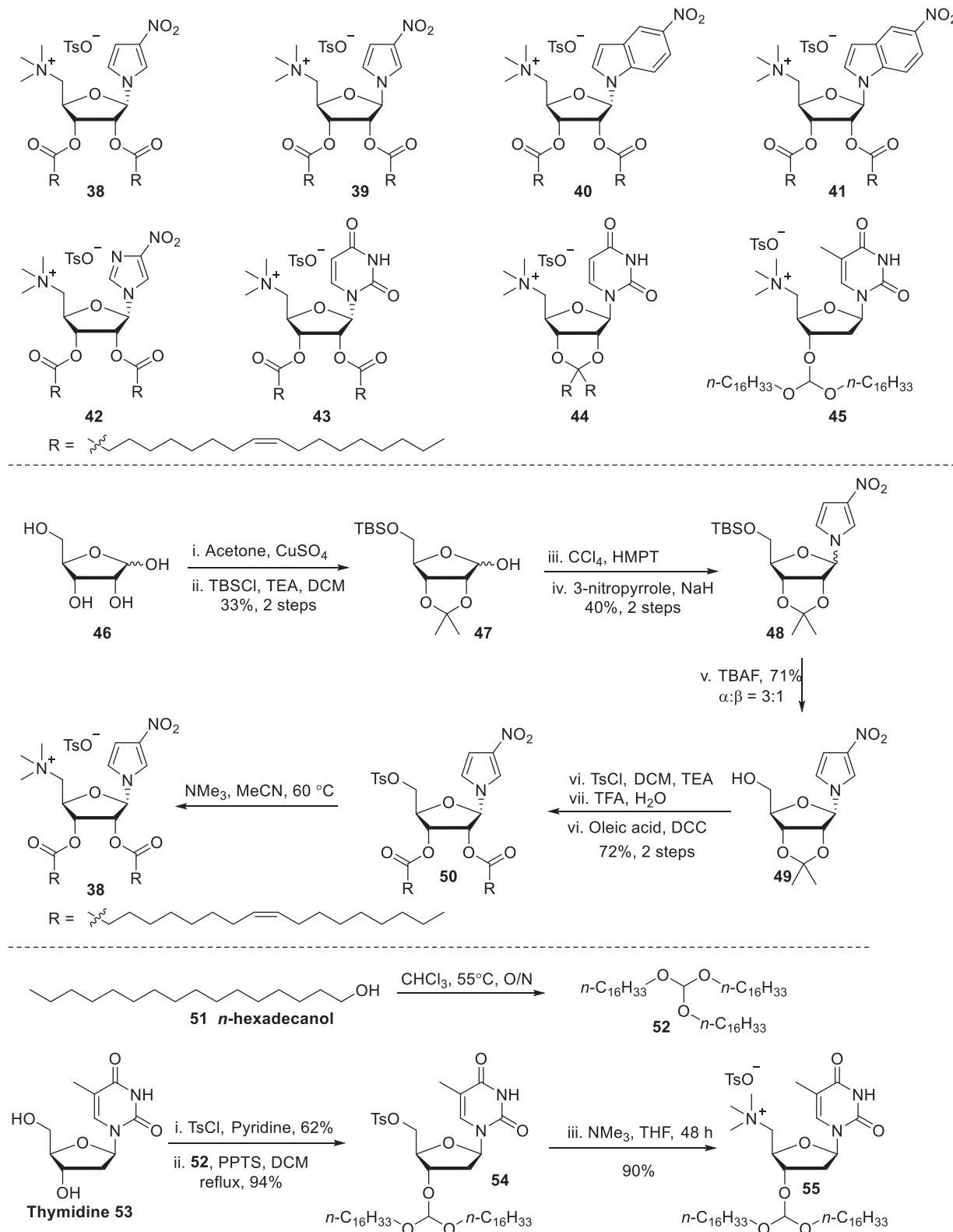
Figure 11. Chemical structures and synthetic routes of nucleoside derived lipids.

orthoester group might promote endosome escape via the *in situ* generation of fatty alcohol.[367,368]

As mentioned in previous sections, ePCs are quaternary cationic lipids with two nonrigid hydrophobic tails, while DC-Chol is an example of a lipid with a rigid cholesterol component that has been reported to be efficient in DNA delivery (Figure

12).[373,374] It was reported that rigid cationic lipids could self-assemble into tightly packed nanoparticles due to limited motional flexibility at the hydrophobic domain.[375] In 2012, Pungente et al. developed five carotenoids-derived single-tailed rigid cationic lipids, which were expected to be capable of self-assembling and delivering siRNA efficiently.[376] As shown in
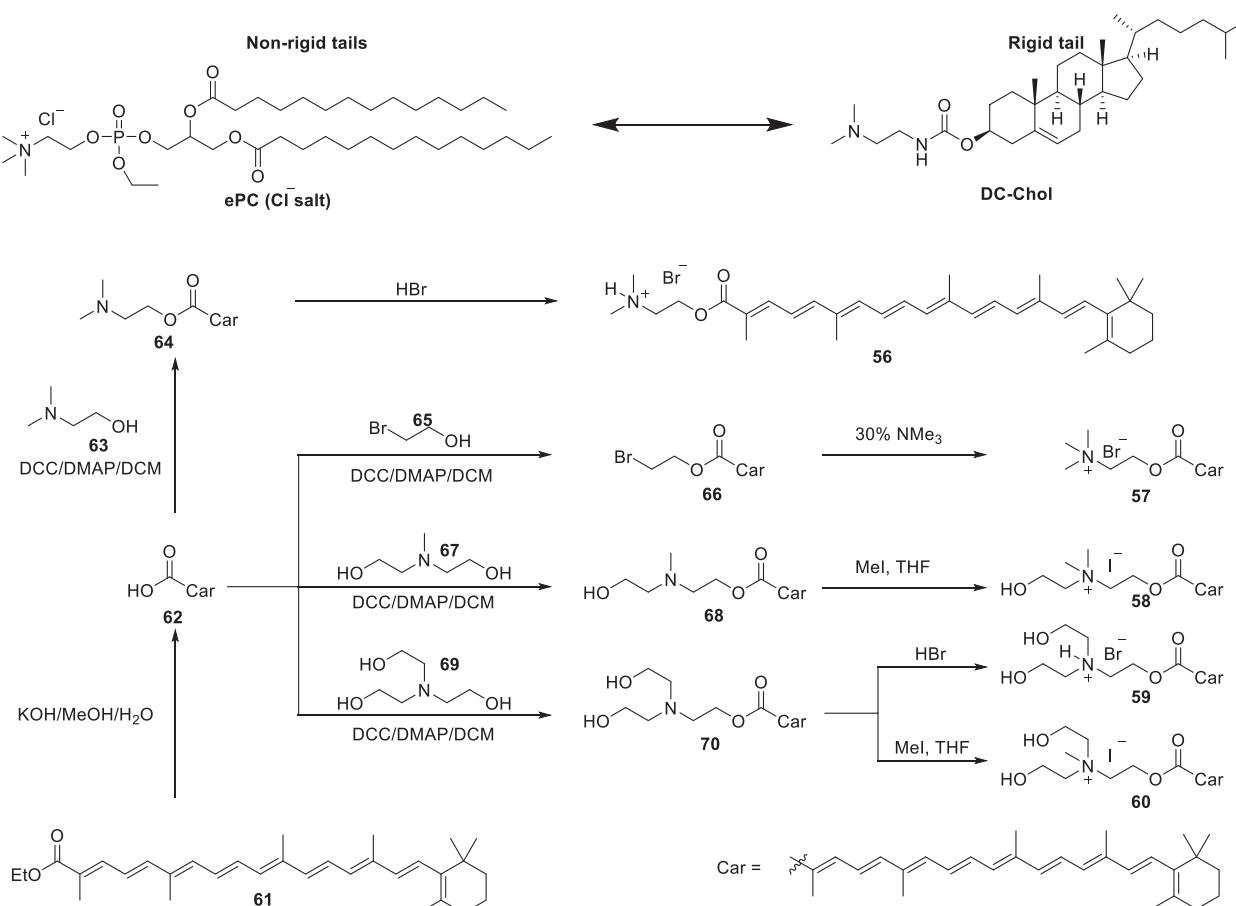
Figure 12. Chemical structures and synthetic route of carotenoid-derived lipids
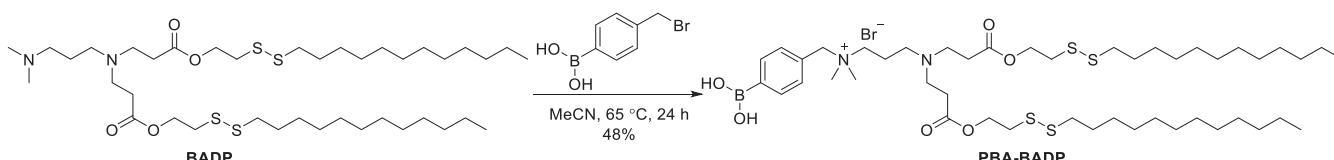

Figure 13. Chemical structure of PBA-BADP.

Figure 12, cationic lipids **56**−**60** contain the same rigid C30-carotenoid hydrophobic tails and different cationic head groups. The synthesis of these lipids started with the hydrolysis of ethyl-β-apo-8′-carotenoate **61**, affording β-apo-8′-carotenoic acid **62**. Then, these lipids were obtained via esterification followed by amination or quaternization. Results indicated that these single-tailed rigid cationic lipids were able to deliver siRNA to eukaryotic cells *in vitro*. Cationic lipids containing quaternary ammonium head groups with a hydroxyethyl moiety (lipids **58**−**60**) showed enhanced siRNA delivery efficiency. Additionally, introducing a second hydroxyethyl group to the head group (lipids **59** and **60**) increased the cytotoxicity without enhancing siRNA delivery efficiency.[376]

It is known that sialic acid (SA) is overexpressed on the surface of a variety of cancer cells[377,378] and phenylboronic acid (PBA) is an effective targeting ligand for improving cancer cell recognition and adhesion via interacting with SA.[379−381] In 2019, Tang et al. synthesized a cationic lipid incorporating a phenylboronic acid ligand, designated as PBA-BADP,[382] via quaternization of the bioreducible ionizable lipid BADP developed by Wang et al. (Figure 13).[383] PBA-BADP LNPs

showed an enhanced delivery of luciferase mRNA compared with BADP NPs in SA-overexpressing HeLa cervical cancer cells. Besides, PBA-BADP LNPs selectively delivered firefly luciferase (FLuc) mRNA to cancer cells including HeLa cells and DU145 cells rather than noncancer HK-2 and CCC-HPF-1 cells. PBA-BADP LNPs encapsulating Cas9 mRNA and HPV18E6 sgRNA induced 18.7% HPV18E6 gene knockout efficiency in HeLa cells.[382]

2.1.2. Guanidinium Lipids. Potential toxicity is one of the challenges for the application of quaternary ammonium-based delivery materials.[384,385] One strategy to overcome this issue is to delocalize the permanently positive charge of the cationic head group. Thus, cationic lipids with amidine, guanidinium, pyridinium, or imidazolium head groups are developed. Lipid diC14-amidine (Vectamidine) with an amidine head group is an early example of this class of lipids to delocalize the positive charge (Figure 14).[386]

Cationic lipids incorporating a guanidinium functional group represent another option for RNA delivery because guanidinium has the following characteristics: (a) The guanidine is a basic functional group with a pK_a value at about 13.5, so it can be

diC14-amidine (Vectamidine™)
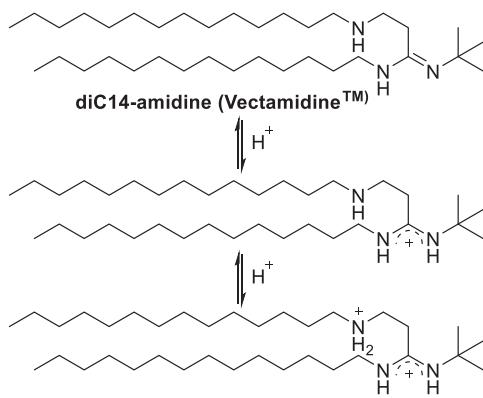
**Figure 14.** Chemical structure of vectamidine and the delocalization of the positive charge.

protonated over a wide range of pH environments, resulting in a permanently positively charged guanidinium head group at physical pH. (b) The guanidinium group interacts with the phosphate backbone of nucleic acids, thus facilitating their encapsulation.[387] (c) Hydrogen bonding between the guanidinium group and the RNA phosphate backbone can also enhance nucleic acids entrapment. (d) The guanidinium group binds with negatively charged proteoglycans on the cell membrane, thus enhancing cell uptake of the nanoparticles.[388−390]

Several groups have chosen arginine, a natural amino acid containing a guanidinium group, as the starting material to develop guanidinium type cationic lipids. In 2006, Santel et al. synthesized an arginine-derived guanidinium lipid AtuFECT01 (Figure 15).[391] Formulated with commercially available helper lipids DPhyPE and DSPE-PEG, the resulting siRNA-Lipoplex was delivered to the tumor endothelial cell following intravenous administration, resulting in reduced Tie2 and CD31 expression in the vasculature of mice.[391,392] Aleku et al. used Atu027 for the inhibition of protein kinase N3 (PKN3) in endothelial cells for the treatment of prostate and pancreatic cancers in mice;[393,394] the favorable preclinical data led to the clinical trial of Atu027.[395,396] A phase Ib/IIa study of synergistic therapy of pancreatic adenocarcinoma with Atu027 and gemcitabine showed this therapy for pancreatic carcinoma was safe and well-tolerated.[396] In 2009, Chen et al. synthesized a series of

guanidinium lipids which contain an L-lysine residue as well as a guanidinium functional group as the head group, such as DSGLA (Figure 15).[397] siRNA encapsulated in the LNPs containing DSGLA showed enhanced cellular uptake and induced stronger gene silencing in H460 tumor cells both *in vitro* and *in vivo* as compared to that formulated with DOTAP.[397] In their following work, they used liposome−polycation−DNA (LPD) nanoparticles containing DSAA to codeliver VEGF siRNA and Dox. Results showed that DSAA acted as an agent that increased the sensitivity of MDR cells to chemotherapy drugs and inhibited the expression of MDR transporters.[398]

In 2010, Mevel et al. reported the synthesis of several cationic lipids comprising a dialkyl glycyclamide or cholesteryl-moiety conjugated with a guanidinium head group (Figure 16).[399] The synthesis of DODAG-9 was accomplished in three steps. The starting *N*-Boc-glycine **71** was coupled with dioctadecylamine **72** via amide bond formation to give amide **73**, which was treated with TFA to remove the Boc protecting group, leading to the key glycine amide intermediate **74**. Conjugation of intermediate **74** with the guanidinylation reagent, 1H-pyrazole-1-carboxamidine monohydrochloride **75**,[400] in ethanol afforded DODAG-9. DODAG-9 was used to deliver anti-hepatitis B virus (HBV) siRNAs to the murine liver *in vivo*.[399]

In 2011, Adami et al. created a library of guanidinium type lipids termed DiLA^2 based on natural and modified arginine for siRNA delivery (Figure 17).[401] The amino groups and carboxyl groups on these compounds are reaction sites for attaching hydrophobic tails with various unsaturation degrees and lengths. A series of DiLA^2 analogs were synthesized based on nornorarginine, norarginine, L-arginine, and homoarginine; two sets of short hydrocarbon tails (C8 and C10) were attached to the α-amino group and the carboxyl group. *In vitro* screening of this panel of DiLA^2 led to the identification of norarginine as the desirable amino acid to build the DiLA^2 library. Then, a series of symmetric norarginine DiLA^2 was synthesized by incorporating hydrocarbon tails from C12 to C18, and the asymmetric C$_{18:1}$-norArg-C$_{16}$ was synthesized by incorporating a C18:1 tail along with a C16 tail.[401] C$_{18:1}$-norArg-C$_{16}$ was identified as the best-performing lipid in this library, which could deliver FVII siRNA efficiently, leading to 90% inhibition of FVII mRNA at a dose of 1 mg/kg after intravenous administration in

AtuFECT01

DSGLA

DSAA
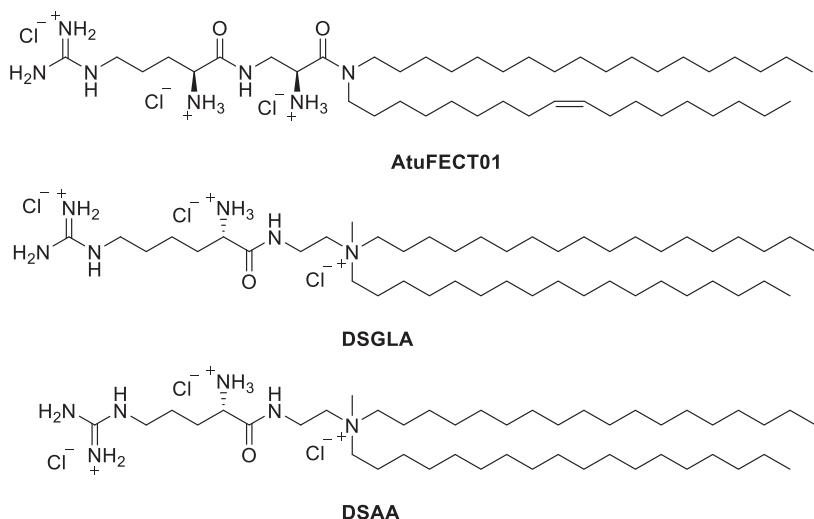
**Figure 15.** Chemical structures of AtuFECT01 and DSGLA.
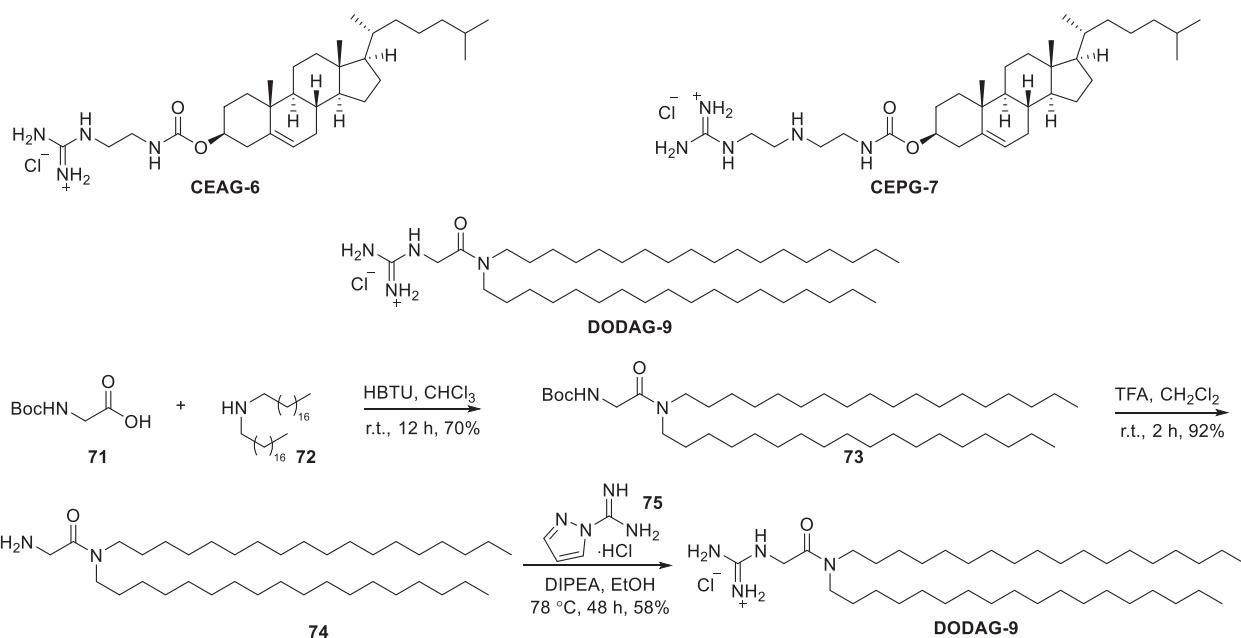
**Figure 16.** Chemical structures of guanidinium lipids and synthetic route of DODAG-9.
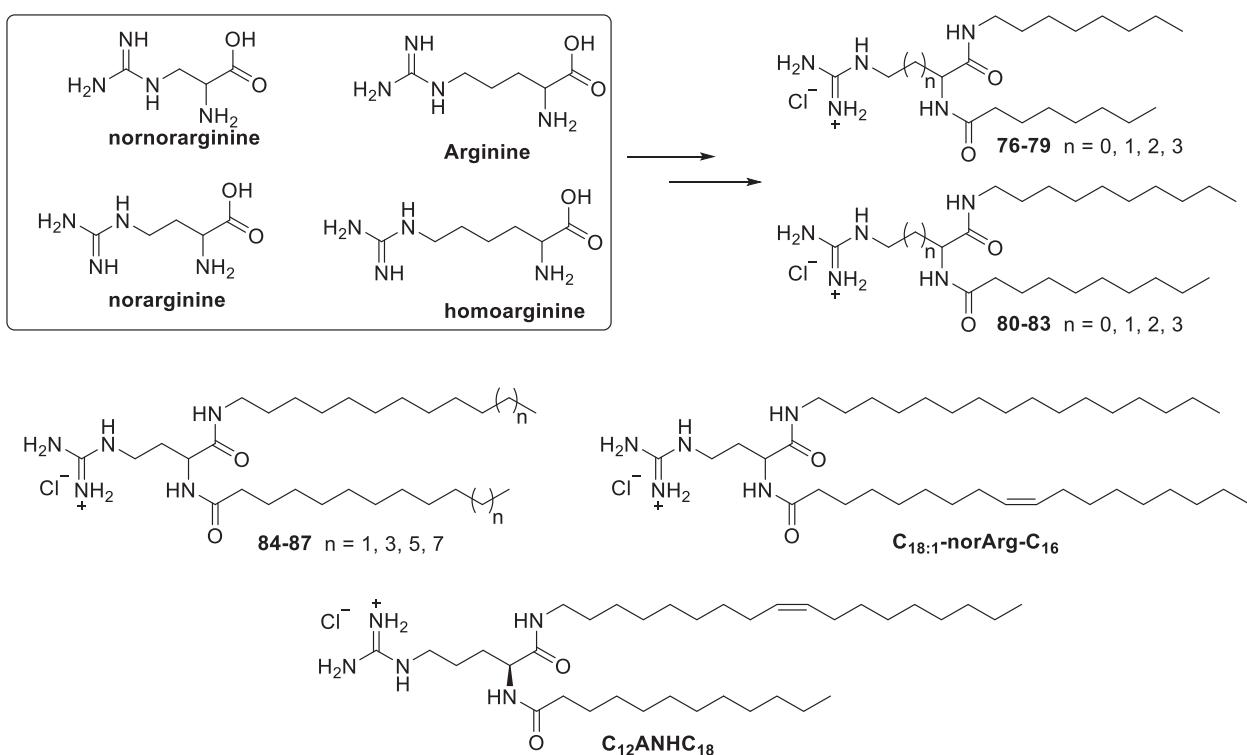
**Figure 17.** Chemical structures of guanidinium DiLA^2 compounds and C$_{12}$ANHC$_{18}$.

mice.[401] In 2020, Sanchez-Arribas et al. synthesized another arginine-based double-chained guanidinium type lipid, designated as C$_{12}$ANHC$_{18}$, which consists of the arginine residue linked to a 12-carbon atom alkyl chain and an unsaturated C18 alkyl chain.[402] The C$_{12}$ANHC$_{18}$ LNPs lipoplexes could deliver GFP siRNA efficiently into HeLa cells and T731 cells *in vitro*.[402]

In 2011, Metwally et al. synthesized four guanidinium derivatives of N^4,N^9-diacylated spermine,[403] based on the symmetrical fatty acid amides of spermine (Figure 18).[404] Starting from the selective protection of the two primary amino

groups of spermine **92** with ethyl 1,3-dioxoisoindoline-2-carboxylate **93**, followed by coupling of amine **94** with oleic acid **95**, amides **96** was obtained. Removal of phthalimides protecting group with ammonium hydroxide gave diamine **97**, which was treated with 1,3-di-Boc-2-(trifluoromethylsulfonyl)-guanidine **98** to install the protected guanidinium group, affording compound **99**. Guanidinium lipid **90** was obtained after deprotection of **99** with TFA in DCM (Figure 18). These guanidinium lipids efficiently bound siRNA and formed the corresponding nanoparticles for siRNA delivery in HeLa cells.
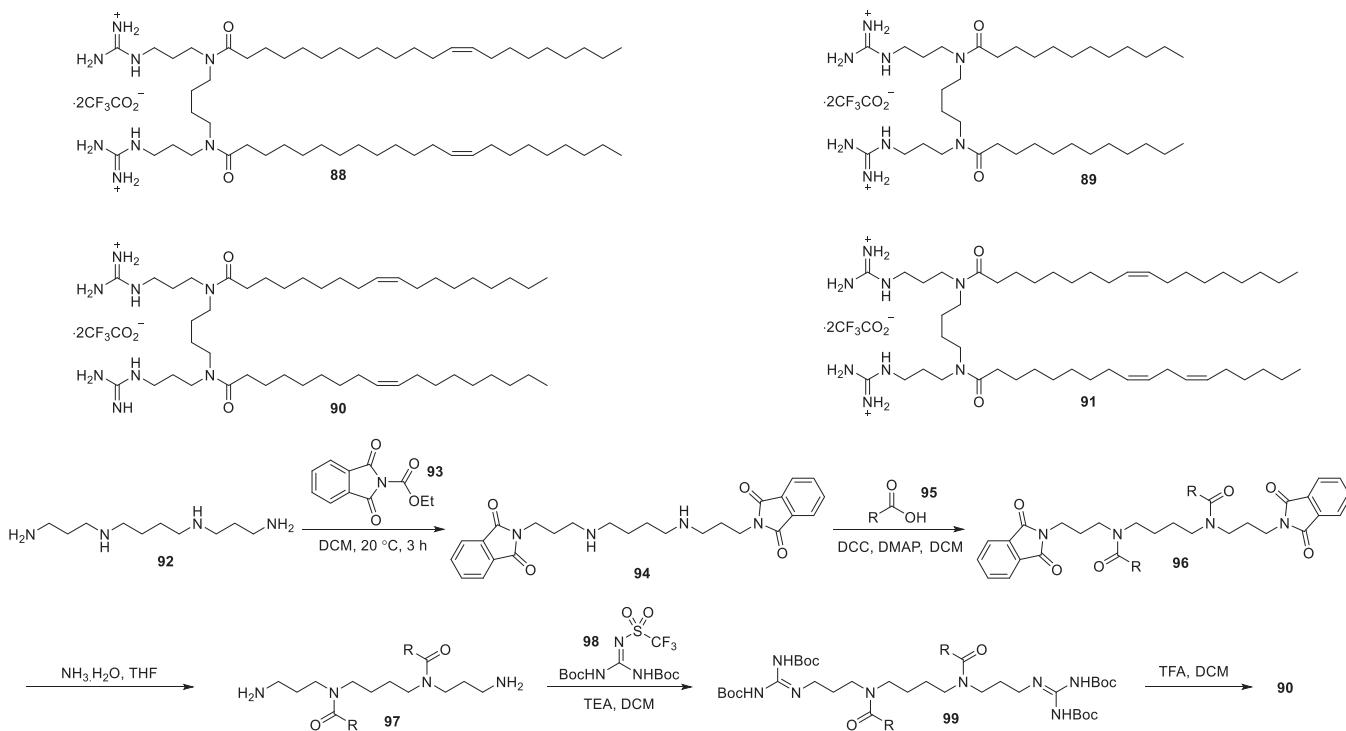
**Figure 18.** Chemical structures and synthesis of N^1,N^{12}-diamidino-N^4,N^9-diacylated spermines.
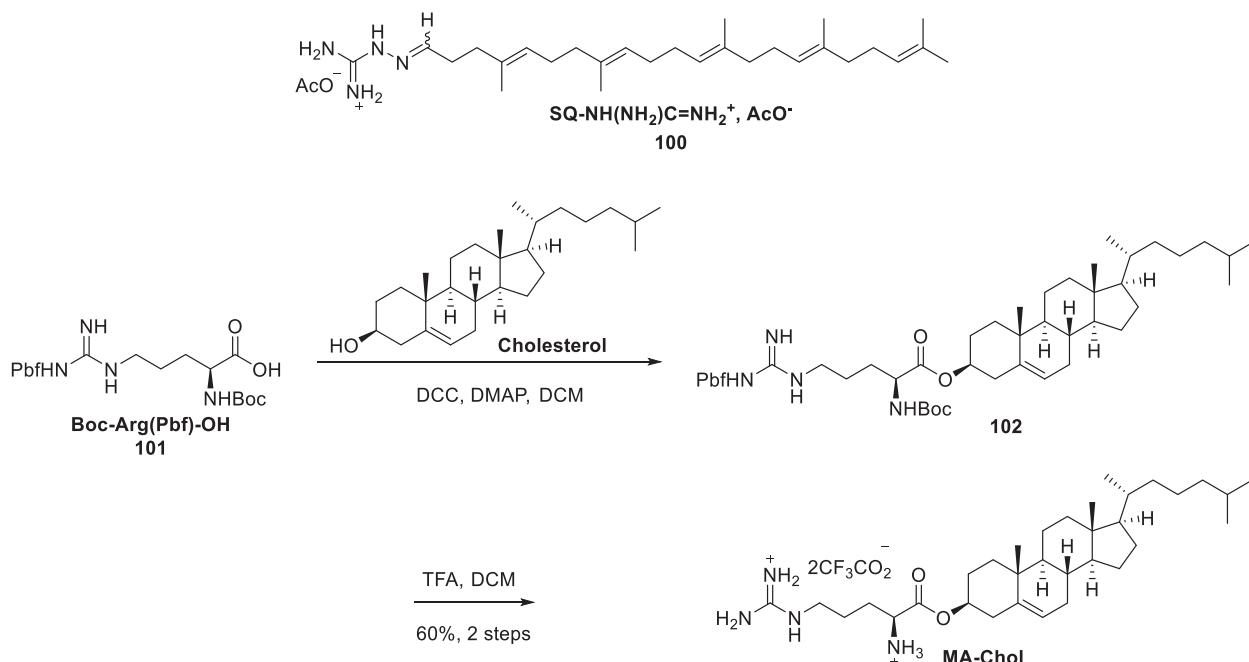
Figure 19. Chemical structures of squalene and cholesterol-derived guanidinium lipids.

LNPs formulated with guanidinium lipid **90** were able to deliver GFP siRNA into HeLa cells, leading to a reduction of GFP expression by 26%.[404]

Squalene, a natural precursor for the synthesis of cholesterol, has low toxicity and is well-tolerated in animal tests.[405] It has been used as a helper lipid, in substitution of cholesterol, in the preparation of cationic niosomes used in gene delivery.[406] Bertrand et al. reported the delivery of anti-EWS/Fli-1 siRNA into A673 cells *in vitro* by taking advantage of squalene-derived guanidinium lipid **100** (SQ-NH(NH$_2$)C=NH$_2^+$, AcO$^-$)

(Figure 19).[407] Cholesterol-derived cationic lipid may function as not only a helper lipid but also a complexation agent of RNA in the preparation of LNPs encapsulating RNA molecules, as cholesterol is an important stabilizer in the preparation of lipid-based nanoparticles. In 2015, Jon et al. reported an arginine–cholesterol-derived guanidinium lipid named MA-Chol (Figure 19).[408] MA-Chol was synthesized via the coupling of the protected form of arginine [Boc-Arg(Pbf)-OH] **101** and cholesterol followed by deprotection with TFA. Systemic administration of anti-PSK siRNA-loaded MA-Chol LNPs
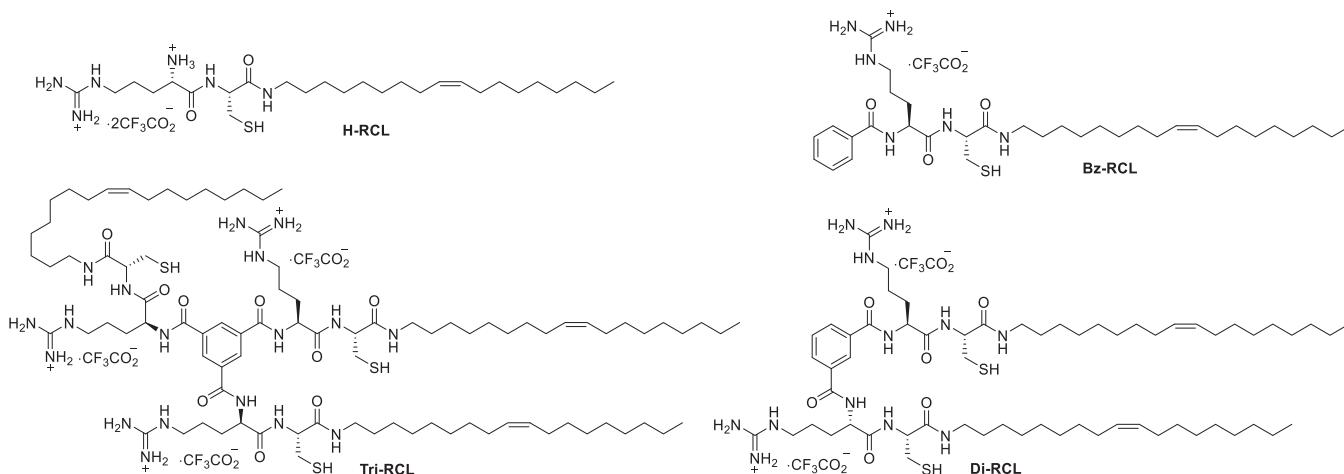
Figure 20. Chemical structures of arginine- and cysteine-derived guanidinium type lipids.
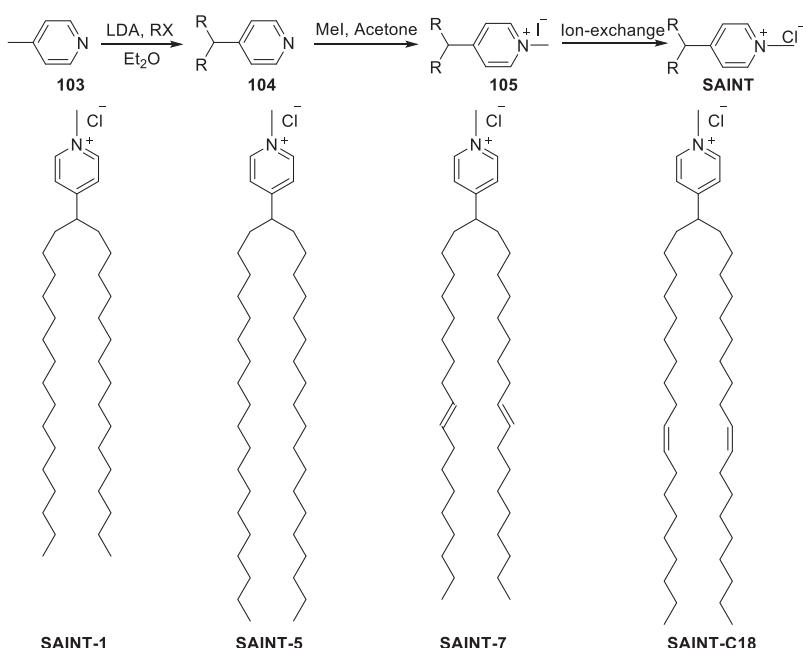
Figure 21. Chemical structure and synthesis of SAINT.

resulted in preferential accumulation of siRNA at the tumor site and ~81% suppression of tumor growth at a dose of 1 mg/kg in mice.[408]

In 2018, Bang et al. prepared a library of guanidinium lipids with arginine, oleyl amine, and cysteine as the building blocks (Figure 20).[409] A cysteine was incorporated in the peptides because cysteine has been shown to increase intracellular delivery.[410] These compounds were composed of two linear peptides (H-RCL and Bz-RCL) and two branched peptides (Tri-RCL and Di-RCL). Results showed that H-RCL and Bz-RCL were able to effectively deliver GAPDH siRNA in HeLa cells, while Di-RCL and Tri-RCL exhibited low delivery efficiency. The incorporation of the hydrophobic benzoyl group in Bz-RCL may improve its interaction with the cell membrane and consequently enhance siRNA delivery compared to H-RCL LNPs.[409]

2.1.3. Pyridinium Lipids. Apart from the guanidinium head group, the permanent positive charge can also be delocalized in heterocyclic rings, such as pyridinium rings and imidazolium

rings. The pyridinium cationic lipids generally have lower cytotoxicity compared to lipids with quaternary ammonium head groups.[300]

In 1997, van der Woude et al. developed a class of pyridinium lipids, termed synthetic amphiphiles interdisciplinary (SAINT) (Figure 21).[299] As shown in Figure 21, the synthesis of SAINT began with the reactions of dialkylation of 4-methylpyridine **103** and various alkyl bromides, giving alkylpyridine **104**, which was quaternized with methyl iodide to afford *N*-alkylpyridinium iodide **105**. Finally, *N*-alkylpyridinium iodide **105** was treated with ion exchange resins (Cl⁻ form) to obtain the *N*-alkylpyridinium chloride salts.[299] SAINT-C18 liposome (SAINT-O-Somes) could selectively and effectively deliver anti-VCAM-1 siRNA and anti-E-selectin siRNA into inflammation activated primary endothelial cells *in vitro*, inducing significant downregulation of target genes.[411,412] In 2014, they further reported that specific PEGylated SAINT-C18 LNPs could deliver anti-VCAM-1 siRNA to activate endothelial cells
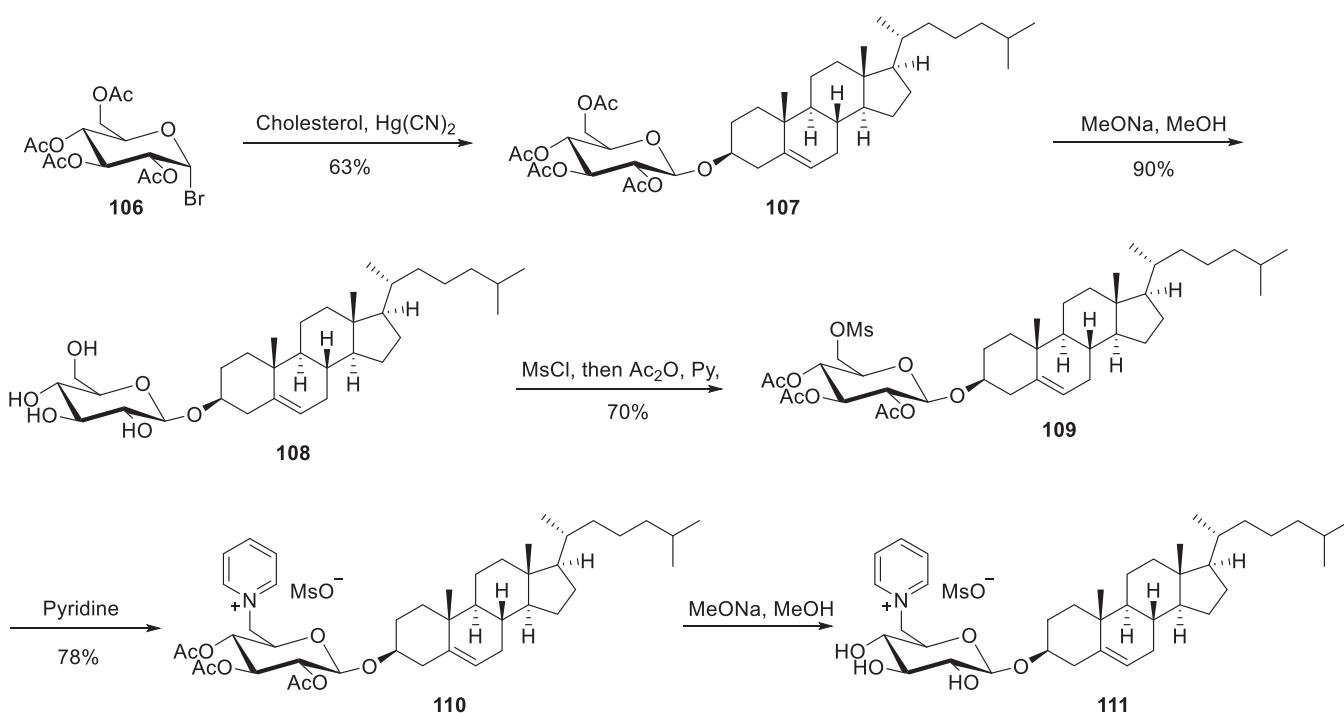
Figure 22. Synthesis of cholesterol-derived pyridinium lipids.
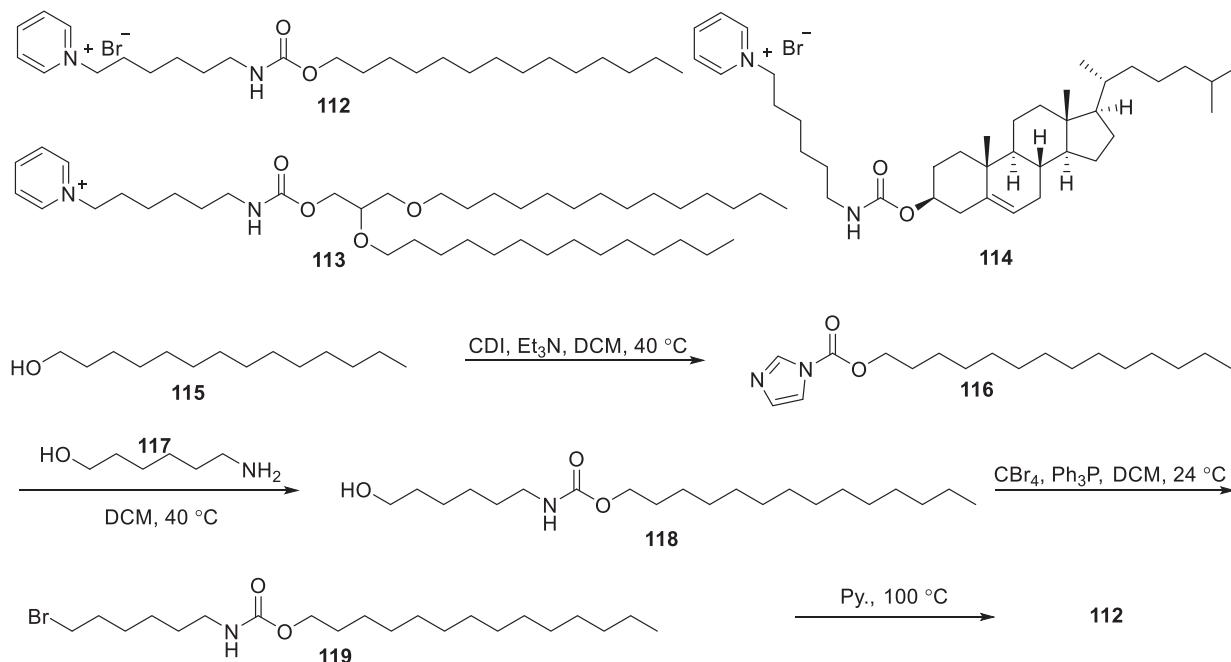
Figure 23. Chemical structures and synthesis of pyridinium lipids

in vivo, resulting in attenuation of VE-cadherin gene expression after intravenous administration.[413]

In 2010, Maslov et al. prepared cholesterol derived pyridinium lipid **111** (Figure 22), in which the pyridinium head group is attached to C-6 of the carbohydrate *β*-glucosyl spacer.[359] Cationic lipids with imidazolium or morpholinium or piperidinium as the head groups were also synthesized. Condensation of acetobromoglucose **106** and cholesterol in the presence of Hg(CN)$_2$ gave glucosides **107**. Removal of the acetyl groups with sodium methoxide gave cholesteryl *β*-D-glucoside **108**, of which the C-6 hydroxy group was

regioselectively mesylated followed by acylation of the other hydroxy groups to afford compound **109**. After direct quaternization of pyridine with **4** and deacylation, pyridinium lipid **111** was obtained. LNPs containing lipid **111** showed effective EGFP siRNA delivery and down-regulation of EGFP in BHK IR780 cells *in vitro*.[359]

In 2013, Maslov et al. developed a series of pyridinium cationic lipids that contained various hydrophobic domains, including tetradecanol, dialkyl glycerol, and cholesterol (Figure 23).[359,360] Carbamates **118** were obtained via coupling of 6-amino-1-hexanol **117** with tetradecanol **115** promoted by *N,N'*-
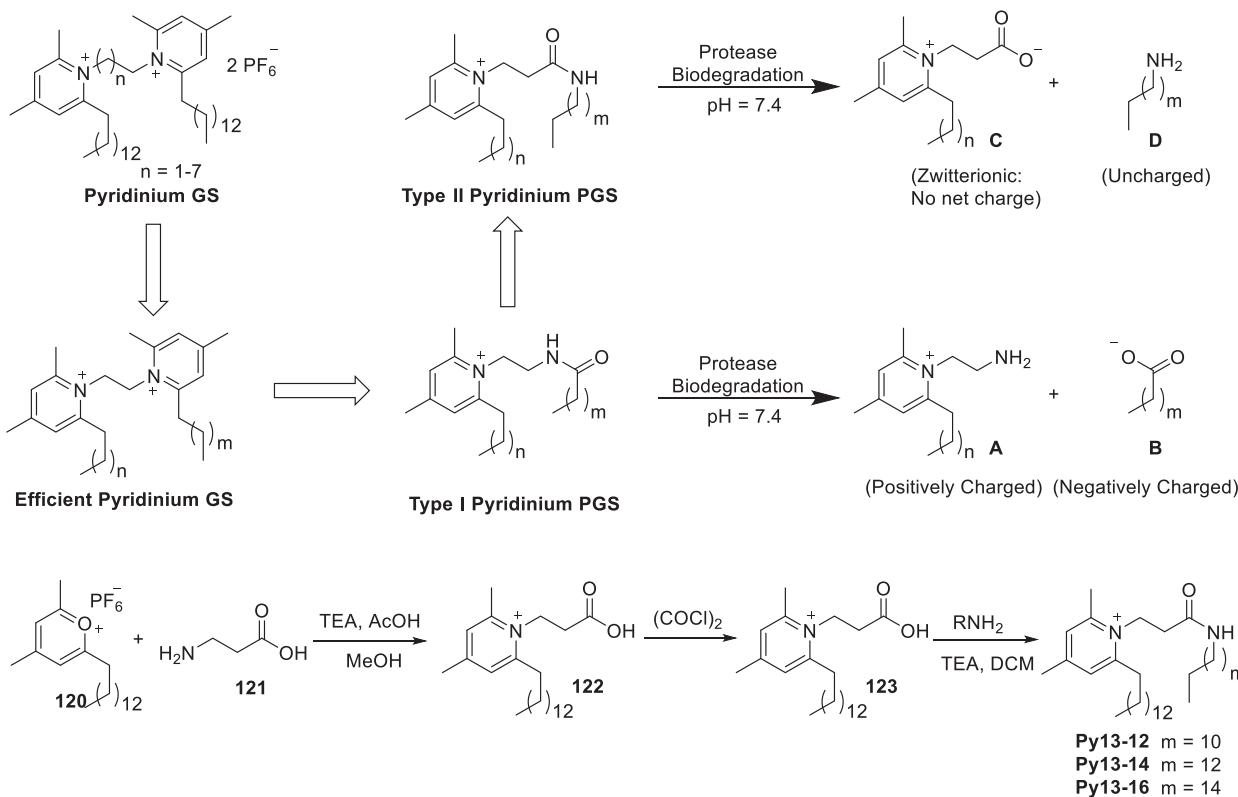
Figure 24. Design, synthesis, and proposed biodegradation pattern of pyridinium psudogemini surfactants.
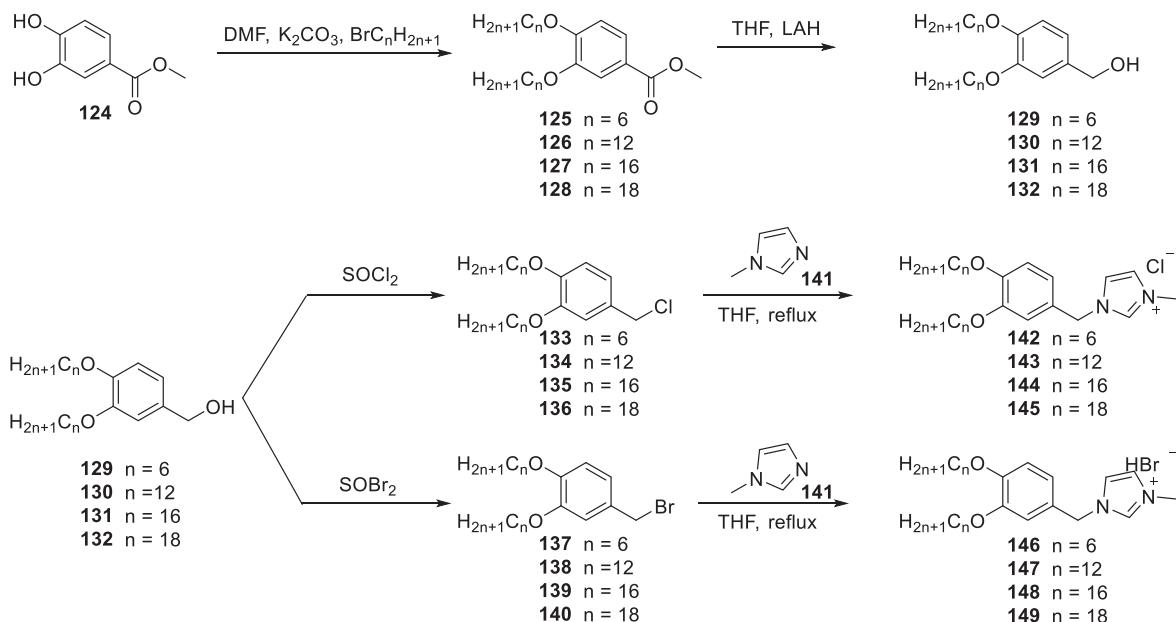
Figure 25. Chemical structures and synthesis of imidazolium lipids.

carbonyldiimidazole (CDI). Bromination of the hydroxy group in compounds **118** followed by quaternization with pyridine gave cationic lipid **112**. Results showed the type of hydrophobic tails determines the delivery activity of siRNA; LNPs containing lipid **113** exhibited better activity in siRNA delivery *in vitro* than that of **112** and **114**.[345]

Pyridinium gemini surfactants (GSs), with a higher charge/mass ratio than pyridinium type lipids, can generate lipoplexes with smaller size.[414,415] Structure−activity relationship studies

of GSs in DNA delivery showed that the gene delivery efficiency of GSs was similar to or higher than that of their pyridinium lipid analogs.[299,416,417] GSs with C2 spacing showed higher delivery efficiency than the other analogs. Although the relatively high molecular curvature of GSs is beneficial for increasing the delivery efficiency, it will increase their cytotoxicity.[418−421] In 2017, Satyal et al. developed a class of analogs of GS, named pyridinium pseudogemini surfactants (PGS), in which one of the pyridinium head groups were replaced by a noncharged
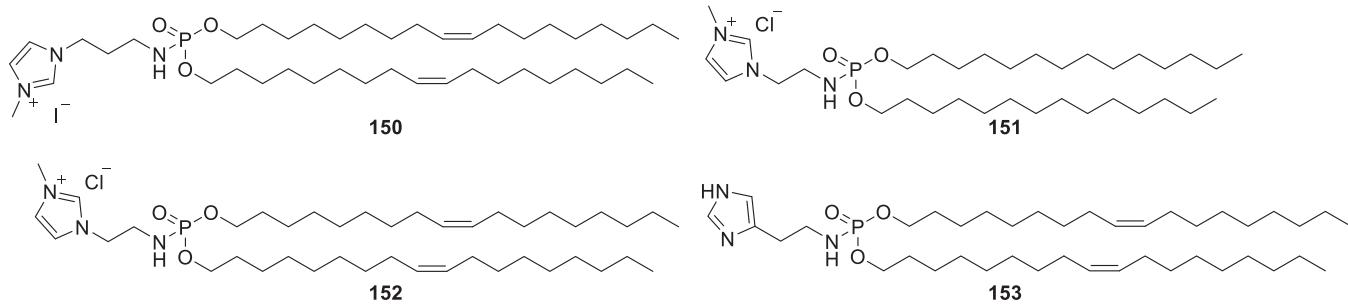
Figure 26. Chemical structures of imidazole/imidazolium lipophosphoramidate lipids.
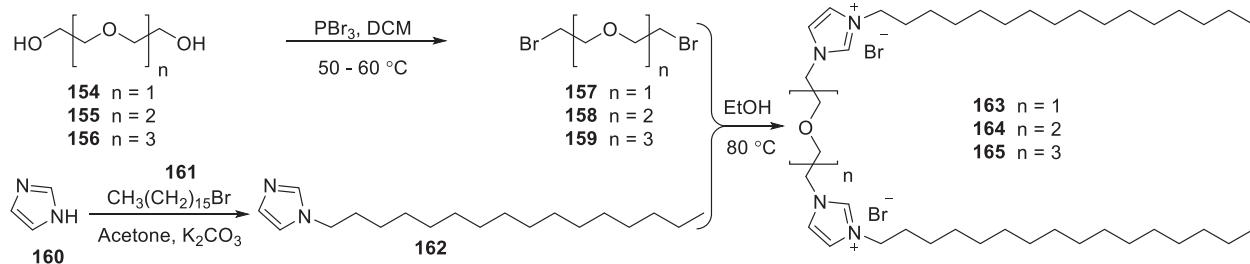
**Figure 27.** Synthesis of gemini imidazolium lipids **163−165**.
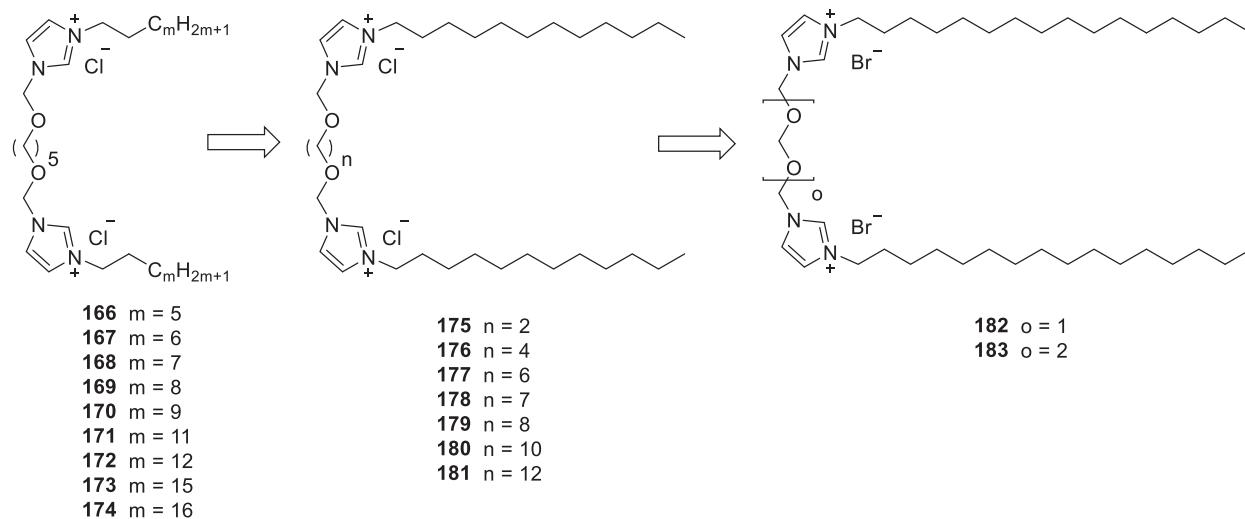
Figure 28. Optimization of imidazolium gemini surfactants.

polar moiety that were capable of biodegradation and hydrogen bonding (Figure 24).[422] The pyridinium pseudogemini surfactants (PGS) can mimic the tapered shape of pyridinium gemini surfactant (GS) with one positive charge.[423] These pyridinium lipids can be hydrolyzed by amidase into neutral components, thus reducing their potential cytotoxicity. Type I PGSs showed efficient delivery of pDNA and siRNA toward several cell lines.[416] To further reduce the cytotoxic effect, type II PGSs are designed in which the position of the amide was switched. Upon biodegradation, type II PGSs generate two species with no net charge and therefore display much lower cytotoxicity. The type II pyridinium PGSs were synthesized via the reaction of pyrylium salts **120** with the amino acid **121** to generate the substituted pyridinium head group followed by amide bond formation (Figure 24). Py13-16/DOPE was shown to be an efficient formulation for the delivery of pDNA, siRNA, and mRNA *in vitro*.[422]

2.1.4. Imidazolium Lipids. In 2009, Dobbs et al. developed two series of imidazolium lipids, chloride (**142−145**) and bromide (**146−149**) derivatives of 1-methyl-3-[3,4-bis(alkoxy)-benzyl]4H-imidazolium with different lengths of hydrophobic tails (Figure 25).[424] These lipids were prepared in three steps, following a slightly modified literature procedure.[425] Methyl 3,4-dihydroxybenzoate **124** was used as the starting material, which was etherified with 1-bromoalkanes **125−128** in the presence of potassium carbonate in DMF followed by reduction of the ester group, giving benzyl alcohols **129−132**. Bromination or chlorination of benzyl alcohols **129−132** was carried out with SOBr₂ or SOCl₂ as the solvent, respectively; the resulting 3,4-bis(alkoxy)benzyl chlorides or bromides were finally converted to the desired imidazolium lipids via quaternization with 1-methylimidazole **141**. Both **143** LNPs and **147** LNPs could induce 80% inhibition of the luciferase gene in A549-Luc cells at the anti-Luc siRNA concentration of 10 nM. Structure−activity relationship analysis showed that imidazolium lipids containing
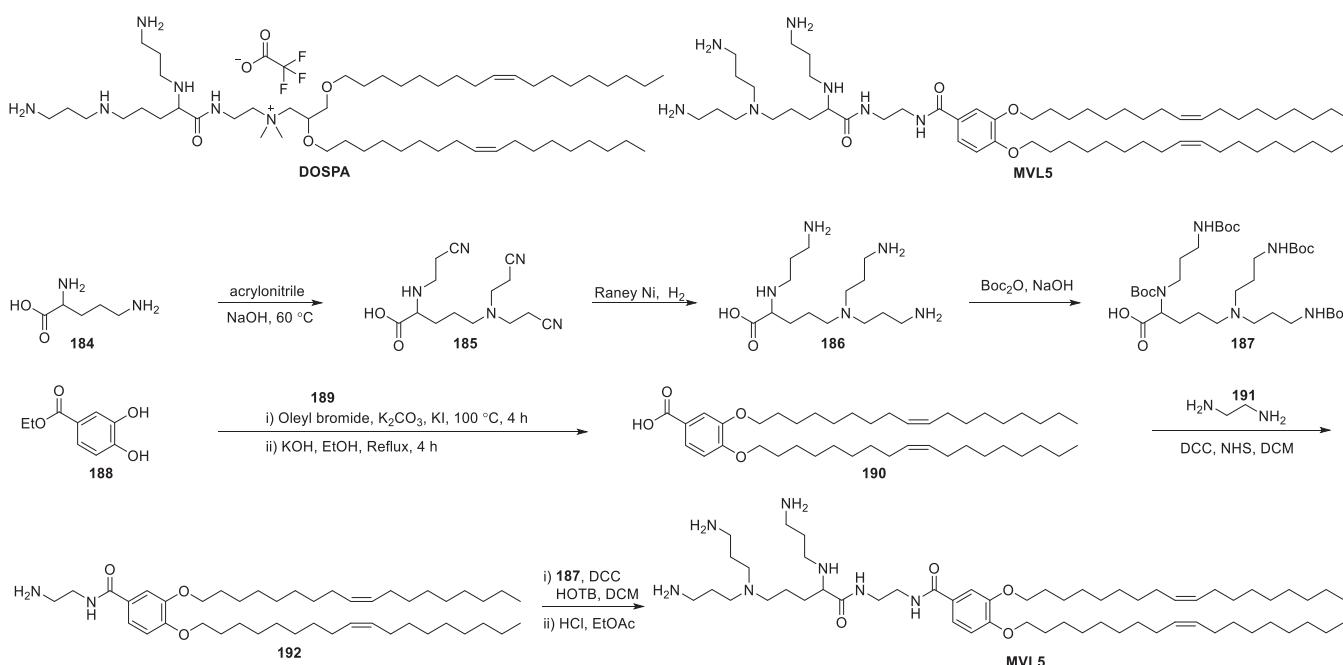
Figure 29. Chemical structures of DOSPA and MVL5 and a synthetic route to lipid MVL5.

dodecyl tails (**143**, **147**) showed enhanced siRNA encapsulation efficiency and higher siRNA delivery efficiency than other analogs.[424]

In 2011, Perche et al. reported the preparation of histidylated LNPs by incorporating imidazole/imidazolium lipophosphoramidate lipids (Figure 26).[426,427] Results showed that histidylated LNPs were an efficient delivery system for the tumor antigen mRNA delivery into splenic dendritic cells.[426,427] In 2013, they developed siRNA-loading LNPs formulated with imidazole/imidazolium lipophosphoramidate and histidinylated polyethylenimine for siRNA delivery into HeLa cells in vitro.[428]

In 2012, Kumar et al. reported the synthesis of imidazolium gemini lipids and evaluated their application in siRNA delivery.[429] The imidazolium gemini lipids were synthesized as shown in Figure 27. Bromination of the diol **154–156** with PBr$_3$ in dichloromethane gave **157–159**; alkylation of imidazole was realized via substitution reaction between imidazole **160** and 1-bromohexadecane **161** according to the reported procedure.[430,431] The imidazolium gemini surfactants **163–165** were synthesized by refluxing the corresponding dibromoalkoxyalkanes **157–159** with N-n-hexadecyl imidazole **162** in ethanol at 80 °C for 3 days.[429] In vitro biological analysis indicated that imidazolium gemini surfactants **163–165** yielded efficient siRNA delivery into HEK 293T cells, H1299 cells, and HeLa cells.[429]

Cationic gemini lipids generally show greatly enhanced properties and lower cytotoxicity as compared to their corresponding monovalent counterparts.[432–434] In 2013, Pietralik et al. prepared a library of imidazolium gemini lipids (**166–174**) with ether type linking groups and hydrophobic tails with different lengths (m = 5, 6, 7, 8, 9, 11, 12, 14, and 16) (Figure 28).[435] Structure–activity relationship analysis showed that the hydrophobic tails should not be shorter than 11 carbon atoms (m > 9) to form complexes with 21 bp DNA and RNA. When the hydrophobic tail was 12 atoms (m = 12) in length, lipids exhibited the highest complexing activity with various nucleic acids.[435] In 2016, Andrzejewska et al. developed another library of imidazolium gemini lipids (**175–181**) with variable

lengths of dioxyalkyl linker groups and dodecyl tails.[436] All of these gemini surfactants can effectively complex siRNA in a P/N ratio ranging from 1.5 to 10. Imidazolium gemini lipids containing dioxyethyl (n = 2, **175**) and dioxyhexyl (n = 6, **177**) spacer groups showed the strongest complexing with siRNA, and they also promoted the formation of an inverted hexagonal (H$_{II}$) phase.[436] In 2016, Martínez-Negro found that gemini lipid with shorter linking spacer (**182**, o = 1) showed higher anti-GFP siRNA delivery efficiency in vitro than gemini lipid with longer linking spacer (**183**, o = 2).[437]

2.2. Ionizable Lipids

In order to overcome the limitation of cationic lipids and further improve the RNA delivery efficiency, numerous ionizable lipids with ionizable amino head groups have been developed as a critical component for the formulation of LNPs.[384,438–440] These ionizable lipids usually contain amino head groups and have an acid dissociation constant (pK_a) less than 7;[441,442] thus, they are protonated and positively charged at acidic pH (pH < 6.0) and neutral at physiological condition (pH = 7.4). They can form LNPs with an overall surface charge close to neutral, which exhibit reduced toxicity and prolonged circulation times as compared with cationic delivery systems after systemic administration, resulting in access to many tissues.[443–445] In 2001, Semple et al. reported that ASO-loading LNPs formulated with DODAP/Chol/DSPC/PEG-CerC14 exhibited a half-life (t$_{1/2}$) of 5–6 h, whereas ASO-loading LNPs containing DODAC/Chol/DSPC/PEG-CerC14 exhibited a half-life of only 15 min.[444] Rapid plasma elimination of DODAC LNPs may be caused by the interactions between the cationic lipid DODAC and the anionic plasma proteins. In an acidified endosome, ionizable lipids are protonated and the resulting positively charged lipids can interact with negatively charged endosomal membranes, leading to the endosomal membrane destabilization and the release of RNA cargo into the cytosol.[292] Optimization of ionizable lipids is explored by combining iterative screening and modification of lipids in one or more domains of their head groups, linkers, or hydrophobic tails.
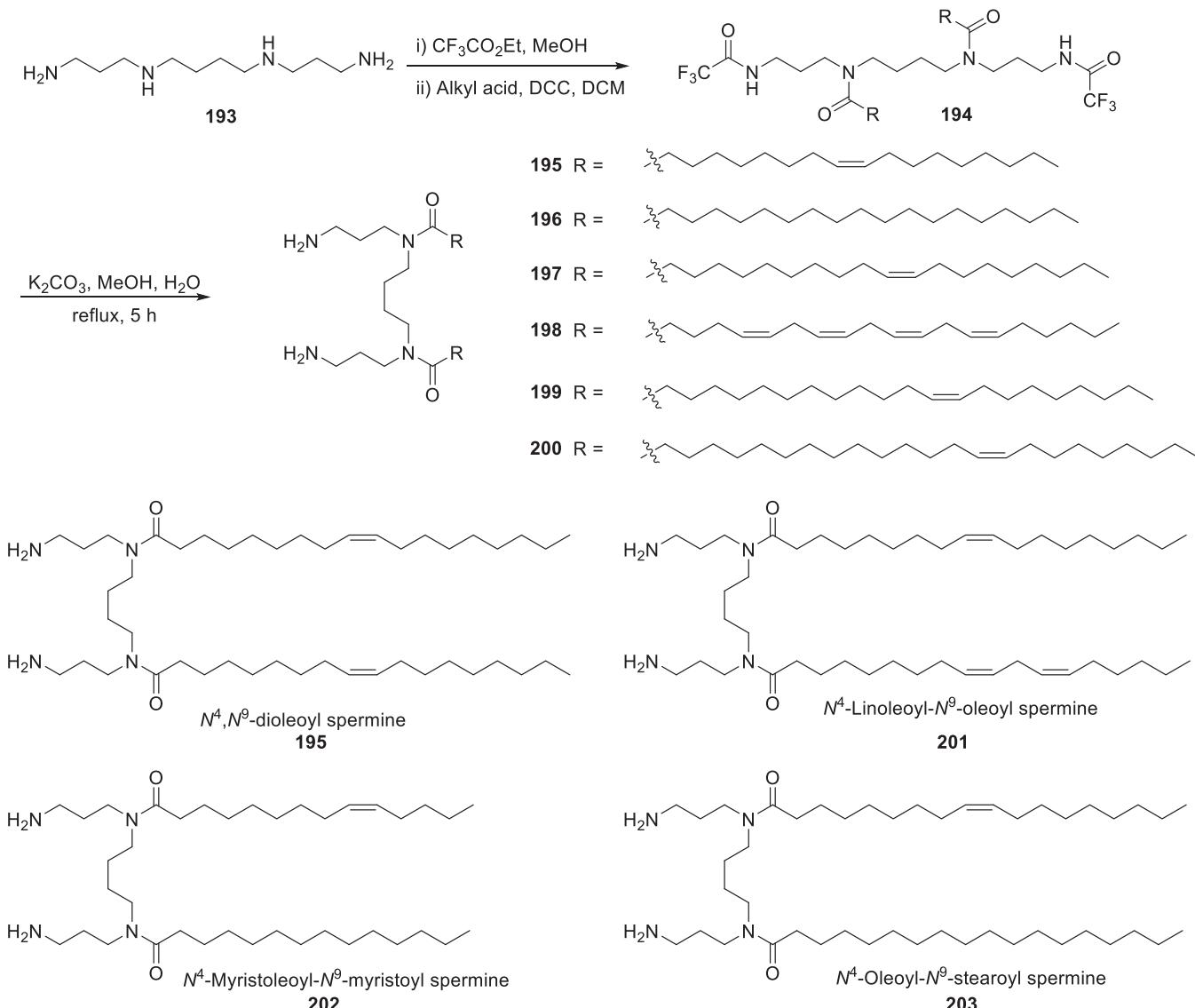
Figure 30. Synthesis of spermine-derived lipids.

2.2.1. Primary and Secondary Amino Lipids. DOSPA is a multivalent lipid in which the spermine head group is linked to the hydrophobic domain that contains two oleyl tails via an amide bond (Figure 29).[446] Lipofectamine is a widely used commercially available transfection reagent for pDNA or RNA delivery that contains DOSPA and DOPE at a molar ratio of 3:1.[447−449] In 2002, Ewart et al. synthesized MVL5, a pentavalent ionizable lipid used for nucleic acid delivery.[450] As depicted in Figure 29, the multivalent building block **186** was prepared by Michael addition of ornithine **184** to acrylonitrile followed by hydrogenation of the resulting nitrile moieties **185**. Boc-protection of all amino groups in compound **186** yielded acid **187**. Benzoic acid **190**[451] was synthesized by etherification of ethyl 3,4-dihydroxy benzoate **188** with oleyl bromide **189** followed by hydrolysis of the ethyl ester. Coupling of benzoic acid **190** and ethylenediamine **191** afforded amine **192**, which was coupled with acid **187**; the resulting product was deprotected with TFA to give MVL5. Compared to the monovalent cationic lipid DOTAP/DOPE, MVL5/DOPE exhibited lower toxicity and higher gene silencing efficiency in mammalian cells.[452] As for a LNP formulated with MVL5 and monooleate glycerol (MOG), the high positive charge density of

MVL5 and positive Gaussian curvature due to MOG facilitated endosomal escape, leading to efficient siRNA delivery and gene silencing *in vitro*.[453]

In 2006, Ghonaim et al. synthesized six symmetric N^4,N^9-diacyl spermines based on spermine, a naturally occurring polyamine, to evaluate the effects of the length and unsaturation degree of hydrocarbon tails on DNA and siRNA formulation.[454] These N^4,N^9-diacyl polyamines with long chains[454] (Figure 30) were synthesized through a three-step route based on their previous work.[455,456] First, the two primary amino groups of spermine **193** were selectively protected as trifluoroacetamides via reaction with ethyl trifluoroacetate.[457] Then aliphatic acyl chains were attached to the remaining secondary amino groups via amide bond formation. Lastly, the desired lipids were obtained after selective removal of the ditrifluoroacetyl protecting groups.[458] By adding two mono-*cis*-unsaturated C20 or C22 chains, the resulting N^4,N^9-dieicosenoyl spermine **197** and N^4,N^9-dierucoyl spermine **199** were shown to be the lead lipids for siRNA delivery in FEK4 and HtTA cells *in vitro*.[454] In 2012, Metwally et al. synthesized seven asymmetric N^4,N^9-diacyl spermines (e.g., **201** and **202**), which contained two different hydrocarbon tails, varying in length from C18 to C24
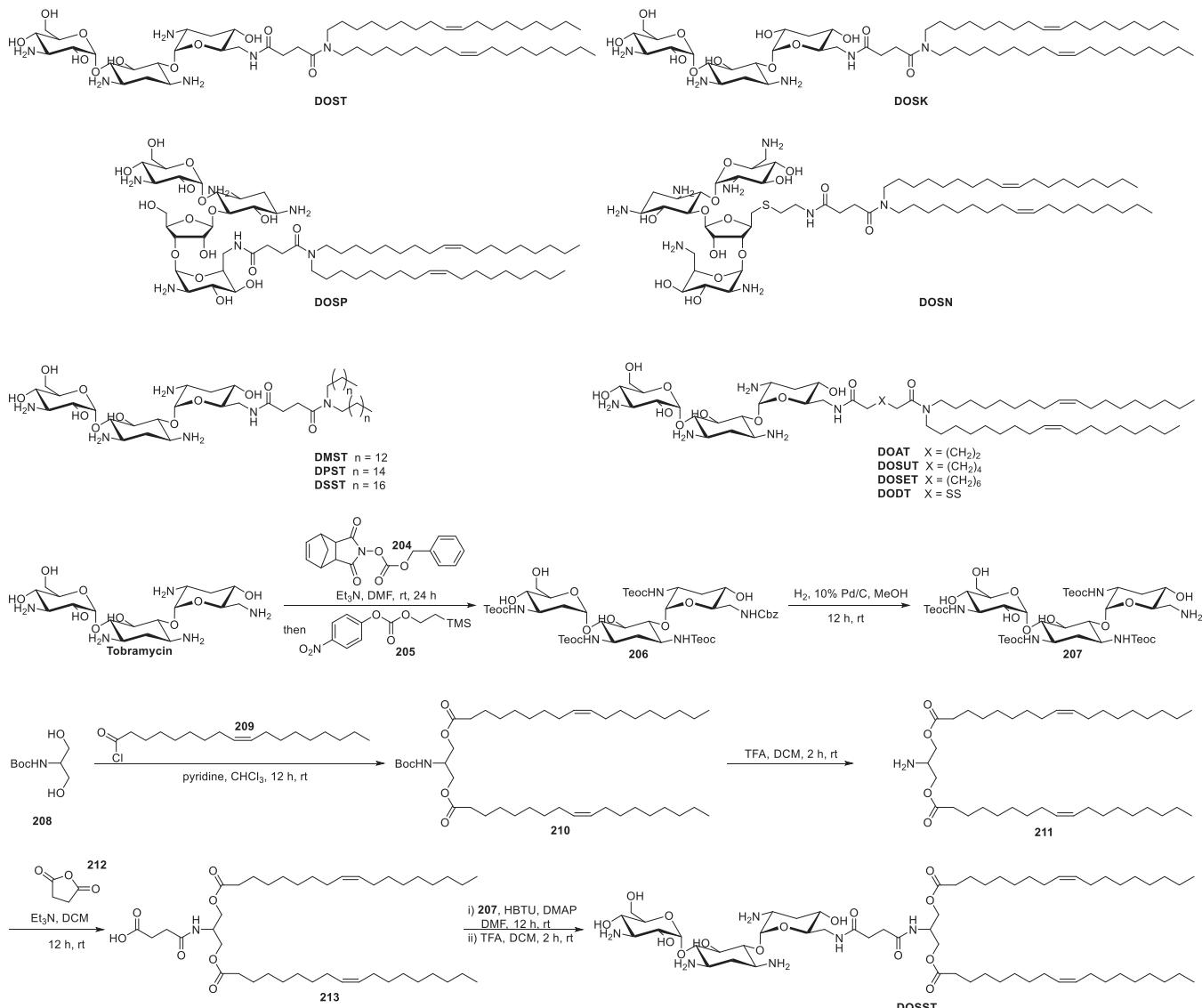
Figure 31. Structure of the aminoglycoside-derived ionizable lipids.

with different unsaturation degrees.[459] Results showed that C18 acyl tails with one or two unsaturation degrees induced effective EGFP gene silencing in HeLa cells. Besides, lipids that improved cell uptake of siRNA-loading LNPs did not necessarily show higher gene silencing activity.[459] Among another six asymmetric N^4,N^9-diacyl spermines, N^4-oleoyl-N^9-stearoyl spermine **203** and N^4-myristoleoyl-N^9-myristoyl spermine **202** were effective in siRNA delivery, leading to 34% EGFP gene silencing in FEK4 primary skin cells *in vitro*. Structure−activity relationship analysis showed that the presence of an unsaturated bond in at least one of the hydrophobic tails is necessary for effective gene silencing.[460]

Aminoglycosides, broadly used as antibiotics, are applied for the synthesis of ionizable lipids for RNA delivery due to their natural affinity for RNA[461−463] as well as their multifunctionality and structural variety. In 2007, Desigaux et al. synthesized a series of primary amino lipids by linking two dioleyl tails to various aminoglycoside head groups via a succinyl spacer (Figure 31).[464] DOST and DOSK are derived from aminoglycosides which contain a 4,6-disubstituted 2-deoxystreptamine (4,6-DDS) ring, whereas DOSP and DOSN are derived from aminoglycosides with a disubstituted 2-deoxystreptamine

(4,5-DDS) ring. Results showed that compared with the other three aminoglycoside-derived lipids,[465] DOSP/siRNA complexes, with smaller particle size and higher colloidal stability, exhibited obvious GFP silencing in d2GFP cells and lamin A/C silencing in HEK293 and Hela cells *in vitro*. The flexibility of DOSP may enhance the endosomal escape of siRNA by forming lamellar microdomains, which can destabilize the endosomal membrane efficiently.[464] Afterward, a structure-activity relationship study was performed to assess the importance of the hydrophobic tails, the spacer between the head group and the tails, and the behavior of stimuli-responsive linkers in delivering different DNA, siRNA, and mRNA.[466] With DOST as the starting point, a set of another seven tobramycin-based lipids (DMST, DPST, DSST, DOAT, DOSUT, DOSET, DODT, and DOSST) were synthesized. As shown in Figure 31, starting with tobramycin, consecutively selective Cbz-protection of the less sterically hindered primary amine and Teoc-protection of the four remaining primary amines were realized by sequential addition of reagents **204** and **205** in one pot, giving the protected tobramycin derivative **206** in 71% yield. Hydrogenation of **206** allowed for the removal of the Cbz protecting group to provide **207**. Diacylation of *N*-Boc serinol **208** with
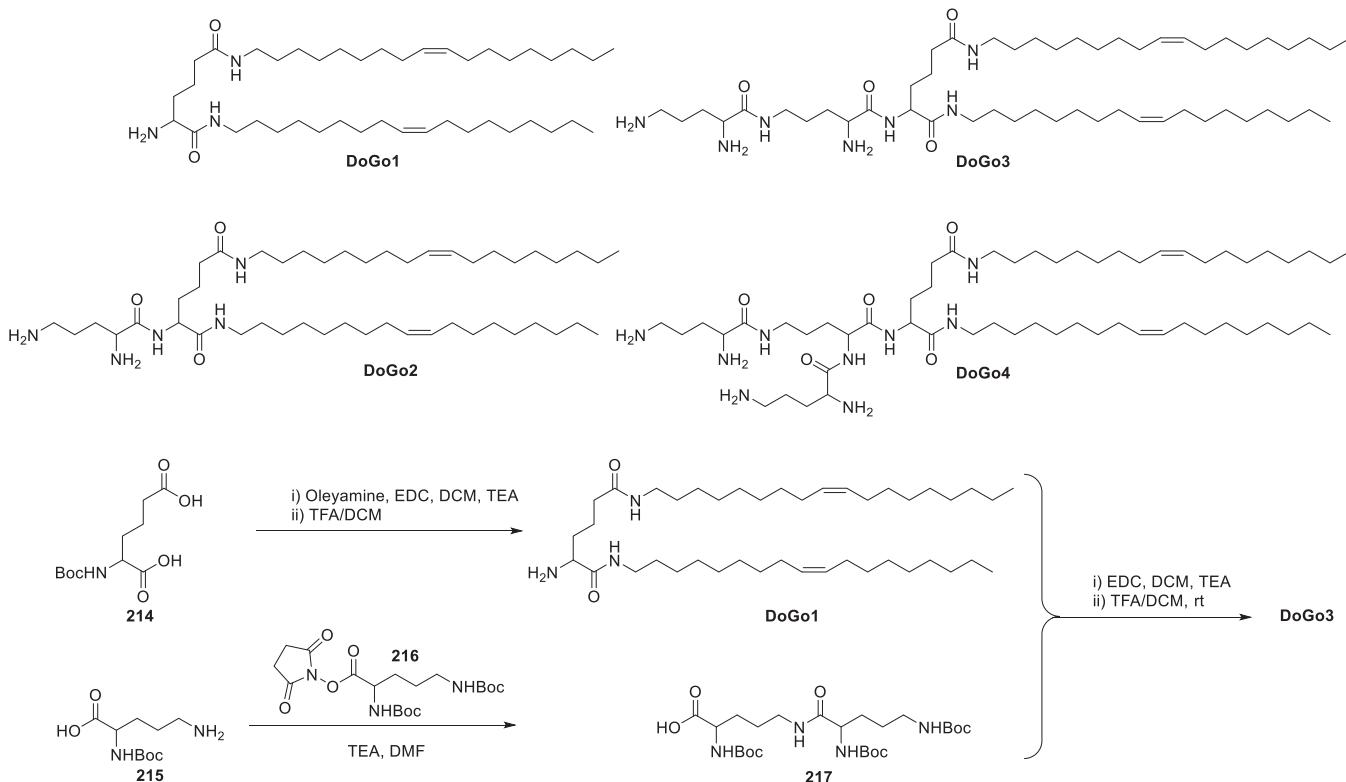
Figure 32. Chemical structure of lysine-derived lipids.

oleoyl chloride **209** yielded **210**, which was treated with TFA to release the free amino group, resulting in amine **211**. Then, amine **211** was reacted with succinic anhydride **212** to give the conjugate **213**. Lastly, **213** and protected tobramycin **207** were coupled via the amide bond formation and afforded DOSST after deprotection of the Teoc groups. DOSST was an effective lipid for the delivery of mRNA, siRNA, and DNA. Structure-activity relationship studies showed that the length of the linker and the properties of the hydrophobic tails were important parameters to be considered in building efficient lipids, with the dioleyl tails suggested to be a better choice compared to shorter or saturated alkyl tails.[466]

Ionizable lipids containing naturally occurring amino acids, such as lysine, with different hydrophobic tails were first reported to be used for DNA delivery.[467,468] In 2009, Suh et al. synthesized an ionizable lipid *N,N′*-dioleylglutamide (DoGo1), which contains two oleylamine tails connecting to the two carboxylic acid groups of glutamic acid through amide bond linkages (Figure 32).[469] Results showed that RFP-specific siRNA formulated with DoGo1 was effective in siRNA delivery *in vivo*, resulting in significant RFP gene knockdown in tumor tissues in mice.[469] To minimize the toxicity of the amino head groups and increase the biostability of the resulting lipids, Xiao et al. synthesized a series of lipids incorporating peptidomimetics based on lysine and glutamine (Figure 32).[470] *N*-Boc-glutamic acid **214** was coupled with oleylamine via amide bonds formation; the resulting amide was treated with trifluoroacetic acid to give DoGo1, which was attached to the Boc-protected diornithine peptide **217** followed by Boc removal to afford DoGo3. A single injection of siRNA-loading DoGO3 LNPs resulted in a 90% knockdown of apolipoprotein B (ApoB) mRNA and a 60% decrease in ApoB protein level in mouse liver at a siRNA dose of 7 mg/kg.[470]

The Lu group developed a family of multifunctional ionizable lipids that are comprised of an ionizable head group, two distal hydrophobic tails, and two peptide-type linkers (Figure 33).[471−473] In their first library, EHCO LNPs showed a high siRNA delivery efficiency into U87-Luc cells.[456] Then they further modified EHCO and SHCO by removing the histidine residue and incorporating hydrophobic tails with various unsaturation degrees. In the secondary library, ECO and ECLn exhibited the highest luciferase gene silencing activity in HT29 cells and CHO cells.[472] These lipids were synthesized using resin-based solid phase synthesis. For example, the synthesis of ECO started with the covalent attachment of one of the amine groups of ethylene diamine **219** to the solid material **218**; then Michael addition of the remaining amine group to methyl acrylate **221** gave compound **222**. Aminolysis of the esters of compound **222** with ethylene diamine **219** afforded diamine **223**, which was coupled with Fmoc-Cys(Trt)-OH **224** via peptide bonds formation. The Fmoc group in the resulting compound was selectively removed with 20% piperidine to afford compound **225**. Finally, the hydrophobic tails were attached to the free amino groups via amide bond formation and ECO was obtained after global deprotection with TFA (Figure 33).[456] These lipids can form stable LNPs with siRNA without the inclusion of other helper lipids. Auto-oxidation of the thiol groups in the cysteine residue incorporated in the linker leads to the formation of intermolecular disulfide bonds, which can further stabilize the LNPs.[474] The disulfide bonds in LNPs are relatively stable in the plasma and can be reduced by the endogenous glutathione to facilitate siRNA release. Besides, the thiol groups can also act as functional groups for modification of LNPs with biocompatible polymers and targeting ligands to minimize the immunogenicity and improve the siRNA delivery efficiency of the LNPs.[475−477]
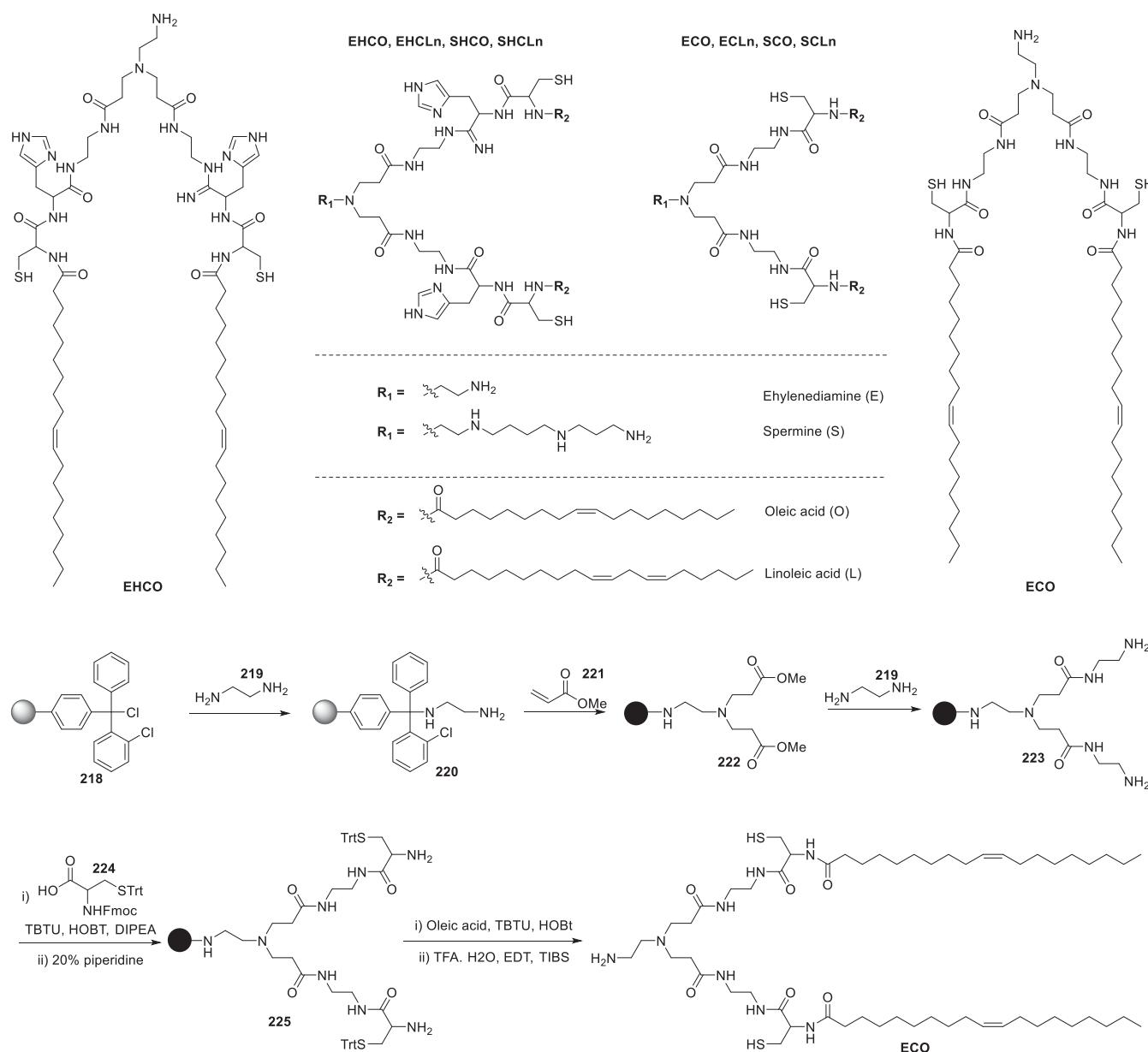
Figure 33. Chemical structures of multifunctional ionizable lipids.
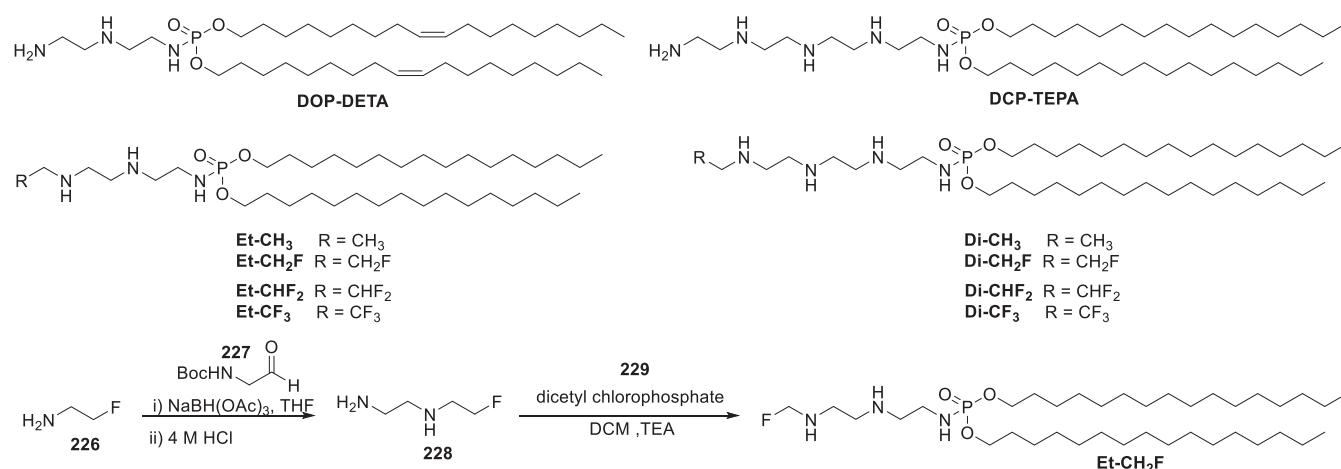
Figure 34. Chemical structures of dialkyl phosphate−polyamine conjugates and synthesis of Et-CH$_2$F.
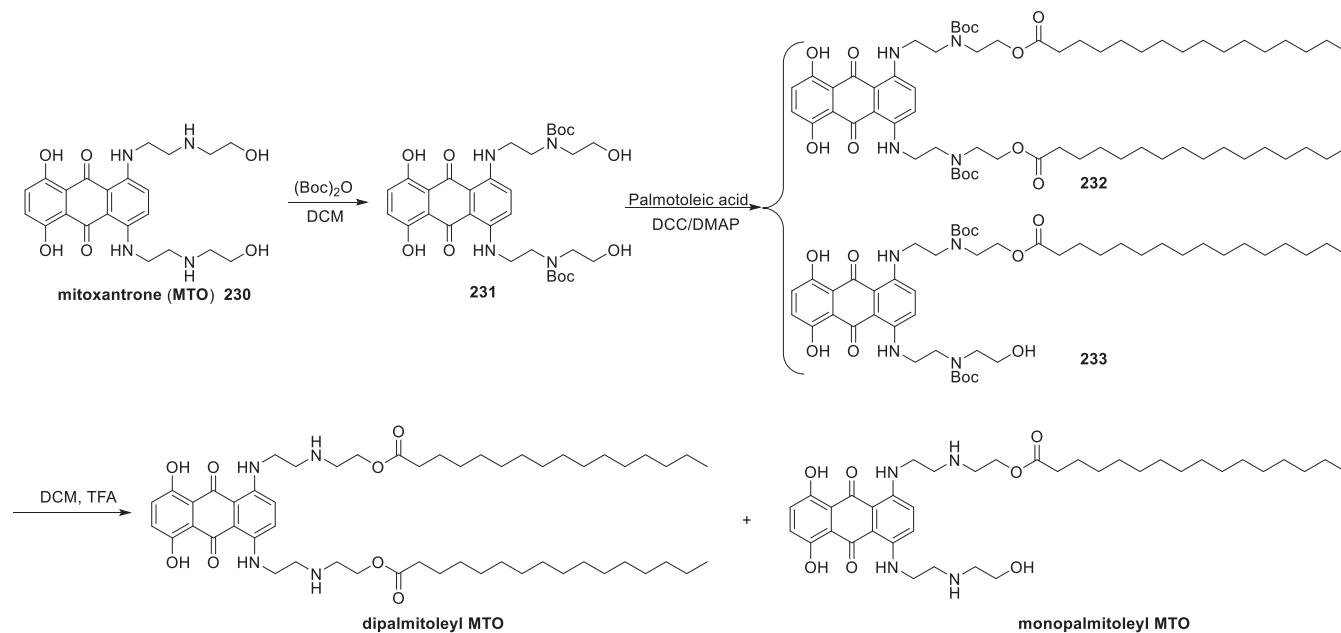
Figure 35. Synthesis of MTO-derived ionizable lipids.
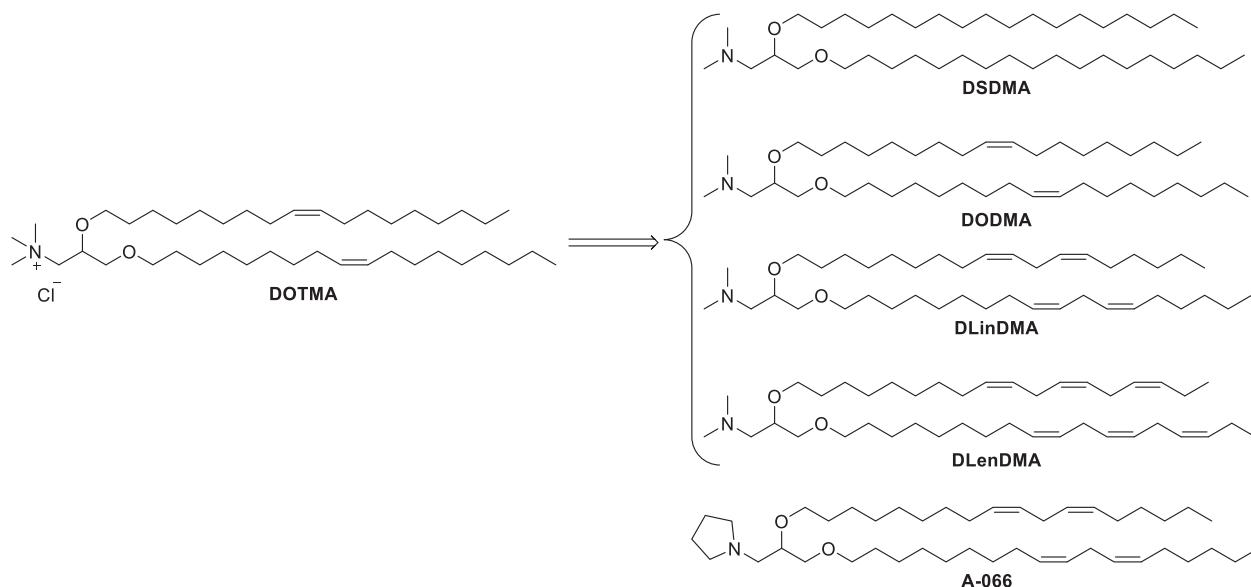
Figure 36. Chemical structures of the first generation of two-tailed amino lipids.

In 2011, Asai et al. synthesized a dicetyl phosphate–tetraethylenepentamine conjugate (DCP-TEPA) that contained four ionizable amino groups attaching to the hydrophobic phosphate portions through a phosphoramide bond (Figure 34). In HT1080 human fibrosarcoma cells, the silencing activity of DCP-TEPA LNPs encapsulating siRNA was closely related to the N/P ratio of DCP-TEPA/siRNA.[478] In 2019, Asai et al. synthesized similar lipids, a dioleylphosphate-diethylenetriamine conjugate (DOP-DETA) (Figure 34). The two oleyl tails offered DOP-DETA higher membrane fluidity and induced membrane fusion. EGFP siRNA-loading LNPs formulated with DOP-DETA/DPPC/cholesterol produced significant gene knockdown in HT1080-EGFP human fibrosarcoma cells *in vitro*. The molar ratio of DOP-DETA to siRNA is the determining factor of the gene knockdown efficiency.[479] In 2019, Okamoto et al. studied the pK_a of ionizable lipids by

replacing hydrogen atom(s) with fluorine atom(s) and evaluated the influence of pK_a on gene silencing efficiency.[480] Two groups of lipids, the EtDA group and the DiETA group, were synthesized (Figure 34). The synthesis of ET-CH2F, for example, started with reductive amination of 2-fluoroethylamine **226** with N-Boc-2-aminoacetaldehyde **227**; the resulting amine was treated with 4 M HCl to remove the Boc protecting group to give diamine **228**. Chemoselective phosphorylation of the primary amine with dicetyl chlorophosphate **229** gave Et-CH$_2$F (Figure 34). Due to the strong electron-withdrawing inductive effect of the fluorine atom,[481] the pK_a's of the ionizable lipids decreased with the increase in the number of fluorine atoms. Results showed that the optimal lipid pK_a for a high gene silencing effect varied according to the number of ionizable amines. LNPs containing Et-CH3 (pK_a = 8.2) showed the highest gene knockdown efficiency in the EtDA group, and
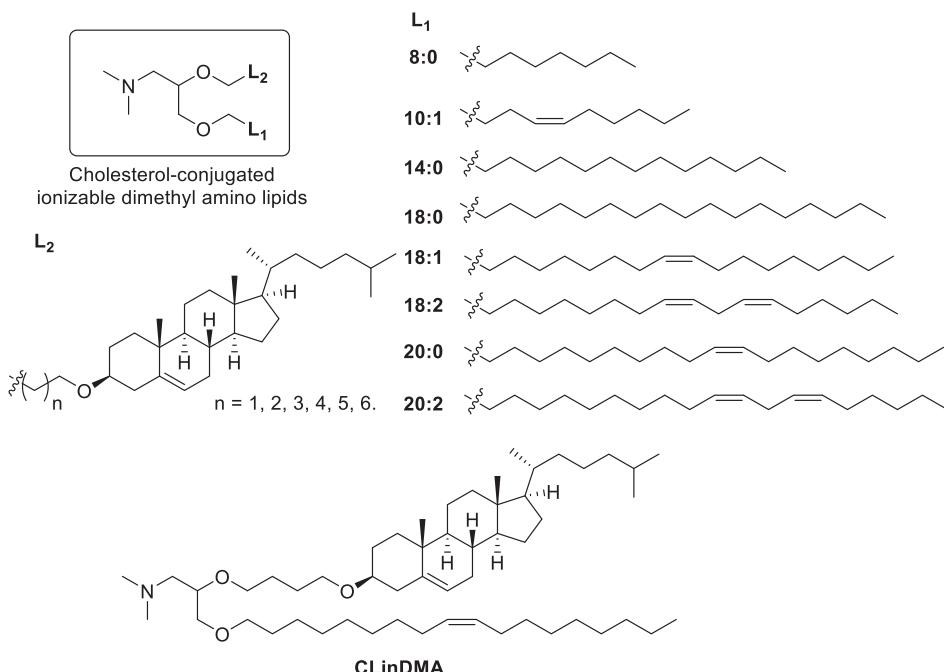
Figure 37. Chemical structures of two-tailed amino lipids with asymmetric tails.

LNPs containing Di-CF3 (pK_{a1} = 7.1, pK_{a2} < 3.0) outperformed other lipids in the DiETA group. These results indicated that the balance between the number of ionizable amines and fluorine atoms was crucial to achieving high gene silencing activity.[480]

In 2011, Chang et al. developed a codelivery system of Mcl-1-specific anticancer siRNA and anticancer drugs mitoxantrone (MTO), in which lipids derived from anticancer drug acted as ionizable lipids that formed the LNPs for siRNA delivery.[482] As shown in Figure 35, palmitoleic acid was conjugated to the mitoxantrone (MTO), generating two palmitoyl MTO (Pal-MTO) lipids: monopalmitoyl MTO **233** and dipalmitoyl MTO **232** (Figure 35). Nanoparticles containing monopalmitoyl MTO **233** and dipalmitoyl MTO **232** at a molar ratio of 1:1 showed effective Mcl-1 siRNA delivery *in vitro*, resulting in a reduction of B16F10-RFP tumor cell viability by 81%. This LNP-siRNA formulation showed stronger anticancer activity after intratumoral administration compared to LNPs alone.[482]

2.2.2. Tertiary Amino Lipids. Tertiary amines are compounds in which a nitrogen atom has three organic substituents. Generally, tertiary amines are less basic than secondary amines, because the steric hindrance of the attached alkyl or aryl groups hinders the protonation of the nitrogen atom. Sometimes primary and secondary amino lipids may exhibit different charge states in specific pH conditions compared to their tertiary amino counterparts. According to the characteristics of the chemical structures and moieties that are incorporated, tertiary amino lipids are divided into two major types in this session: (i) two-tailed amino lipids and (ii) bioactive molecules derived lipids.

2.2.2.1. Two-Tailed Amino Lipids. In 2005, Heyes et al. synthesized two-tailed dimethyl amino lipids by modifying DOTMA.[105] The trimethylammonium head group was replaced by a dimethylamino head group, and four hydrophobic tails with incremental degrees of saturation were conjugated via the ether linkers, leading to four amino lipids (DSDMA, DODMA, DLinDMA, and DLenDMA) (Figure 36). They showed that the unsaturation degree of the hydrophobic tails affected

fusogenicity, lipid pK_a, cellular uptake, and intracellular RNA delivery efficiency. The ^{31}P-NMR analysis indicated that the increase of the saturation degree of hydrophobic tails from 2 to 0 double bonds compromised the fusogenicity of the LNPs. They also found that DLinDMA that contains two 18-carbon hydrocarbon tails with two unsaturated degrees showed the highest luciferase gene silencing effect in Nuro2A cells. They speculated that the linoleyl chains could produce a lipid with an inverted conical shape and tend to adopt the membrane-destabilizing inverted hexagonal (H_{II}) phase.[105] In 2006, LNPs formulated with DLinDMA/DSPC/cholesterol/PEG-C-DMA were used to deliver siRNAs, resulting in significant silencing of ApoB (>90%) when administrated systemically in nonhuman primates at a dose of 2.5 mg/kg.[483] LNPs containing DODMA could also deliver CDK4 siRNA to MDAMB-468 cells and Hela cells, inducing a significant reduction of CDK4 protein expression.[484] In 2013, DODMA-based LNP was used for systemic delivery of R-122 miRNA, a liver-specific tumor suppressor miRNA, leading to growth suppression of hepatocellular carcinoma (HCC) xenografts by 50% in mice.[485] A-066 is an analog of DODMA that contains a pyrrole head group. A-066 LNPs encapsulating TetR siRNA could induce significant antitumor efficacy in orthotopic hepatocellular carcinoma models.[486]

Researchers have shown that lipids containing asymmetric tails may improve delivery efficiency compared to lipids with symmetric tails, owing to the increased fusogenesity of lipids and improved membrane fluidity of LNPs.[487−490] For example, Tao et al. developed a library of dimethylamino lipids with asymmetric tails that were a unique modification of DLinDMA (Figure 37).[491] Different from DLinDMA, the CLinDMA series incorporated a cholesteryl ether hydrophobic domain in the lipid tail. They evaluated the siRNA delivery efficiency of these lipids to elucidate their structure−activity relationship and found that the interaction between ionizable lipid and biomembrane was closely related to the pK_a value of the lipid and the environmental pH, and highly charged ionizable lipids
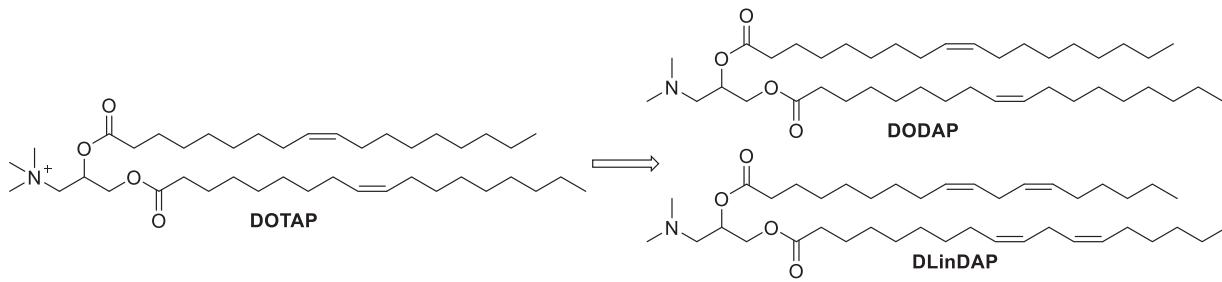
Figure 38. Chemical structures of DODAP and DLinDAP.
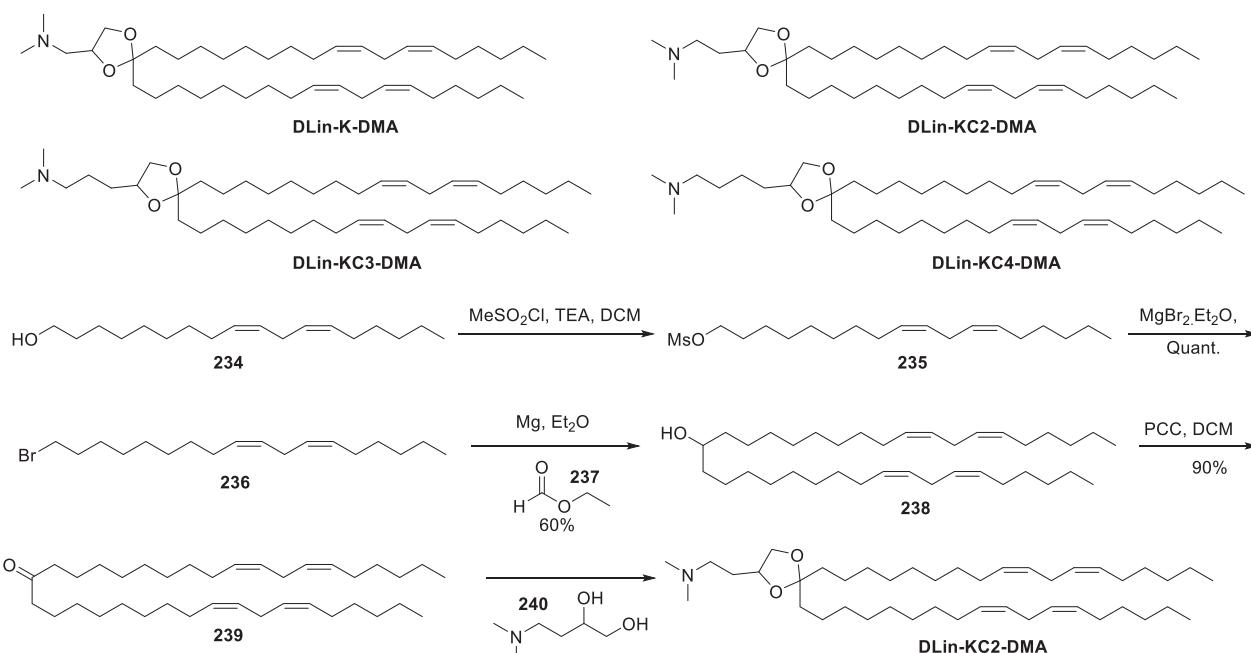
Figure 39. Chemical structures and synthesis of latently biodegradable two-tailed lipids.

lead to stronger interaction.[492−495] Results also showed that the enhanced fusogenesity and membrane destabilizing capability of the ionizable lipids with asymmetric tails appeared to correlate with the overall lipid volume.[496] Besides, the inclusion of a rigid cholesterol ether tail in lipids could produce LNPs that more mimic cell membrane and decrease protein adsorption to lipid membranes.[497]

Other modifications include replacing the ether linkers in DODMA and DLinDMA with esters, leading to DODAP and DLinDAP (Figure 38). 3-(Dimethylamino)propane-1,2-diyl-dioleate (DODAP), an ionizable variant of DOTAP with a pK_a of 6.6, is one of the early biodegradable ionizable lipids used for the delivery of nucleic acids.[498−500] In 2016, LNPs formulated with DODAP/DSPC/cholesterol/C16-PEG2000-Ceramide were used to deliver RVG-9r siRNA, leading to efficient silencing of mutant ataxin-3 gene and reduction in behavior deficits in two Machado−Joseph disease (MJD) mice following intravenous administration.[501]

In 2010, Semple et al. described the synthesis of latently biodegradable cationic lipids by introducing a ketal ring into the linker domain, named as a class of DLin-K-DMA (Figure 39).[502] As shown in Figure 39, at the beginning of the synthesis of DLin-KC2-DMA, linoleyl alcohol **234** was converted into linoleyl bromide **236** via methanesulfonylation followed by bromination. Then addition reaction between ethyl formate **237** and Grignard reagent prepared from **236** gave secondary alcohol

238, which was oxidized to afford ketone **239**. Finally, the head group was attached via ketalization of ketone **239** with diol **240** to furnish DLin-KC2-DMA.[502] These ionizable lipids, with pK_a values in a range between 6.2 and 6.7, displayed potent gene silencing activity *in vivo*. Particularly, DLin-KC2-DMA was identified as the lead candidate among the four lipids for siRNA delivery. DLin-KC2-DMA showed tolerability in both rodent and nonhuman primates, LNPs formulated with DLinKC2-DMA/DSPC/cholesterol/PEG-lipid at a molecular ratio of 40:10:40:10 exhibited activity at anti-TTR siRNA doses as low as 0.01 mg/kg in rodent and 0.1 mg/kg in nonhuman primates, respectively.[502] In 2012, a DLin-KC2-DMA formulation with an androgen receptor (AR) siRNA was developed as an AR inhibitor to treat prostate cancer. This formulation showed strong suppression of AR both *in vitro* and *in vivo*.[503] In 2013, Lin et al. examined *in vivo* gene silencing activities of LNP-siRNA systems incorporating DLinDAP, DLinDMA, DLin-K-DMA, and DLinKC2-DMA and found that their activity varied over 3 orders of magnitude with the following order DLinKC2-DMA > DLin-K-DMA > DLinDMA ≫ DLinDAP.[504] This tendency was in accordance with results of previous *in vivo* gene silencing studies in human prostate tumor tissue[503] and primary antigen-presenting cells (APC) of the spleen and peritoneal cavity.[505]

Based on the studies on DLin-K-DMA, additional biodegradable two-tailed dimethyl amino lipids were then synthesized
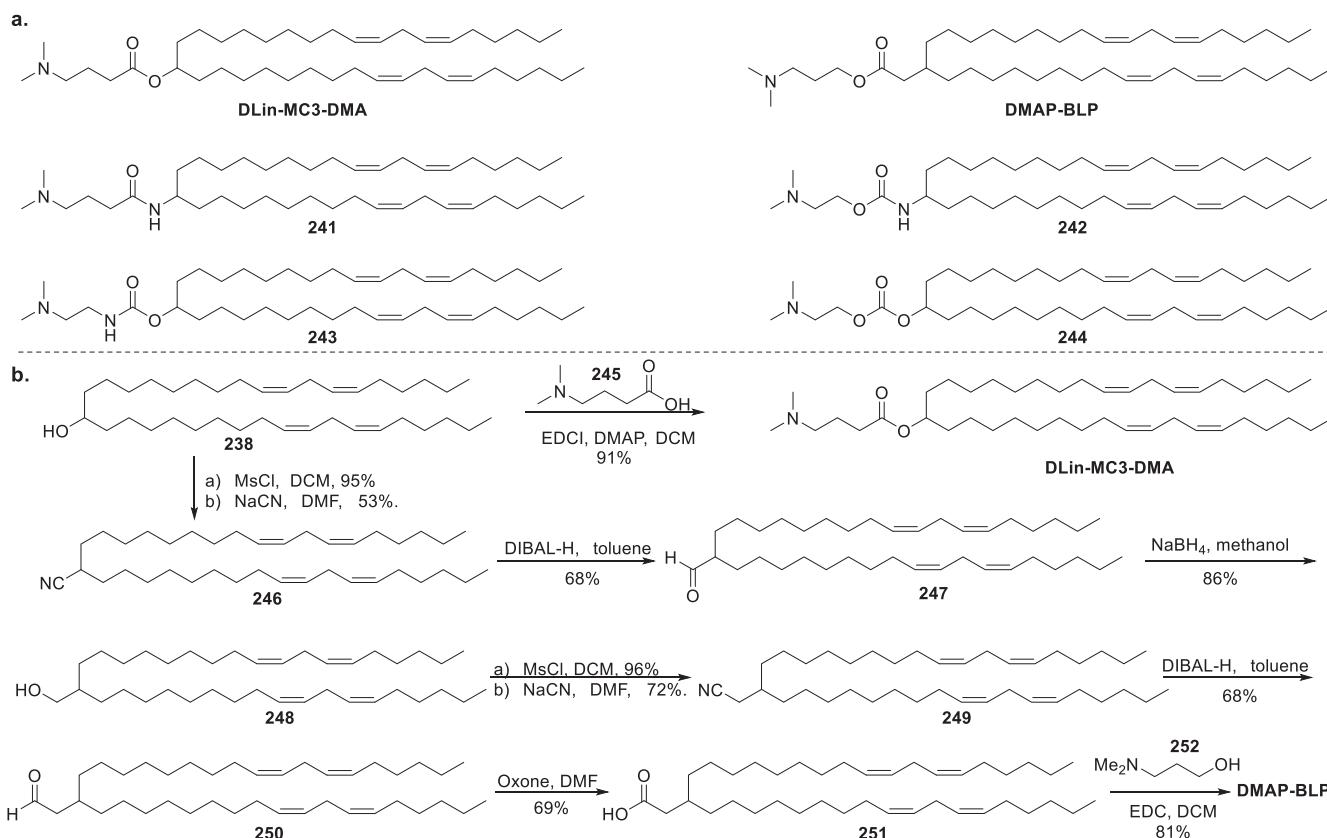
Figure 40. Representative chemical structures and synthetic routes to biodegradable two-tailed lipids.

(Figure 40).[441] The linker domain of the amino lipid was further modified by introducing biodegradable ester, amide, carbamate, or carbonate groups to modulate the lipid pK_a (Figure 40a). 53 amino lipids with pK_a values between 4.17 and 8.12 were prepared and complexed with siRNA to form LNPs. Interestingly, gene silencing potency was closely related with pK_a value: ionizable lipids with pK_a between 6.2 and 6.5 had the lowest ED$_{50}$. The lead compound identified in this study was named DLin-MC3-DMA (MC3), which contains a dilinoleic acid tail and an ester linker group and enables potent hepatic gene silencing with an ED$_{50}$ of 0.005 mg/kg for rodents and less than 0.03 mg/kg for nonhuman primates (a molecular ratio of 50:38.5:10:1.5 for MC3/cholesterol/DSPC/PEG-lipid).[441,502] This LNPs formulation has been applied in Onpattro (patisiran), the first FDA approved LNP-based siRNA drug, which targets liver hepatocytes, the primary site for the synthesis of TTR protein.[506] In 2016, Nabhan et al. used MC3 LNPs encapsulating human frataxin (FXN) mRNA for supplementing FXN protein in dorsal root ganglia in mice following intrathecal administration.[507]

In 2013, Rungta et al. synthesized 3-(dimethylamino)propyl-(12Z,15Z)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yl]henicosa-12,15-dienoate (DMAP-BLP) (Figure 40b), an analog of DLin-MC3-DMA.[508] The synthesis of DMAP-BLP began with sulfonylation of secondary alcohol **238** with methanesulfonyl chloride followed by S$_N$2 reaction with sodium cyanide, giving cyanide **246**. Cyanide **246** was reduced with DIBAL-H, and the resulting aldehyde **247** was treated with NaBH$_4$ to afford primary alcohol **248**, which was converted to cyanide **249**. Then, reduction of cyanide **249** gave aldehyde **250**, which was oxidized with oxone to generate acid **251**. Finally, the combination of the head group with the hydrophobic tails via an esterification reaction between acid **251** and amino alcohol **252** promoted by EDCI provided DMAP-BLP (Figure 40).[508] Rungta et al. used LNPs formulated with DMAP-BLP to deliver GRIN1 siRNA, resulting in efficient silencing of neuronal gene expression *in vitro* and selective reduction of synaptic *N*-methyl-D-aspartate receptor (NMDAR) currents in the brain in mice through intracranial injection.[508] In 2016, DMAP-BLP-based LNPs were used for investigating the effect of particle size on the *in vivo* activity of siRNA-loaded LNPs. Results showed that siRNA-loading LNPs with diameters < 45 nm were considerably less potent in gene silencing as compared to LNPs in larger sizes. This is partially attributed to the rapid dissociation of lipid components which leads to a decrease in the stability of smaller LNPs.[509] DMAP-BLP has also been used to formulate mRNA vaccines in several preclinical studies[510,511] and clinical studies.[510,512] In a series of influenza preclinical studies, DMAP-BLP LNP delivered nucleoside-modified mRNA encoding hemagglutinin (HA) immunogens intradermally, resulting in full protection of mice against a lethal challenge with a single dose of 0.4 μg per mouse.[510] In a phase 1 trial using DMAP-BLP LNPs to deliver two distinct mRNA-encoded HA immunogens, all of the 23 participants had HAI titers > 1:40 at a dose of 100 μg via intramuscular injection.[512] In 2017, a DMAP-BLP-based LNP formulated with DMAP-BLP/cholesterol/DSPC/PEG-lipid at a weight ratio of 50:38.5:10:1.5 was used for delivering IgEsig-prM-E mRNA encoding immunogen for the Zika virus. This LNP-mRNA formulation was capable of protecting mice lacking type I and II IFN signaling against a lethal challenge with a single dose of 10 μg or two 2 μg doses in a prime-boost approach.[513,514] In 2018, Jhon et al. prepared DMAP-BLP LNPs
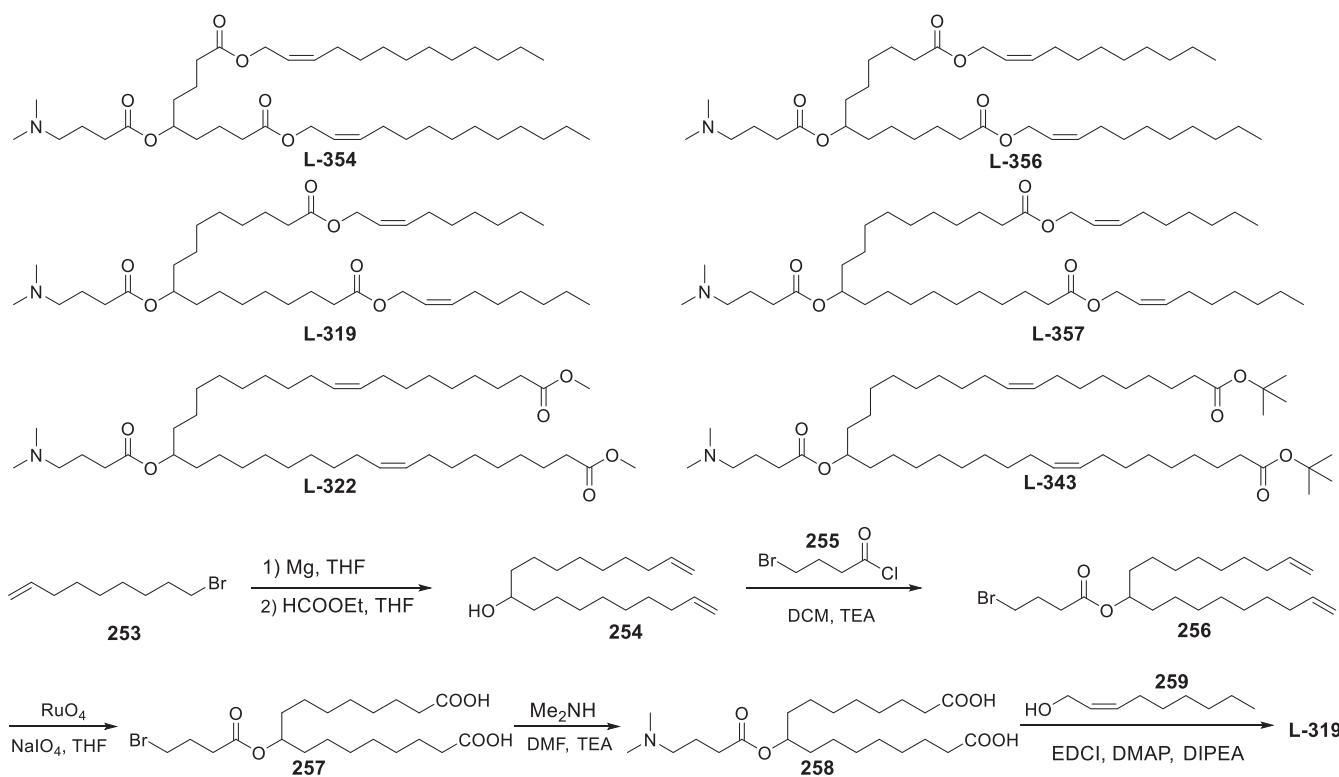
Figure 41. Synthesis of biodegradable dimethyl amino lipids.
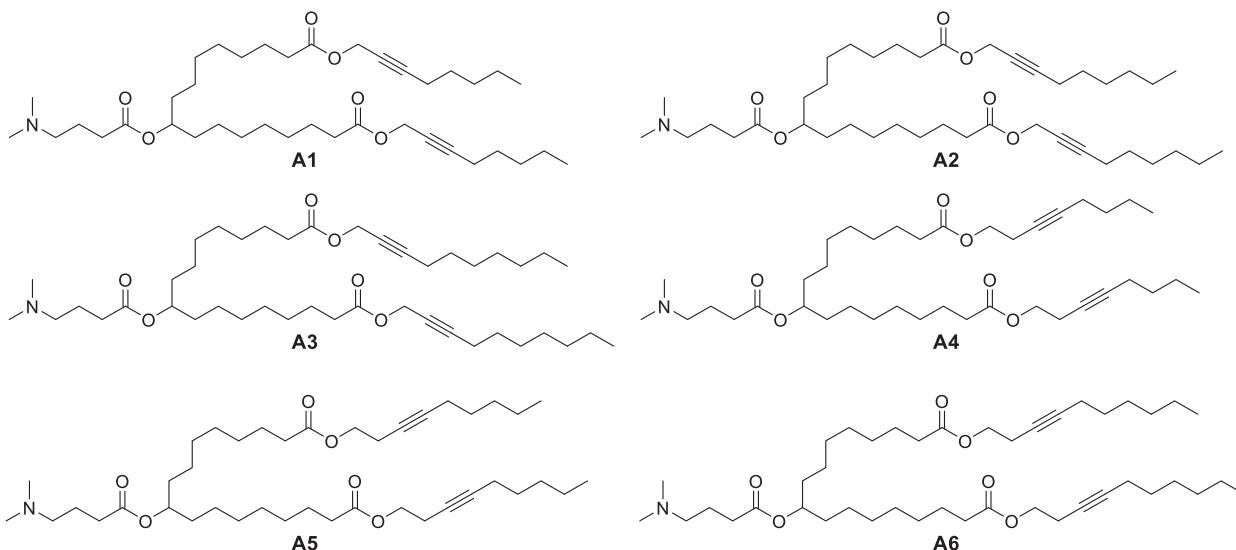
Figure 42. Chemical structures of biodegradable alkyne analogs of DLin-MC3-DMA.

encapsulating mRNAs encoding the human cytomegalovirus (CMV) glycoprotein B (gB). The DMAP-BLP/mRNA vaccine was efficiently delivered in mice and nonhuman primates (NHPs) following intramuscular injection, resulting in broadly neutralizing antibodies and potent immune responses.[515]

It is challenging to introduce biodegradable functionality in lipids and maintain high RNA delivery efficacy at the same time. In 2013, Maier et al. further modified DLin-MC3-DMA by incorporating a biodegradable primary ester linker in place of one of the double bonds in the hydrophobic tail (Figure 41).[516] The synthesis of L-319 started with the addition of Grignard reagent prepared from 9-bromo-1-nonene **253** to ethyl formate,

giving secondary alcohol **254**, which underwent acylation with 4-bromobutanoyl chloride **255** to provide ester **256**. Oxidization of the terminal alkene groups in **256** with RuO$_4$ led to bis-acid **257**. Then, the dimethylamino head group was installed via substitution of the bromine atom to afford amino bis-acid **258**. L-319 was finally obtained via bis-esterification of bis-acid **258** with (Z)-2-nonen-1-ol **259** with EDCI as the carboxyl activating agent. Structure-activity relationship analysis showed that the position of the ester bond was critical to the delivery efficiency and clearance rate of lipids. Moving the ester bond closer toward the amine head group led to decreased delivery efficacy in vivo, which might be related to the decreased pK_a. When the ester
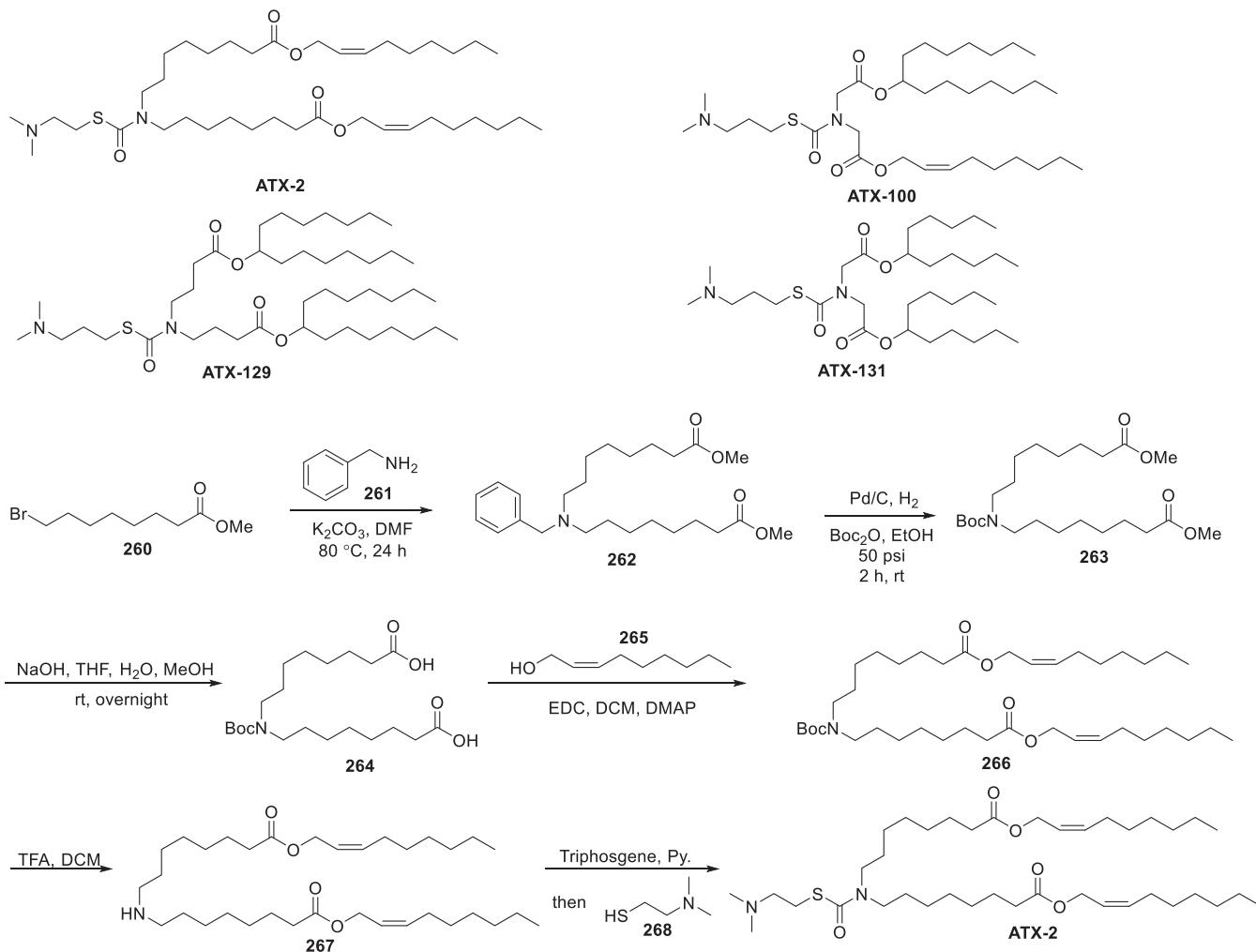
Figure 43. Chemical structures and synthesis of ATX lipids.

bond was positioned further away from the amine head group, the lipids were more resistant in the mouse liver. L-319 was identified as the lead biodegradable amino lipid from the screening, and L-319 LNPs had an FVII ED_{50} of less than 0.01 mg/kg in mouse studies. Results from pharmacokinetic studies in mice suggested that the resulting water-soluble metabolites from ester cleavage of L-319 were rapidly cleared from plasma and tissues without significant toxicity.[516]

In 2020, Miao et al. synthesized a library of biodegradable analogs of DLin-MC3-DMA by introducing ester and alkyne groups into the hydrophobic tails (Figure 42).[517] They observed that the incorporation of alkyne groups in the hydrophobic tails of the ionizable lipids could enhance their fusion with the endosomal membrane, thus facilitating endosomal membrane destabilization, endosomal escape, and the release of the mRNA payload. Particularly, A6 showed an 8.5-fold higher production of human erythropoietin (hEPO) than MC3 LNPs in mice. The enhanced endosomal fusion might be caused by the tail protrusion and the lateral diffusion of the outer layer lipids into the endosomal membrane. Additionally, coformulation of A6 and cKK-E12[518] synergistically boosted mRNA delivery into hepatocytes.[517]

Rajappan et al. synthesized a library of biodegradable ionizable lipids (referred to as ATX) that contain a central nitrogen atom connecting with two esterase-sensitive hydrophobic tails and the amino head group via a thiocarbamate union

(Figure 43).[519,520] ATX with diverse chemical structures were obtained by changing the acid chain length, altering the ester-forming alcohol, and tuning the distance between the sulfur atom and the head group. The synthesis of ATX-2, for example, started with the combination of methyl-8-bromooctanoate **260** and benzylamine **261**, giving benzylic amine **262**, which was hydrogenated in the presence of Boc-anhydride, affording diester **263**. Hydrolysis of bis-ester **263** led to diacid **264**, which was bis-esterified with (Z)-2-nonenol **265** to provide diester **266**. The Boc protection was removed, and the resulting Bis-ester **267** was treated with triphosgene and 2-(dimethylamino)-ethanethiol **268** in pyridine to yield ATX-2. The lipophilicity (indicated by cLog$D$) and basicity (indicated by pK_a) of the lipids were measured, and lipids were formulated into LNPs encapsulating anti-FVII siRNA for evaluating their activities in the FVII knockdown assay *in vivo*. Results showed that the *in vivo* activity of lipids is related to a multivariable equation with strong ties to not only its pK_a but also its lipophilicity. For example, ATX-131 LNP, with a measured pK_a of 6.21, showed no FVII gene knockdown activity due to its low lipophilicity (cLog$D$ < 10).[519] In 2017, ATX-2 LNPs encapsulating human FIX (hFIX) mRNA were used for treating a Factor IX (FIX)-deficient mouse model of hemophilia B, resulting in 2-fold more hFIX protein expression than MC3 LNPs at an mRNA dose of 2 mg/kg following intravenous injection.[61] One of the ATX was used to deliver mRNA in the development of the SARS-CoV-2
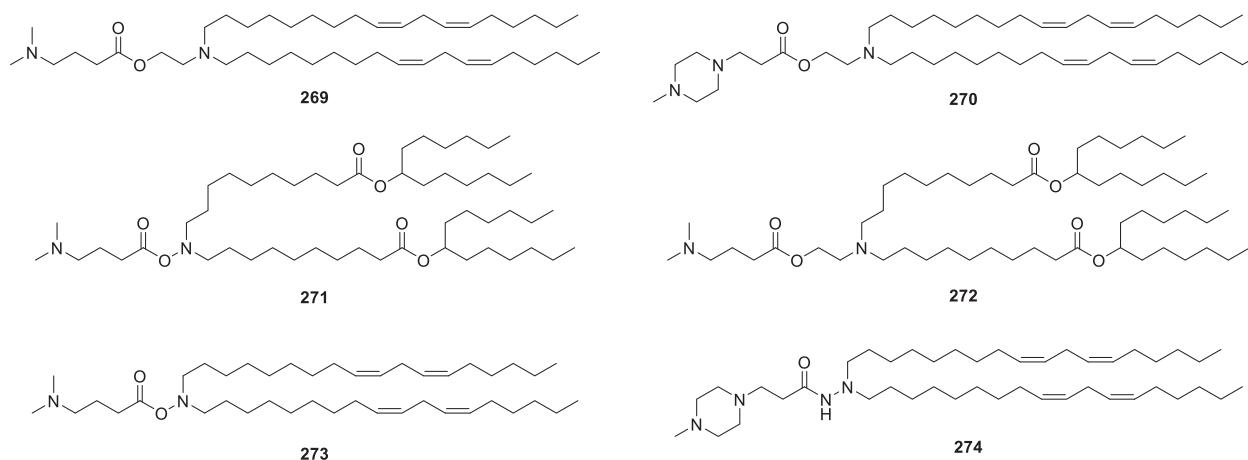
Figure 44. Chemical structures of two-tailed lipids with other linkers.
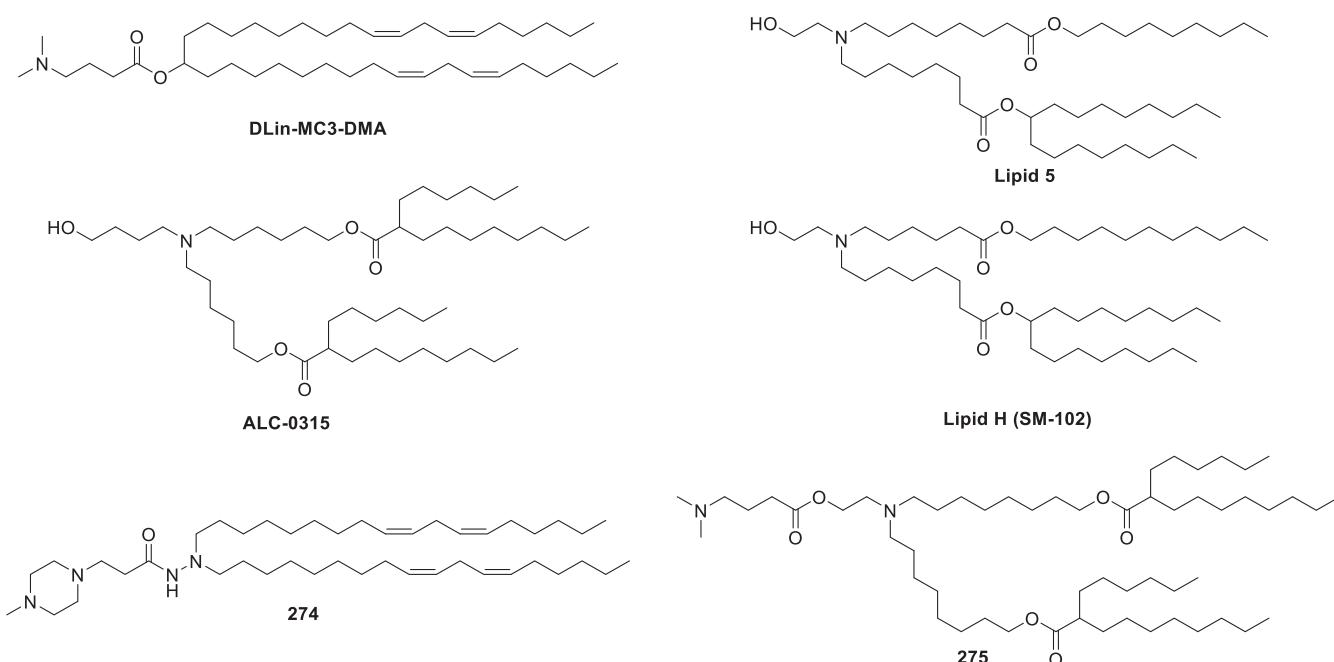
Figure 45. Chemical structures of ionizable lipids used in the development of COVID-19 mRNA vaccines.

vaccine (LUNAR 36-COV19), of which a single dose of 2 μg per mouse protected mice from both mortality and infection following wild-type SARS-CoV-2 challenge.[521]

In 2020, Ramishetti et al. synthesized a library of analogs of MC3 that contained other linker groups such as hydrazine, ethanolamine, and hydroxylamine for gene silencing in leukocyte subsets (Figure 44).[522] Lipids incorporating hydroxyl-amine and ethanolamine linkers were more efficient in gene silencing in CD8+ and CD4+ T leukocytes compared to that with hydrazine linkers. Additionally, lipids with a piperazine head group (lipid **270**) accumulated more in the spleen than the liver, while lipids with a dimethylamino head group (lipid **269**) resulted in more accumulation in the liver than the spleen. Moreover, lipids with a branched ester as the tail (lipids **271** and **272**) were less effective for siRNA delivery than those with linoleyl chains (lipids **273** and **269**). Systemic administration of lipid **269** LNPs encapsulating CD45 siRNA resulted in signification reduction of the CD45 level in both CD4+ and CD8+ T lymphocytes.[522]

The results of clinical trials of BNT162b2[523−525] and mRNA-1273[526−528] vaccines successfully led to their approval in clinical application,[7−9] providing a powerful approach to counteract the worldwide COVID-19 pandemic. Ionizable lipids used in the BNT162b2 and mRNA-1273 formulations were disclosed as ALC-0315[529,530] and SM-102 (Lipid-H),[530,531] respectively (Figure 45). ALC-0315 has two degradable branched ester tails, and SM-102 contains a primary degradable ester tail and a degradable branched ester tail. ALC-0315 LNPs showed approximately 6-fold higher firefly luciferase level than MC3 LNPs at a FLuc mRNA dose of 0.3 mg/kg in mice,[529] whereas Lipid 5, an analog of SM-102 (Lipid-H), displayed a 3−6-fold increase in protein expression or immune responses in mice compared to MC3 when delivering an mRNA encoding influenza immunogen.[531] These results indicated that the branching of tails may create a more cone-shaped structure and facilitate the endosomal escape of RNA molecules.[532,533] BNT162b2 uses LNPs formulated with ALC-0315/cholesterol/DSPC/PEG-lipid to deliver mRNA encoding immunogen that
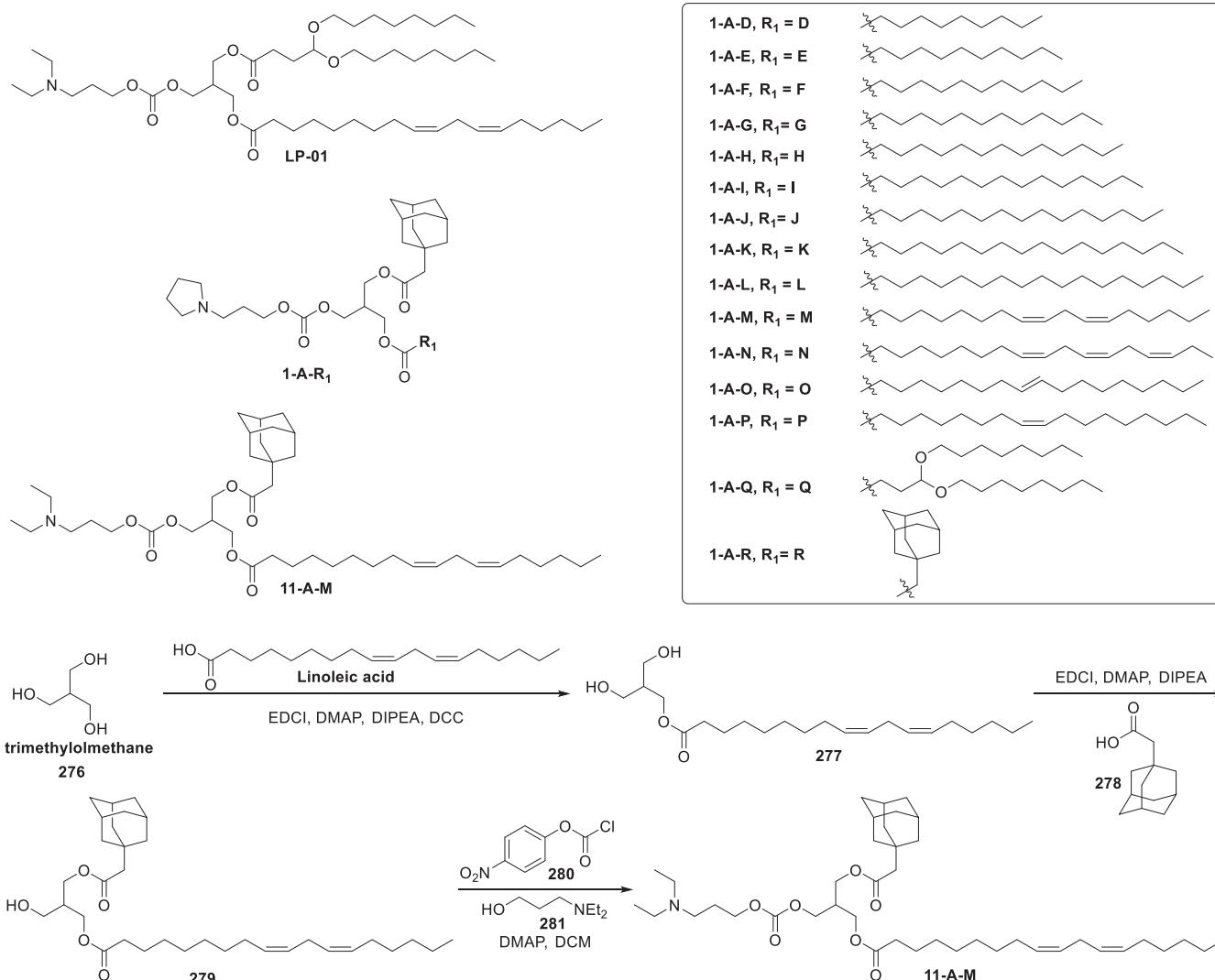
Figure 46. Chemical structures of constrained lipids with small head groups.

is a diproline-stabilized, membrane-bound spike protein.[525] mRNA-1273 uses SM-102 LNPs to deliver mRNA encoding transmembrane-anchored diproline-stabilized prefusion spike with a native furin cleavage site. SM-102 (lipid H)[531] and lipid 5[534] belong to a class of biodegradable ionizable lipids that were synthesized by incorporating greater branching rather than the dilinoleic alkyl tails of MC3.[531,534] Both lipid 5[534] and SM-102 (lipid H)[531] contain an ethanolamine head group, a saturated linear tail with a primary ester linker and a second saturated branched tail with a less biodegradable secondary ester linker. Lipid 5 LNPs displayed luciferase expression 3-fold higher as compared to MC3 LNPs in mice and induced hEPO expression 5-fold higher than MC3 LNPs in nonhuman primates following intravenous administration. The enhanced protein expression may possibly be due to a high endosomal release of the mRNA cargo in lipid 5 LNPs. Pharmacokinetic studies revealed that lipid 5 LNPs decreased liver accumulation and were fully degraded within 24 h in both rats and nonhuman primates. SM-102 (lipid-H), which structurally differs from lipid 5 by a two-carbon displacement of the primary ester tail, was identified as the most promising lipid following intramuscular (IM) administration. The pK_a of SM-102 LNPs is slightly higher than that of lipid 5 LNP (6.68 vs 6.56).[531] Results of a phase 3

clinical trial showed that two 30 μg doses of BNT162b2 induced 95% protection against COVID-19 (95% credible interval, 90.3 to 97.6),[525] and mRNA-1273 conferred 94.1% protection against COVID-19 illness after receiving two 100 μg doses in another phase 3 clinical trial.[527] In 2021, Elia et al. used lipid 274 LNPs and lipid 275 LNPs to deliver mRNA encoding SARS-CoV-2 human Fc-conjugated receptor binding domain (RBD-hFc mRNA).[57] While both lipid 274 LNP RBD-hFc mRNA and lipid 275 LNP RBD-hFc mRNA induced equal cellular and humoral responses in mice at an mRNA dose of 5 μg, only lipid 275 LNP RBD-hFc mRNA exhibited strong immunogenicity following intradermal administration. Both intradermal administration and intramuscular administration of lipid 275 LNPs could activate antigen presenting cells (APCs), thus inducing cellular responses.[57]

The stability of LNP−RNA formulations is related to the properties of both RNA molecules and LNPs. For example, chemically modified siRNA molecules are double-stranded and relatively stable, while mRNA molecules are prone to be degraded via hydrolysis of the phosphodiester bonds[535] and oxidation of the nucleobases.[536] The chemical and physical features of LNPs can greatly affect their stability. The unsaturated hydrocarbon tails of lipids or cholesterol can be
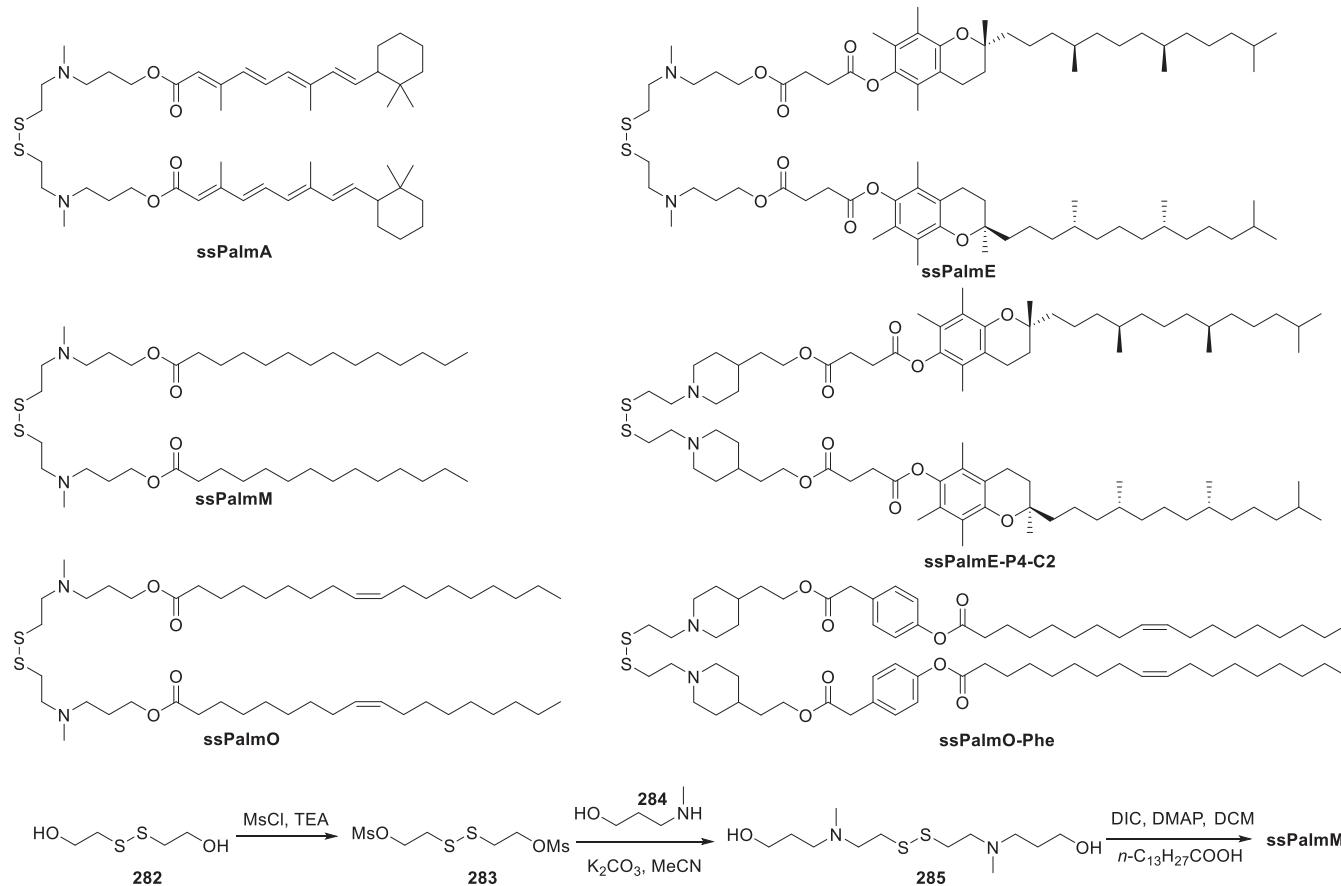
Figure 47. Chemical structures of representative ssPalm and a synthetic route to ssPalmM.

oxidized by various enzymes or agents.[537,538] Aggregation and fusion can lead to physical destabilization of LNPs.[539,540] Generally, PEG-lipids are incorporated in LNPs to prevent the aggregation of nanoparticles and to improve their circulation stability.[541] At 2−8 °C, mRNA-1273 and BNT162b2 can be stored for 30 days and 5 days, respectively. Both of them need to be stored in a frozen state at extremely low temperature for long shelf life (6 months) and thawed before injection.[542] The shelf life of the LNP−siRNA formulation of patisiran is as long as three-years when kept between 2 and 8 °C. The main difference of lipid components between the two LNP−mRNA vaccines and the LNP−siRNA formulation of patisiran lies in the ionizable lipids (SM-102, ALC-0315 vs MC3); therefore, it may be the stability of mRNA, rather than that of LNP, which determines the storage conditions and shelf life of LNP−mRNA formulations.[543]

In 2017, Fin et al. reported the simultaneous delivery of Cas9 mRNA and sgRNA for transthyretin gene editing *in vivo* by using LP-01 LNPs, resulting in a significant transthyretin gene editing in the liver in mice and a reduction in serum transthyretin levels by >97% after a single administration (Figure 46).[544] In 2017, Dahlman et al. developed an efficient method to measure the biodistribution of many barcoded nanoparticles in a single mouse.[545] This barcoded nanoparticle system can facilitate the characterizations of a large number of LNPs targeting specific tissues and cells and aid understanding of the structure−activity relationship of lipids *in vivo*. In 2019, they found that certain LNPs could deliver sgRNA and siRNA into splenic T cells *in vivo*.[546] They synthesized a library of 16 constrained ionizable lipids containing small head groups (diethylamino head group

or 1-pyrrodinyl head group), carbonate linkers, and two hydrophobic tails. As shown in Figure 46, the two hydrophobic tails were attached to two hydroxyl groups trimethylolmethane **276** via successive esterification, and the amino head group was conjugated to the remaining hydroxyl group via a carbonate linker to afford the desired lipids. 55 LNPs carrying anti-GFP siRNA and unique barcodes were formulated from 15 ionizable lipids, C14-PEG2000, cholesterol, and DSPC at four different ratios. Results showed that the top-performing 11-A-M LNPs were enriched more than other LNPs at a dose of 0.5 mg/kg in mice. The biodistribution of 11-A-M LNPs was in splenic CD8+ T cells, CD4+ T cells, and B cells. 11-A-M LNPs were also used to deliver chemically modified sgRNA targeting GFP to downregulate the expression of GFP in CD8+ and CD4+ T cells in mice.[546]

ssPalm are a class of gemini ionizable lipids, in which the two tertiary amino head groups are connected via a bioreducible disulfide linker and each amino head group is connected with a methyl group and hydrophobic tails (Figure 47).[547−552] The starting material for the preparation of ssPalmM was 2,2′-dithioldiethanol **282**, which was methanesulfonylated to give methanesulfonate ester **283**. The head groups were installed via the substitution reaction between methanesulfonate ester **283** and 3-(methylamino)-1-propanol **284**, affording diol **285**. Diol **285** was esterified with myristic acid to give ssPalM.[547] Prior studies reported that ssPalm was protonated after internalization of ssPalm LNPs into cells, thus facilitating the endosomal escape of ssPalm LNPs.[504] Besides, the disulfide bridge cleavage induced by intracellular glutathione (GSH) in lysosomes facilitated the disassembly of LNPs and the release of the
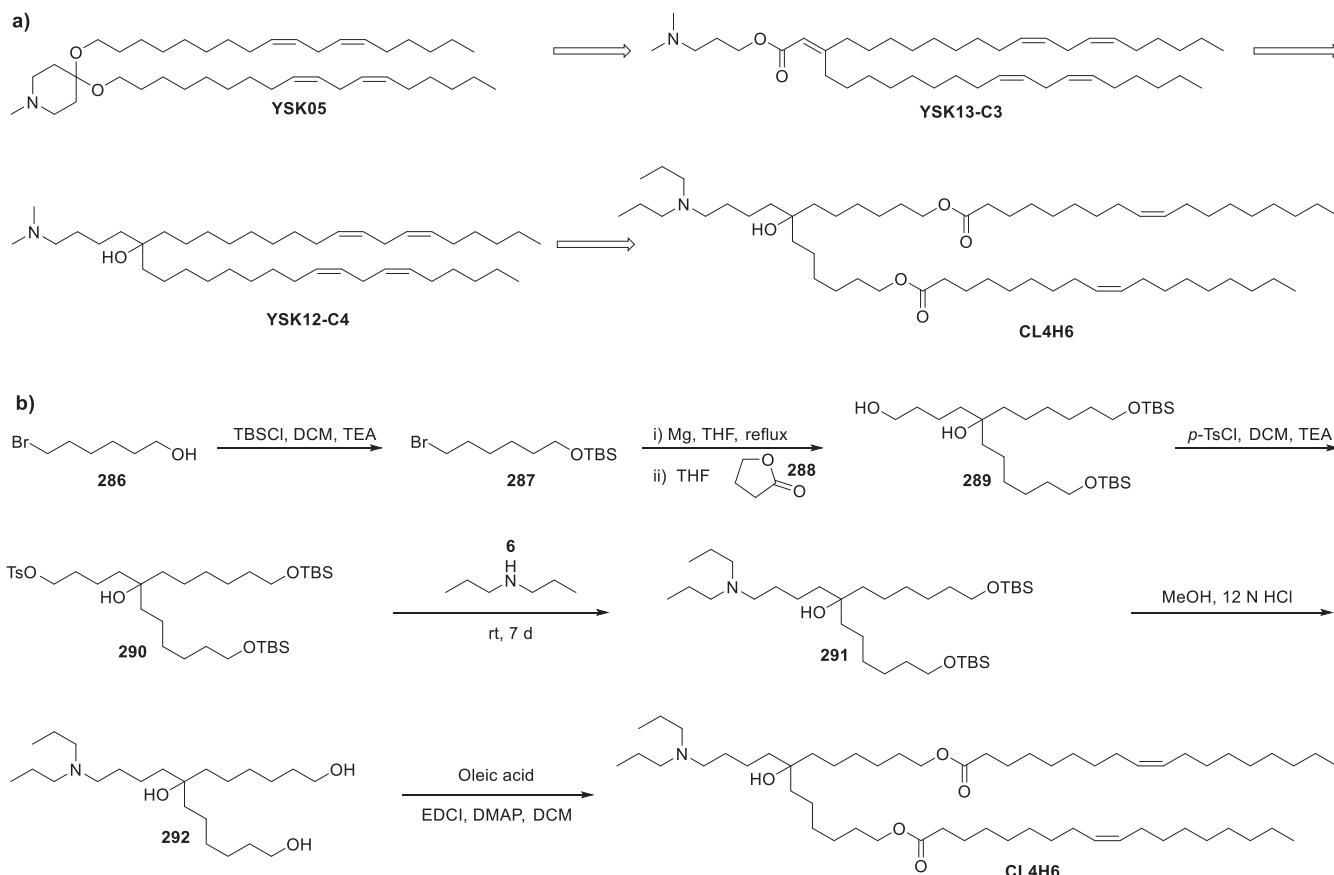
Figure 48. Chemical structures of YSK series lipids and a synthetic route to CL4H6.

encapsulated RNA molecules.[547,553] In 2015, Akita et al. found that ssPalmE LNPs could deliver FVII siRNA to hepatic cells more efficiently as compared to ssPalmM or ssPalmA after intravenous administration at a siRNA dose of 4 mg/kg in mice.[554] ssPalmA LNPs were used to deliver siRNA against Col1a1, resulting in an inhibitory effect on hepatic fibrosis in mice following systemic injection, with an ED_{50} of 0.25 mg/kg.[555] In 2015, they further modified ssPalmE by incorporating more flexible piperidine and systemically prolonging the distance between the hydrophobic domains and the ionizable nitrogen atoms, resulting in the pK_a being increased from 6.03 to 6.18, thus allowing the tertiary amine groups more readily to be pronated. The increased pK_a of the ssPalmE-P4-C2 led to the ED_{50} value for the ssPalm-P4-C2 LNPs in FVII knockdown as low as 0.035 mg/kg in mice.[554] In 2018, by integrating the oleic acid scaffold into the ssPalm, Tanaka et al. prepared ssPalmO and its derivatives, which exhibited high mRNA delivery efficiency in the colon and less immune stimulation.[556,557] In 2019, Tateshita et al. reported the *ex vivo* transfection of DCs using ssPalmE LNPs encapsulating ovalbumin (OVA) mRNA; the transfected DCs induced strong cytokines production in mouse bone marrow-derived dendritic cells (BMDCs).[558] In 2020, Tanaka et al. synthesized a self-degradable analog of ssPalmO, ssPalmO-Phe, by introducing a phenyl ester linker. The self-degradation of ssPalmO-Phe involved in the mechanism of "hydrolysis accelerated by intra-particle enrichment of reactant (HyPER)".[550] In this reaction, disulfide bonds are cleaved under a reducing environment, generating enriched hydrophobic thiols, which can attack the phenyl ester linker group and break them down. Additionally, codelivery of Cas9

mRNA and sgRNA targeting TTR by ssPalmO-Phe LNPs allowed the editing of 55% of the TTR-encoding genome and led to a 95% reduction in serum TTR level after systemic administration in mice.[550]

The Harashima group developed the YSK series of ionizable lipids; among them several lead lipids have been identified, such as the first generation YSK05,[202] the second generation YSK13-C3,[559,560] as well as the third generation YSK12-C4[561] and CL4H6[562] (Figure 48). In 2012, DODAP-based lipid YSK05 was developed, which contained an ionizable cyclic tertiary amino head group, a ketal linker, and two linoleyl chains. YSK05, with a pK_a value of 6.4, could promote endosomal membrane fusion and endosomal escape. YSK05 LNPs encapsulating anti-PLK1 siRNA showed efficient and durable PLK1 gene silencing activity after intravenous administration in mice.[202,563] YSK05 LNPs have also been used in siRNA delivery for the treatment of hepatitis C virus (HCV) infections in mice with humanized livers[564] and the codelivery of sorafenib (SOR) and anti-midkine gene (anti-MK) siRNA to treat hepatocellular carcinoma (HCC).[565] To enhance the tissue clearance as well as RNA delivery efficiency, biodegradable YSK13-C3 was then developed.[559,560] The ED_{50} of gene silencing in mice following intravenous administration of YSK13-C3 LNPs encapsulating blood-clotting factor VII (FVII) siRNA was 0.015 mg/kg, which was about 4-fold more active than that of YSK05 LNPs.[560] Intravenous injection of HBV siRNA-loading YSK13-C3 LNPs into mice could simultaneously reduce the levels of the HBV DNA and HBV antigens (HBsAg and HBeAg).[560] In 2016, they developed YSK12-C4, which contained a hydroxy group near the amino head group. The use of YSK12-C4 LNPs
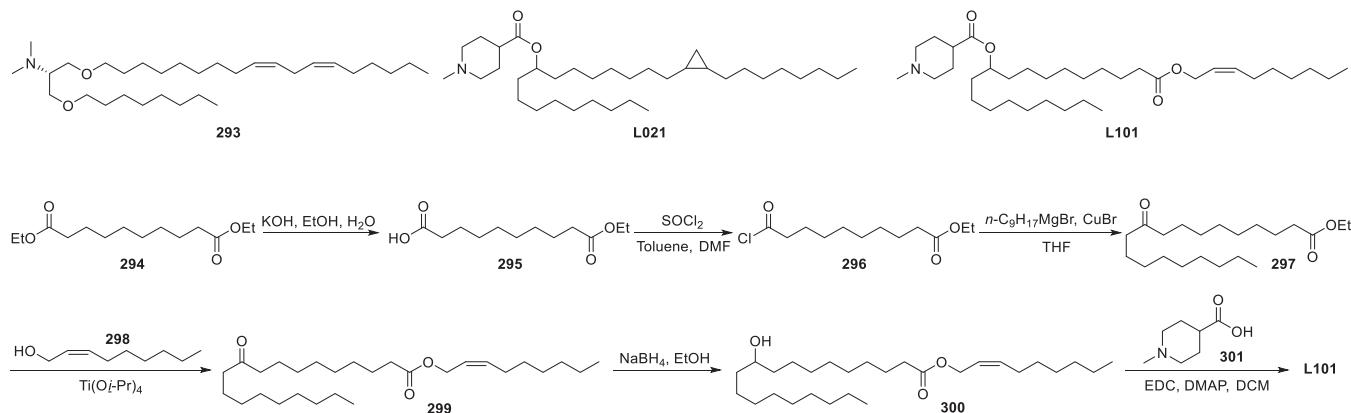
Figure 49. Chemical structures of lipid **293**, L021, and L101 and a synthetic route to L101.
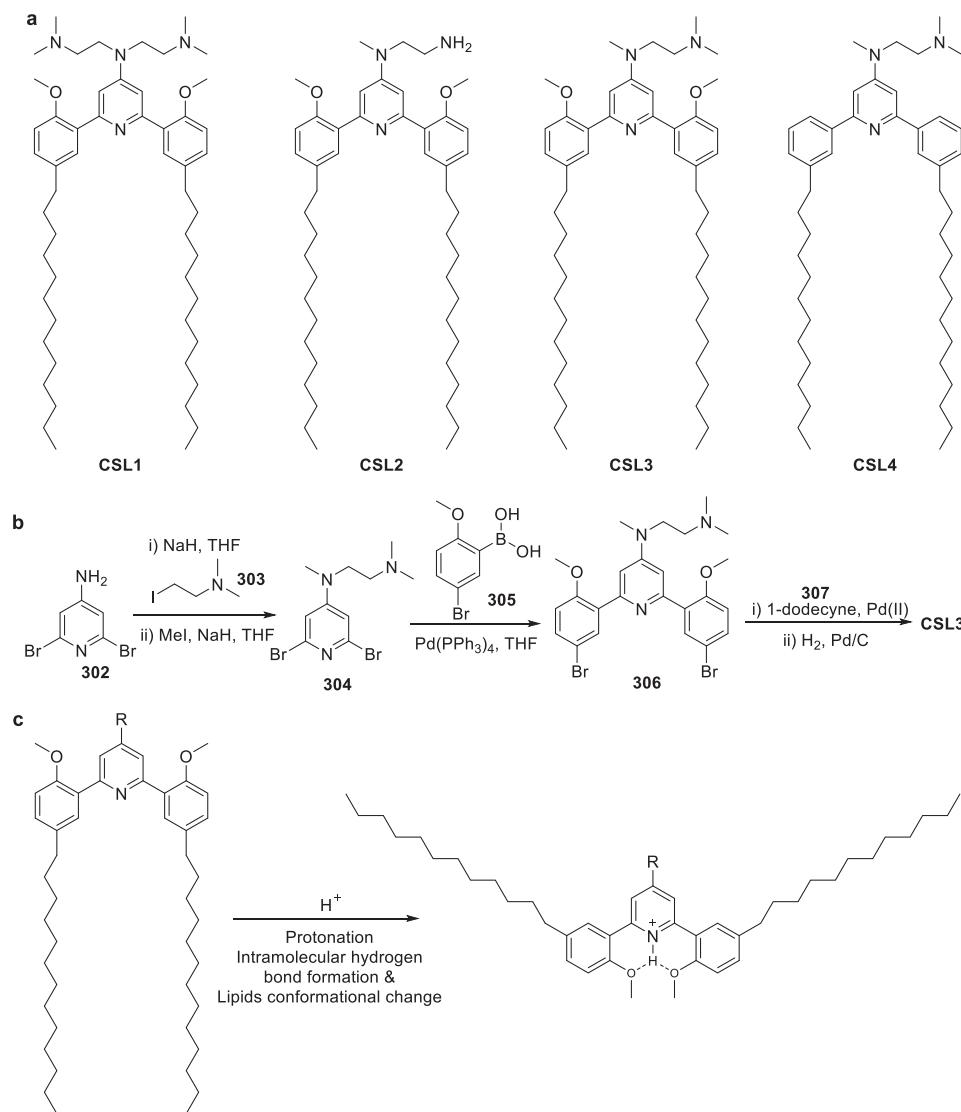
Figure 50. (a) Chemical structures of ionizable switchable lipids. (b) Synthetic route to CSL3;. (c) Protonation-induced conformational change of the ionizable switchable lipids.

encapsulating scavenger receptor class B type 1 (SR-B1) resulted in a gene silencing in mouse BMDCs, with an ED_{50} of 1.5 nM. Additionally, downregulation of the suppressor of cytokine signaling 1 led to an elevation of the cytokine level and significant inhibition of tumor growth.[561] Based on the study of

YSK12-C4, CL4H6, a lipid that contained oleic acid esters in the hydrophobic tails and had a pK_a value of 6.25 was identified as another lead lipid (Figure 48).[562] Structure−activity relationships showed that the structure of the head group was key to the apparent pK_a's of ionizable lipids and played an important role in
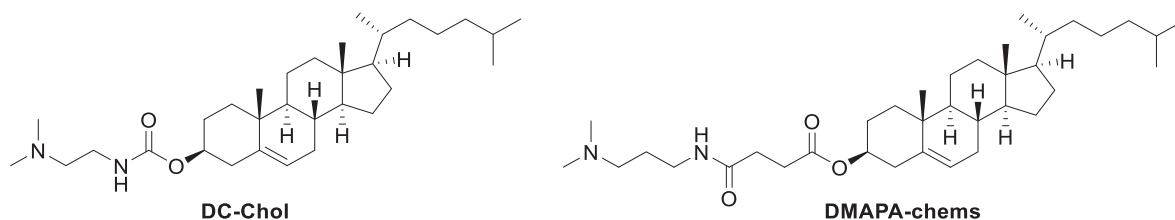
Figure 51. Chemical structures of cholesterol-derived ionizable lipids.

the intrahepatic distribution and endosomal escape of LNPs. Intravenous administration of CL4H6 LNPs encapsulating FVII siRNA into mice resulted in significant FVII gene silencing with a hepatic ED_{50} of 0.0025 mg/kg.[562] As shown in Figure 48, the starting material for the synthesis of CL4H6 was 6-bromo-1-hexanol **286**, which was treated with *tert*-butyldimethylsilyl chloride (TBSCl) to protect the hydroxy group, giving compound **287**. Grignard reagent prepared from compound **287** was reacted with δ-valerolactone **288** to afford diol **289**. The amino head group was installed via chemical selective sulfonylation of the primary hydroxy group of diol **289** followed by a substitution reaction with dipropylamine **290**, providing compound **291**. Removal of the two TBS protecting groups led to triol **292**, which underwent bis-esterification of the two primary hydroxy groups with oleic acid to obtain EL4H6 (Figure 48).[562]

In 2014, Gindy et al. reported that LNPs containing lipids with asymmetric tails were well-tolerated as they were rapidly cleared *in vivo*.[566] Asymmetric lipid-formulated LNPs favor the adoption of destabilizing nonbilayer structures to facilitate the release of RNA cargo. However, these LNPs suffer from physical instability during storage, due to their weak hydrophobic interactions between the lipid components in LNPs, which may be driven by the asymmetric lipids.[566] Based on this work, Suzuki et al. developed a series of ionizable lipids that contained two asymmetric hydrophobic tails (e.g., L021) (Figure 49). *In vitro* screening and structure−activity relationship studies identified L021 that contained an *N*-methylpiperdine head group and a C9 short tail, as the lead candidate for siRNA delivery (Figure 49).[567] Intravenous injection of L021 LNPs encapsulating FVII siRNA induced significant FVII gene knockdown with an ED_{50} of ∼0.02 mg/kg in mice. Additionally, L021 LNPs were stable to be stored at 4 °C for 1.5 years as a liquid without an increase in LNPs size or degradation of lipids. The cyclic *N*-methylpiperidine head group may increase hydrophobic interactions between the lipid components in the LNPs, thus enhancing the bilayer packing and conferring LNPs with sufficient stability.[567] In 2016, they also revealed that both low-density lipoprotein (LDL) receptor and Apolipoprotein E (ApoE) played significant roles in *in vivo* intracellular uptake of L021 LNPs. Besides, *in vivo* biodistribution of L021 LNPs was significantly influenced by ApoE.[568] In 2017, they synthesized a biodegradable analog of L021, L101, by introducing an ester linker into the long hydrophobic tail (Figure 49).[569] As shown in Figure 49, monohydrolysis of diethyl decanedioate **294** gave decanedioic acid monoethyl ester **295**, which was treated with oxalyl chloride to afford acyl chloride **296**. Substitution reaction between acyl chloride **296** and *in situ* generated organocuprate reagent led to ketone **297**. The biodegradable tail was installed via transesterification between **297** and (*Z*)-non-2-en-1-ol **298** catalyzed by Ti(O*i*-Pr)$_4$, giving compound **299**. Finally, the ketone **299** was reduced and coupled with amino acid **301** to furnish lipid L101. L101 LNPs encapsulating anti-FVII siRNA

induced significant FVII gene silencing *in vivo* in mice in a dose-dependent manner with an ED_{50} of 0.02 mg/kg in terms of siRNA. They also found that L101 was resistant to mouse or human serum esterase but could be degraded by intracellular hepatic enzymes (e.g., lysosomal esterases). Compared to biodegradable L101, nondegradable L021 showed a 100-fold more accumulation in mouse liver.[569]

In 2017, Viricel et al. developed a series of ionizable switchable lipids that contained a tricyclic ionizable head group and two dodecyl hydrophobic tails.[570] Various ionizable amino groups can be attached to the 4′-position of the central pyridine ring to modify the head group (Figure 50a). The synthesis of CSL3 began with a substitution reaction between 2,6-dibromopyridin-4-amine **302** and 2-iodo-*N*,*N*-dimethylethan-1-amine **303** followed by methylation of the resulting amine, giving amine **304**. The tricyclic core **306** was formed via Suzuki coupling[571,572] between compound **304** and phenylboronic acid **305**. Finally, the hydrophobic tails were installed via Sonogashira coupling[573] followed by hydrogenation of the carbon−carbon tribonds (Figure 50b). As reported in their previous work, lipids with this type of backbone can change their conformation upon protonation of the central pyridine at endosomal pH values (pH 5) (Figure 50c), and lipids with alkyl tails of 12 carbons showed the highest siRNA delivery efficiency *in vivo*.[574] Once the central pyridine is protonated, intramolecular hydrogen bonding within the tricyclic core will lead to an orientation change of the two hydrophobic tails. Conformation change of the hydrocarbon tails destabilizes LNPs and prompts the release of the siRNA payload (Figure 50c).[570,574] Results showed that siRNA-loading CSL2 LNPs and siRNA-loading CSL3 LNPs showed dose-dependent gene knockdown activity *in vitro*, whereas siRNA-loading CSL1 LNPs was ineffective. The failure of CSL1 LNPs in gene knockdown may be attributed to the bulky head group that can inhibit intramolecular hydrogen bond formation. CSL4, lacking the two methoxy groups, remained in endosomes as it could not undergo a conformational change.[570]

2.2.2.2. Bioactive Molecules Derived Lipids. Lipids incorporating various bioactive molecules, such as oligopeptides/amino acids, sugar,[575] and small molecular drugs,[59,576,577] have been developed as formulation components for RNA delivery. Cholesterol, for example, is biologically compatible and generally used as a helper lipid to stabilize the LNPs.[578,579] Many cholesterol-based ionizable lipids have been synthesized and used in the applications of RNA delivery.[496,580,581]

In 1991, Gao et al. reported the synthesis of DC-Chol, a cholesterol-derived ionizable lipid in which the dimethylamine head group is attached to the cholesterol moiety via a biodegradable carbamate linker (Figure 51).[581] In contrast to cationic liposome formulated with fully charged cationic lipids, such as DOTMA and DOTAP, LNPs formulated with DC-Chol/DOPE are only partially charged at pH 7.4,[582] thus inhibiting the aggregation of LNPs.[583] In 2011, Zhang et al.
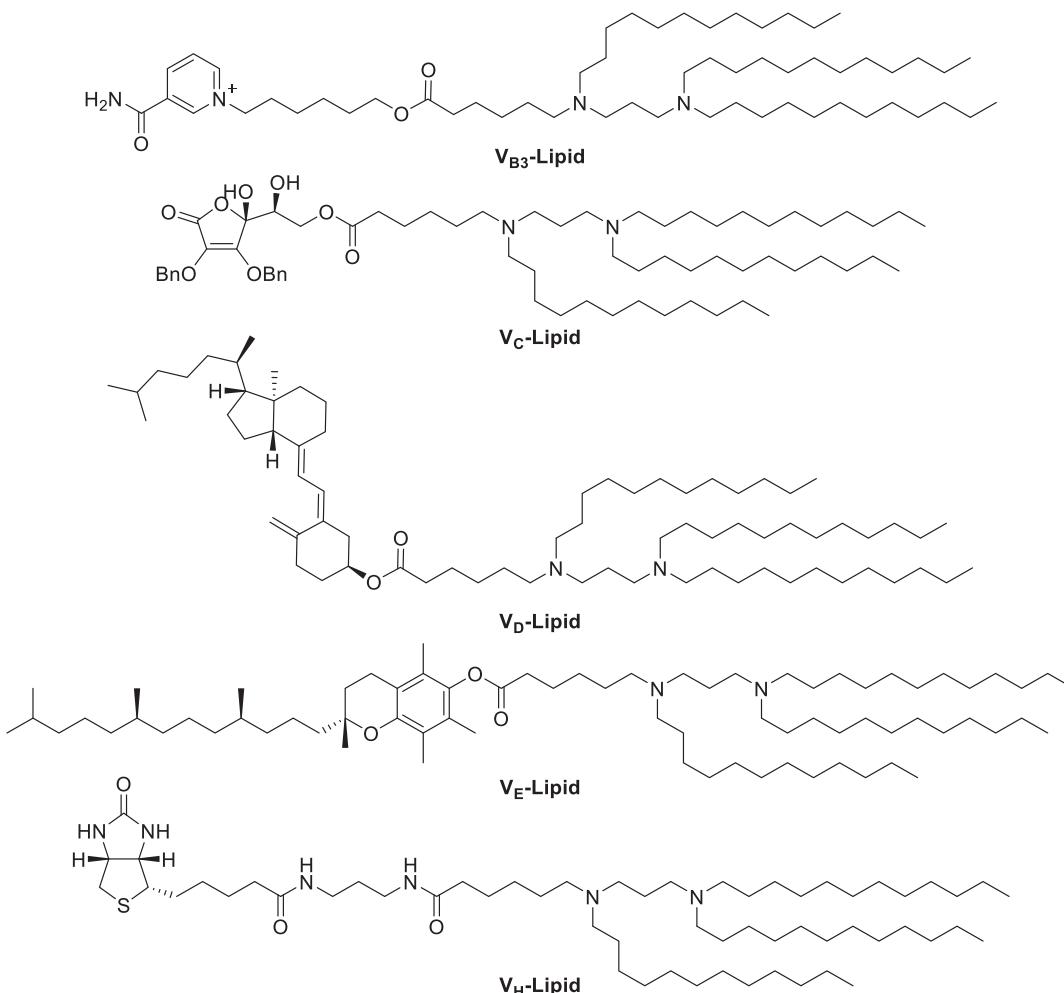
Figure 52. Chemical structures of vitamin-derived ionizable lipids.

found that at a DC-chol/siRNA weight ratio as high as five or ten, DC-Chol/DOPE LNPs showed the highest siRNA delivery efficiency into SK-BR3 cells *in vitro*.[584] In a mouse tumor model, PEGylated DC-Chol/DOPE LNPs containing kinesin spindle protein (KSP) siRNA led to a significant KSP gene silencing at tumor sites and a strong inhibition of tumor growth after systemic administration in mice at a siRNA dose of 1 mg/kg.[585] The DC-Chol/DOPE LNPs have also been used in encapsulating alpha-1-antitrypsin (AAT) mRNA, resulting in high transfection efficiencies and sustained AAT protein expression in A549 cells *in vitro*.[586] In 2016, Zhao et al. synthesized another cholesterol-based ionizable lipid, designated as DMAPA-chems, in which the head group and cholesterol moiety were connected through a 1,4-succinic acid linker with biodegradable ester and amide linkage bonds (Figure 51).[587] DMAPA-chems LNPs encapsulating Notch1 siRNA showed high Notch1 gene knockdown efficiency in SKOV3 cells at an N/P ratio of 100.[587]

In 2019, Hou et al. reported a library of vitamin-derived lipids: V$_{B3}$-Lipid, V$_C$-Lipid, V$_D$-Lipid, V$_E$-Lipid, and V$_H$-Lipid (Figure 52).[59] These vitamin-derived lipids were prepared through the conjugation of vitamins or their derivatives with an amino lipid with a carboxylic acid group through ester or amide bond formation. LNPs formulated with V$_C$-Lipid were optimal in delivering mRNA encoding an antimicrobial peptide IB367 along with a cathepsin B (CatB) via a cleavable linker into macrophages. The V$_C$-LNPs engineered macrophages provide a

promising therapeutic against multidrug-resistant bacteria-induced sepsis.[59]

Given the fact that phospholipids and glycolipids are natural components of the cell membrane, Zhang et al. synthesized a library of phospholipid and glycolipid-derived ionizable lipids (PLs and GLs) and used these materials to formulate biomimetic nanoparticles for mRNA delivery (Figure 53).[588] The synthesis of PL1 started with phosphorylation of 3-bromo-1-propanol **309**, giving phosphate triester **310**, while the synthesis of GL1 began with glycosylation of β-D-galactose-pentaacetate **314** with 3-bromo-1-propanol **309** promoted by a boron trifluoride diethyl etherate complex. The remaining chemical conversions include the substitution of bromide in compounds **310** and **315** with monoprotected 1,3-propanediamine **311**, removal of the Boc protecting group using TFA, and reductive amination reaction.

2.2.3. Lipidoids. Combinatorial chemistry approaches permit the rapid synthesis of large libraries of ionizable lipids, which are termed lipidoids or lipid-like compounds.[518,589−592] An important advantage of combinatorial chemistry approaches is that a vast number of lipids with large diversities can be synthesized by assembling variant building blocks. The combination of this method with high-throughput screening can lead to the quick discovery of ionizable lipids with good RNA delivery efficiency.[518,589−591] Many types of reactions can be applied to produce lipidoids, including aza-Michael addition
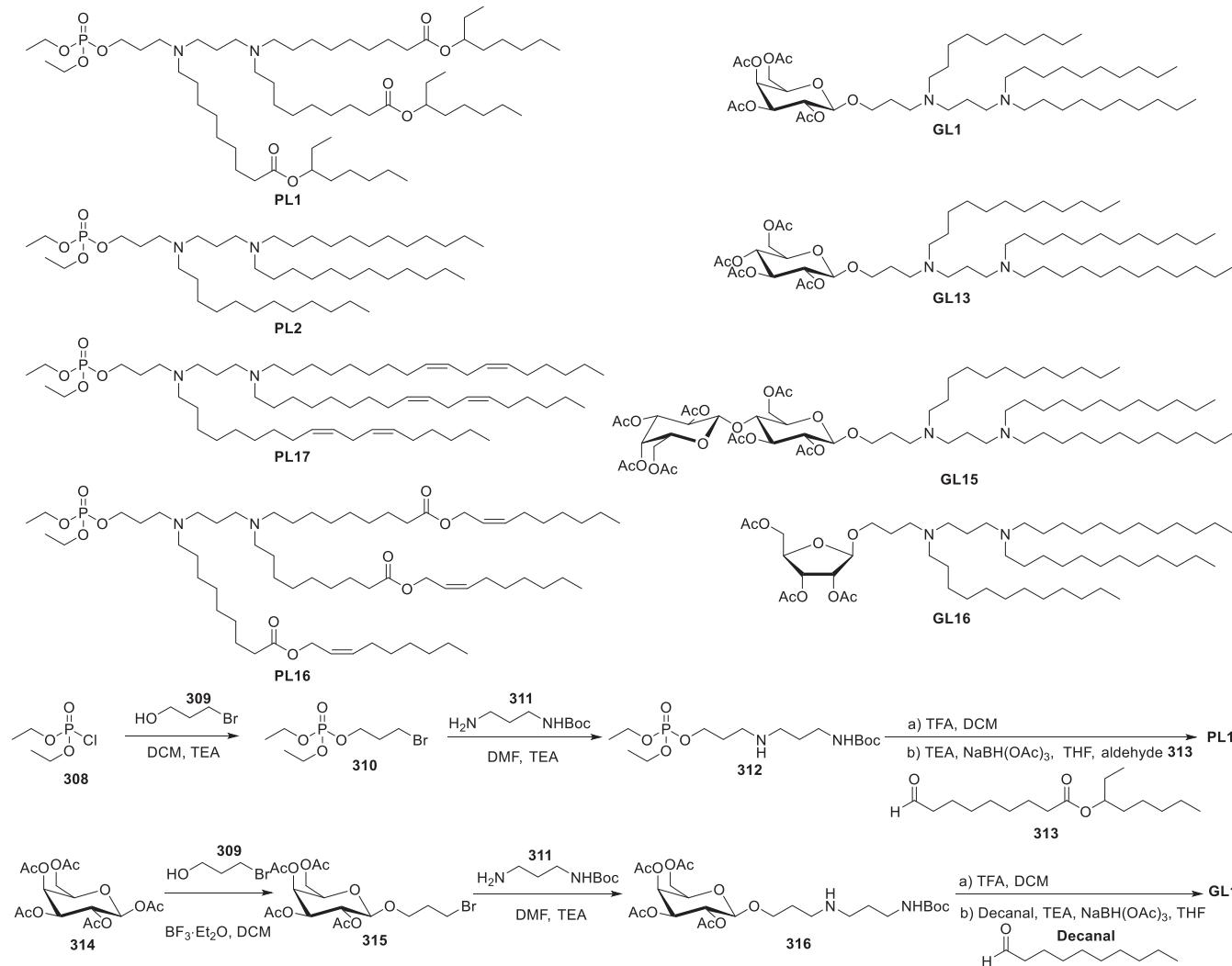
Figure 53. Chemical structures and synthesis of representative phospholipids and glycolipids.
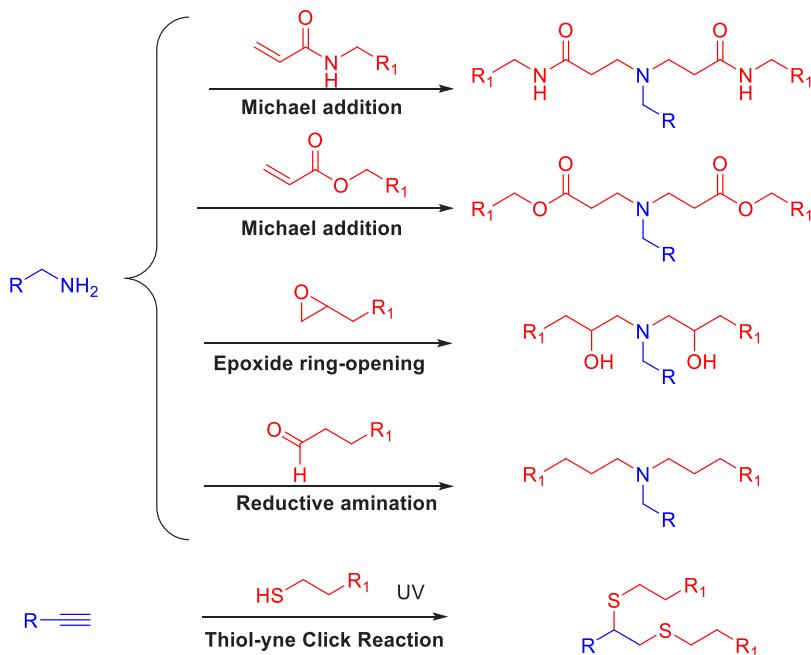
Figure 54. Representative reactions for the preparation of lipidoids.
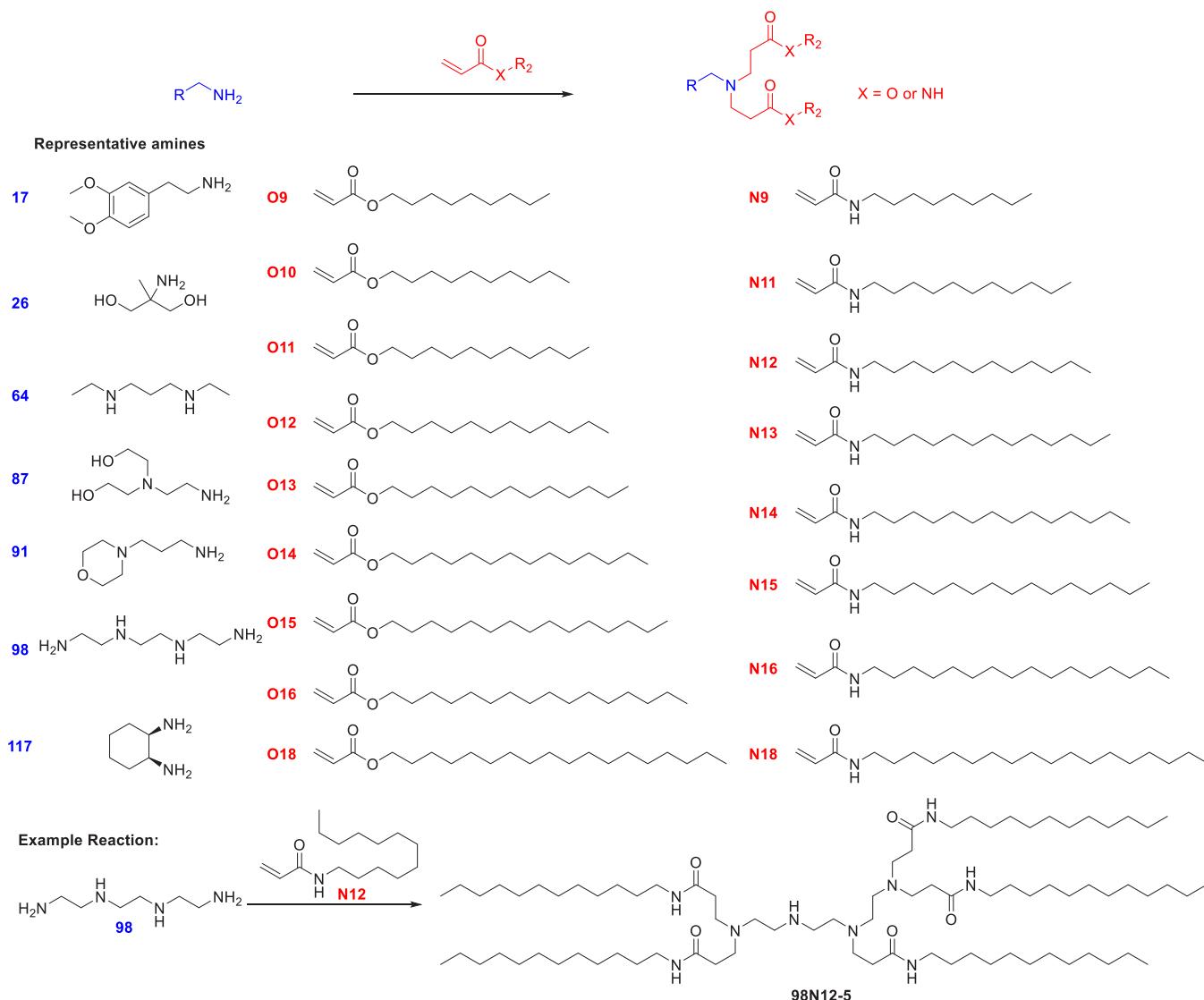
Figure 55. Synthesis of lipidoids via Michael addition of amines with acrylamides and acryl ester.

of amines with acrylamides or acrylesters,[589,593,594] ring-opening of epoxides with amines,[590,595,596] and reductive amination of aldehydes[597−599] (Figure 54). Thiol−yne click reaction[600,601] and multicomponent reactions[602−604] can also be used in the synthesis of lipidoids (Figure 54).

2.2.3.1. Lipidoids Synthesized via Michael Addition. In 2008, the Anderson and Langer group reported a library of lipidoids composed of 1200 lipids by conjugating 54 polyamines with 17 acrylamides and acryl ester with varying length of the alkyl group (between 9 to 18) (Figure 55).[589] 98N-12 with 5 tails attached (98N12-5) turned out to be a lead lipid when tested for gene silencing in HeLa cells. 98N12-5 LNPs encapsulating anti-FVII siRNA induced a 90% reduction of expression of the blood clotting Factor VII at a siRNA dose of 5 mg/kg in murine liver.[589] In 2009, Nguyen et al. found that 98N12-5 LNPs encapsulating siRNA targeting the influenza nucleoprotein gene could lead to effective antiviral activity *in vivo* following systemic administration in mice.[605] Pharmacodynamic studies of 98N12-5 LNPs showed they specifically targeted the liver following intravenous and intraperitoneal injection,[606] and tissue biodistribution data indicated that >90% injected dose was distributed in the liver.[607] PCSK9 siRNA-

loading LNPs formulated with 98N12-5/cholesterol/DMG-mPEG2000 at a molar ratio of 42:48:10 led to significant silencing of PCSK9 gene in mice following systemic administration.[608]

In 2014, Whitehead et al. evaluated the antifactor VII siRNA delivery efficacy of a library of 1400 lipids prepared from amines and acrylate tails and identified 304O13 as the lead candidate, which induced factor VII gene silencing with an ED_{50} of 0.01 mg/kg in mice (Figure 56).[609] Structure−activity relationship studies showed that lipids containing three or more O13 tails and at least one tertiary amine may exhibit strong siRNA delivery efficacy. Then a secondary library of lipids was prepared from 12 polyamines and O13, and 503O13 was shown to be the best lipid for siRNA delivery *in vivo*.[609] In 2016, the Whitehead group studied another library of lipidoids synthesized from the combination of 6 amines and 13 tails (Figure 56) and found that lipidoids synthesized from alkyl acrylate were shown to be more effective in siRNA delivery than lipidoids prepared from the methacrylate tail.[610] Isodecyl acrylate (Oi10)-conjugated lipidoids exhibited the highest siRNA delivery efficiency in the whole library. The chemical structure of the tails significantly affected the surface pK_a values of LNPs.[610] In 2018, Ball et al.
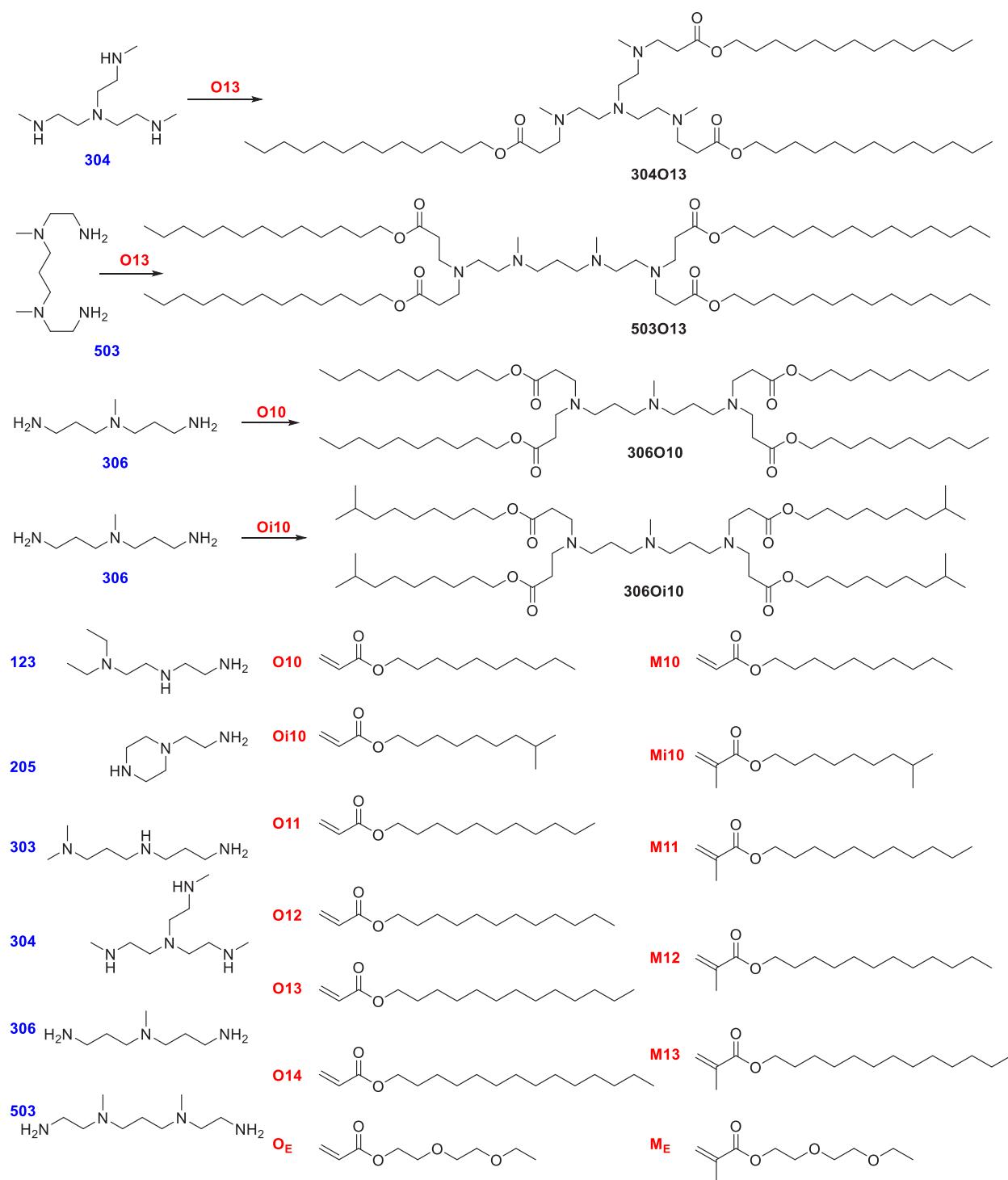
Figure 56. Lipidoids synthesized via Michael addition of alkyl-amines (blue) to alkyl-acrylate or methacrylate tails (red).

reported that 306Oi10-based LNPs can codeliver Factor VII siRNA and luciferase mRNA *in vivo*, which may be a potential method for treating diseases associated with both aberrant upregulation and downregulation of genes. Co-formulation of siRNA and mRNA in a single LNP substantially enhanced their delivery efficiency compared to LNPs encapsulating individual RNAs.[611] In 2019, Hajj et al. found that 306Oi10, with a one-carbon branch, increased luciferase mRNA delivery 10-fold compared to the linear tailed 306O10 (Figure 56).[612] The improvement may be due to the branch in the Oi10 tail that

increases the distance between lipidoid molecules within the LNP bilayer, thus facilitating protonation of the lipidoid molecules at the late stage of the endosome.[612] Additionally, 360Oi10 LNPs could deliver Cy5-labeled luciferase mRNA to hepatocytes, endothelial cells, and Kupffer cells in the liver following intravenous administration. Besides, 360Oi10 LNPs were capable of sufficiently encapsulating and codelivering functionally three different mRNAs (mCherry, firefly luciferase, and erythropoietin) in mice *in vivo*.[613]
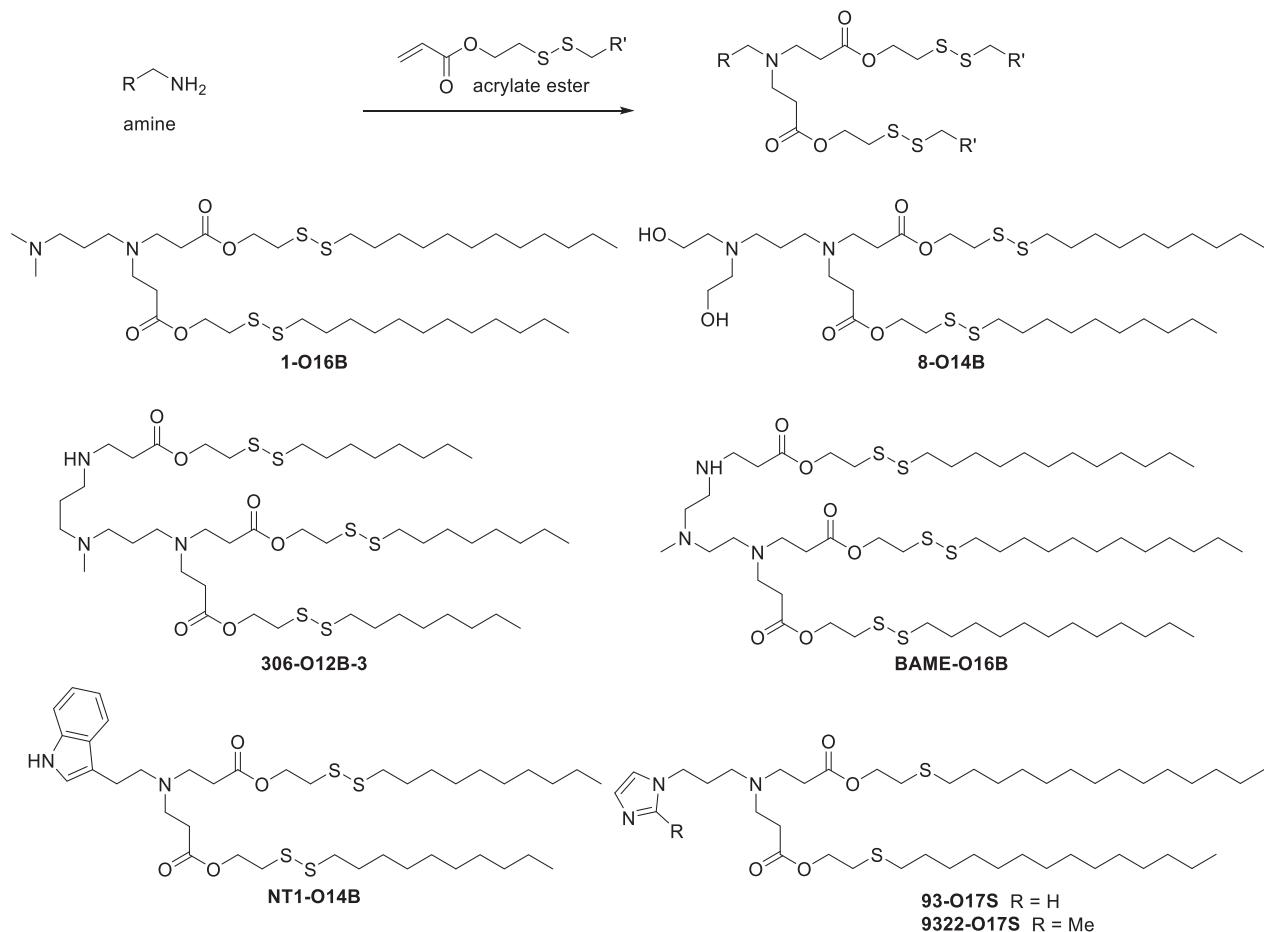
Figure 57. Chemical synthesis of bioreducible lipids.
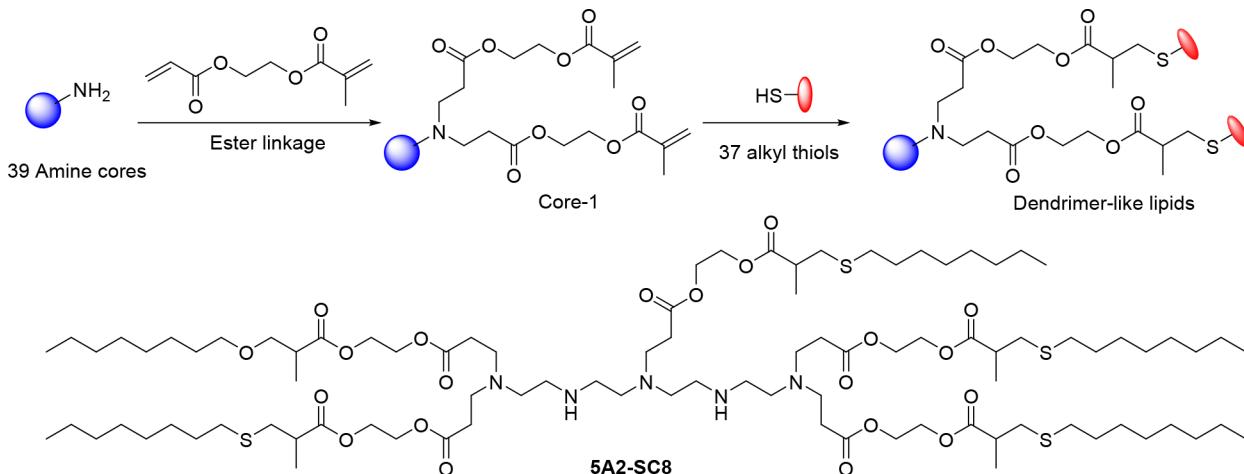
Figure 58. Modular strategy for the synthesis of dendrimer-like lipids and chemical structure of 5A2-SC8.

By utilizing the combinatorial strategy, the Xu group has developed many bioreducible ionizable lipids, of which the hydrophobic tails contain both disulfide bonds and ester bonds (Figure 57).[614−621] Disulfide bonds are cleavable under the intracellular GSH condition, and esters can be hydrolyzed by esterase, thus facilitating degradation of lipidoids and RNA release. In 2014, 1-O16B was found as a lead candidate, which can deliver GFP-targeting siRNA to downregulate GFP in MDA-MB-231 cells.[383] In 2016, by screening another library of bioreducible ionizable lipids, 8-O14B was shown to be capable

of delivering Cas9 and sgRNA into GFP-HEK cells, resulting in greater than 70% knockdown of GFP expression with efficiency. Structure−activity relationship study suggested that lipids containing hydrophobic tails with a tail length between 14 to 18 carbons were more efficient in Cre protein delivery than lipids with 12-carbon tails.[622] BAME-O16B LNPs were used in codelivery of Cas9 mRNA and sgRNA, leading to knockout of cellular GFP expression with efficiency up to 90% in human embryonic kidney cells and reduction of serum PCSK9 by 80% in C57BL/6 mice.[618] In 2019, LNPs formulated with 306-

a.

Example Reaction:

Representative amines   **Alkyl epoxides**

b.

G0-C14

Figure 59. Synthesis of lipids from ring-opening reaction between amines and epoxides.

O12B-3/cholesterol/DOPE/DSPE-PEG2000 at a ratio of 16:4:1:1 (w/w) were used to deliver ASOs targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) in mice, resulting in significant PCSK9 silencing with the ED_{50} as low as 0.034 mg/kg

Figure 60. Chemical structures of aminoglycosides, epoxides, and acrylic esters and a representative schematic reaction between aminoglycoside and epoxide.

following systemic administration.[623] In 2020, Ma et al. synthesized a class of neurotransmitter-derived lipidoids (NT-lipidoids)[615] for brain delivery of RNA, as neurotransmitters (e.g., tryptamine) can traverse the blood−brain barrier (BBB) effectively.[624,625] LNPs formulated with 306-O12B-3 and NT1-O14B at a ratio of 7:3 (w/w) could cross the BBB and deliver Tau-ASO into neuronal cells, leading to a reduction of tau mRNA by ∼50% and a reduction of tau protein by ∼30% at a Tau-ASO dose of 20 μg following five times intravenous administration.[615] In 2021, Zhao et al. reported two top lipidoid candidates, 93-O17S and 9322-O17S, for delivering mRNA into the primary T lymphocytes via a rough-to-detail screening approach.[614] Structure−activity analysis showed that the amine

head group, heteroatom substitution, spacer length, and linker type in the tail are all critical factors that influenced the RNA delivery efficiency. Both 93-O17S LNPs and 9322-O17S LNPs could deliver Fluc mRNA to the spleen efficiently in mice *in vivo*. Systemic delivery of Cre mRNA using 93-O17S LNPs could result in ∼8.2% and ∼6.5% of delivery efficacy into CD4+ and CD8+ T cells in mice, respectively.[614]

In 2016, Zhou et al. prepared a library of over 1500 degradable dendrimer-like lipids via Michael addition between various amines and acrylate esters followed by thiol−ene reaction with alkyl thiols.[626] 5A2-SC8, a lipid with five nitrogen atoms and five short alkyl chains, was identified as the top-performing lipid in the library (Figure 58). LNPs encapsulating let-7g miRNA
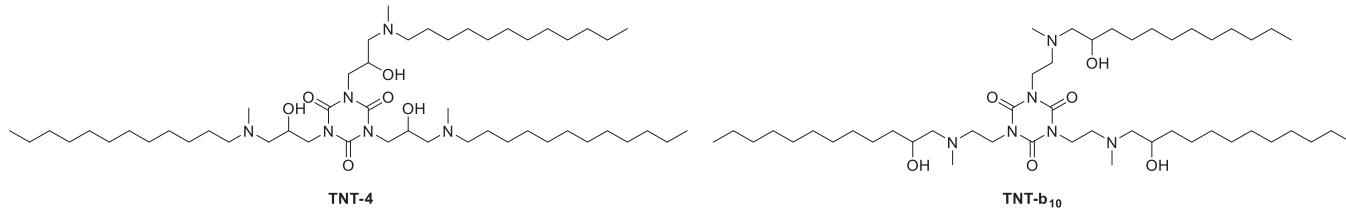
Figure 61. Chemical structures of TNT-4 and TNT-b$_{10}$.
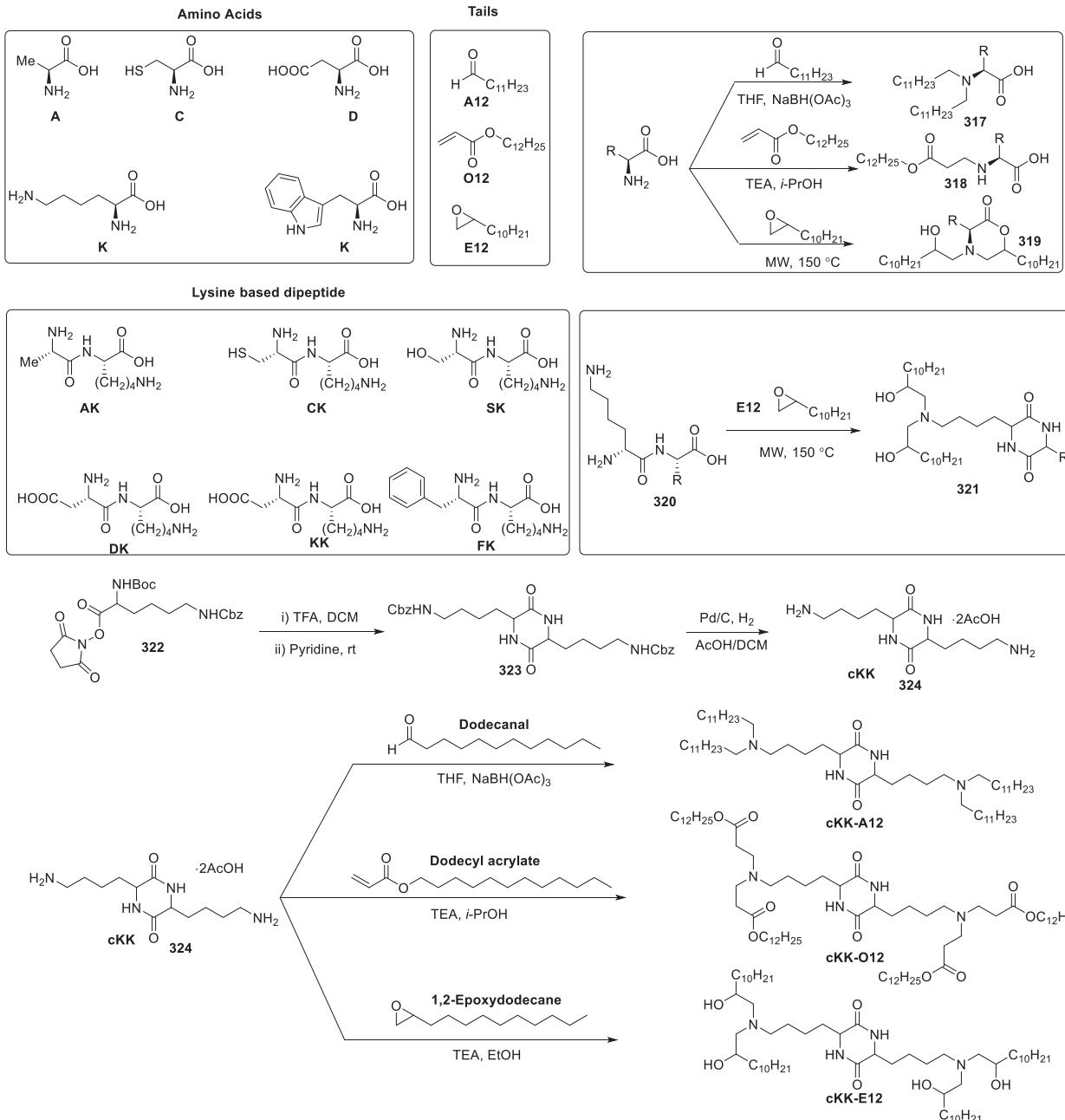
Figure 62. Synthesis of amino acid-derived lipidoids.

prepared from 5A2-SC8/DSPC/cholesterol/PEG-lipid induced strong let-7g gene silencing effects, inhibited tumor growth, and prolonged the survival time of mice with liver cancer at a miRNA dose of 1 mg/kg following weekly intravenous administration.[626] In 2018, Zhou et al. optimized the formulation of 5A2-SC8 LNPs by lowering the mole fraction of 5A2-SC8 to 24% and

using DOPE instead of DSPC; the optimized 5A2-SC8 LNPs showed strong luciferase mRNA delivery efficacy in mice.[627] In FAH−/−mice, 5A2-SC8 LNPs encapsulating fumarylacetoacetate hydrolase (FAH) mRNA induced equivalent levels of ALT, TBIL, and AST compared to wild type mice following intravenous injection.[627]
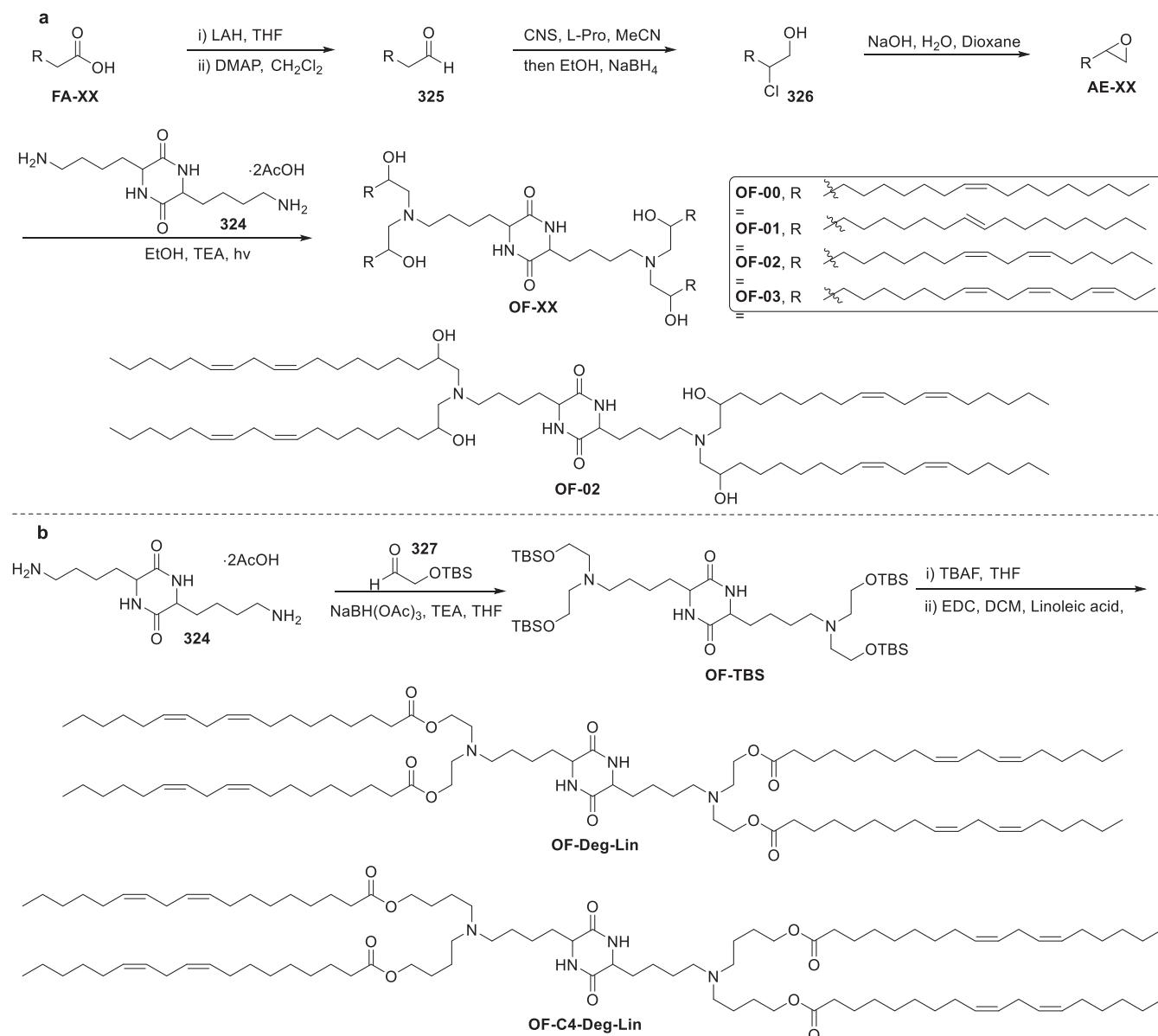
Figure 63. Chemical structures and synthesis of OF-XX and OF-Deg-Lin.

2.2.3.2. Lipidoids Synthesized via Epoxide Ring-Opening. In 2010, Love et al. synthesized a library of amino alcohol-derived lipids via a ring-opening reaction of epoxides with varying tail lengths with amines (Figure 59a).[590] C12-200 LNPs encapsulating FVII siRNA induced effective silencing of factor VII in mouse hepatocytes at low doses after intravenous administration.[590] In 2013, Sahay et al. confirmed that siRNA delivery by C12-200 LNPs was mediated by Cdc42-dependent micropinocytosis.[628] In a later study, a formulation of C12-200/DOPE/cholesterol/C14-PEG2000 at a molar ratio of 35:16:46.5:2.5 was used to prepare the optimized mRNA-loading C12-200 LNPs, which induced 7-fold more EPO protein expression than the initial formulation (C12-200/DSPC/cholesterol/C14-PEG2000).[629] C12-200 LNPs prepared in this formulation have been used in the study for mRNA-mediated human α-galactosidase protein replacement therapy in mice and nonhuman primates.[630] In a later study, direct intramyocardial injection of C14-113 LNPs encapsulating GFP mRNA in mice induced rapid and transient GFP

expression in a dose-dependent manner with limited off-target biodistribution.[631] In 2013, Xu et al. synthesized G0-C14[632] via epoxide ring-opening of alkyl epoxide by generation 0 of poly(amidoamine) (PAMAM) (Figure 59b); this ionizable lipid has been used in the delivery of both siRNA[632] and mRNA[633,634] for the treatment of cancer in mice.

Aminoglycosides are a class of antibiotics that contain three to five amino-substituted sugars.[635,636] The general mechanism of their antibacterial bioactivities is to selectively bind to bacterial 30S rRNA (rRNA).[637,638] Besides, aminoglycosides are capable of penetrating the cell membrane of bacteria by disrupting the lipopolysaccharide components[639] which might facilitate endosomal escape. In 2013, Zhang et al. synthesized a family of lipid-modified aminoglycosides for *in vitro* and *in vivo* siRNA delivery.[640] Eight aminoglycosides were reacted with terminal epoxides bearing tail lengths of 10−16 carbons (Figure 60). Their results indicated that C11 and C12 tails were the preferred chain length for siRNA delivery, whereas lipids with tails longer than C13 were generally inefficient in siRNA delivery, which
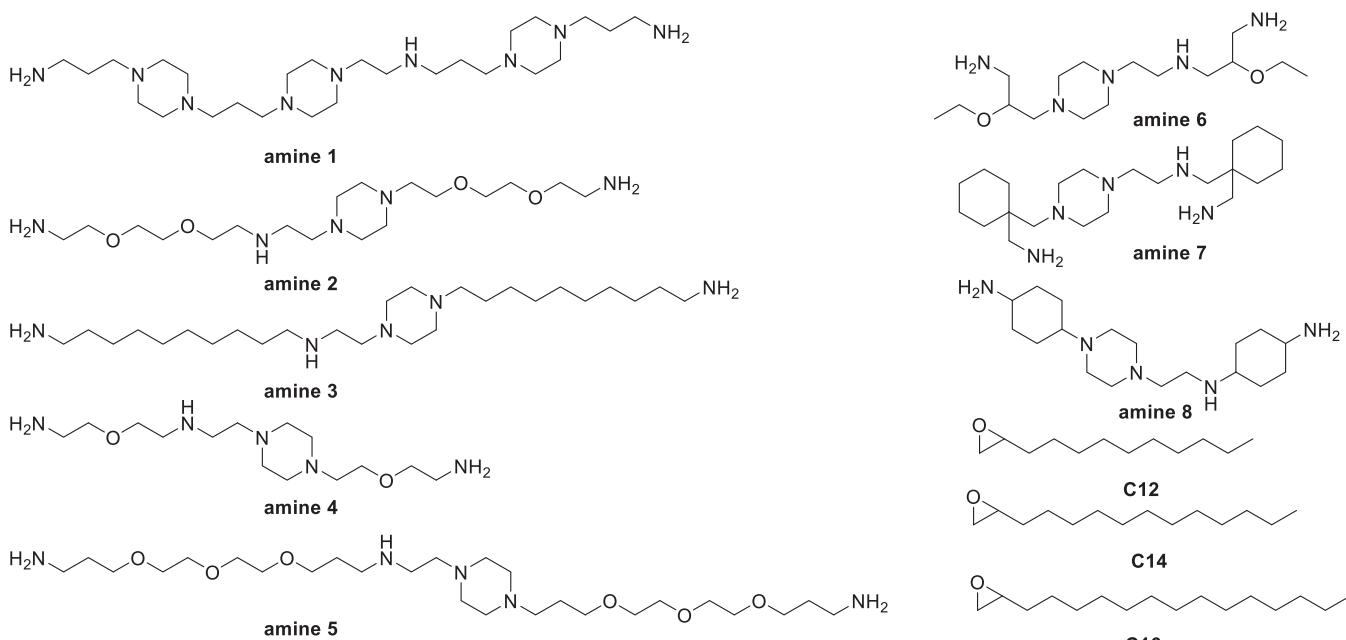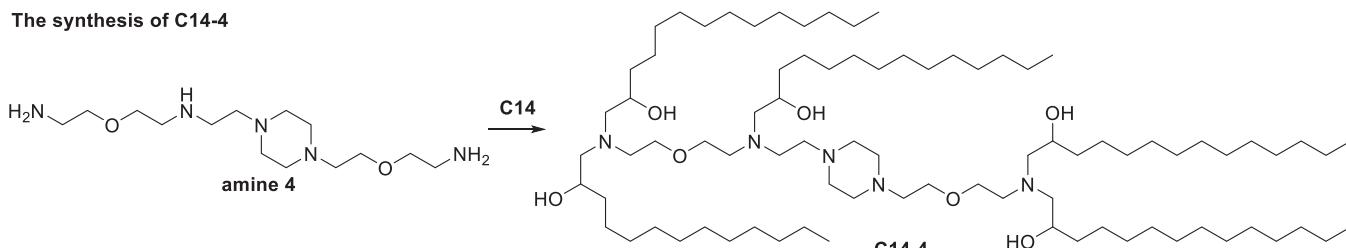
Figure 64. Synthesis of lipidoids via epoxide ring-opening of alkyl epoxides with polyamines.

may be caused by their decreased solubility and lower fluidity. HG and GT are favored core scaffold aminoglycosides for the synthesis of lipids with high siRNA delivery efficiency, while AP-, PR-, NO-, and RB-derived lipids generally showed low siRNA delivery efficiency. HG-C11 LNPs encapsulating anti-FVII siRNA were able to induce effective Factor VII knockdown in mice with an ED_{50} of 0.04 mg/kg.[640] In 2020, Yu et al. synthesized another library of aminoglycoside-derived lipids and evaluated their potency as mRNA formulation components.[641] Four aminoglycosides, namely hygromycin, amikacin, gentamycin, and Geneticin, were reacted with seven epoxides bearing hydrocarbon tails and with five acrylic esters through epoxide ring-opening and Michael addition, respectively. GT-C10, obtained from the reaction of gentamycin and epoxide C10, was able to deliver luciferase mRNA at a dose of 0.05 mg/kg to produce a 10^7 average luminescence intensity in mouse liver following an intravenous administration.[641]

In 2014, Dong et al. developed a series of 1,3,5-triazinane-2,4,6-trione (TNT)-derived lipids that contained a six-membered core and three hydrophobic tails (Figure 61). TNT-4 was identified as a lead lipid, which showed efficient delivery of pDNA and siRNA both *in vitro* and *in vivo*.[642] In 2016, Li et al. synthesized four analogs of TNT-4 by exchanging the positions of the tertiary amino group and secondary hydroxyl group relative to the TNT ring (Figure 61) and evaluated the effects of spacing around the trazinane trione (TNT) ring on mRNA delivery.[643] The TNT-b10 luciferase mRNA LNPs induced 2-fold higher luciferase level than that of TNT-4 *in vitro*.

Intravenous and intraperitoneal administration of TNT-b10 LNPs encapsulating luciferase mRNA induced the luciferase expression in the mouse spleen.[643]

In 2014, Dong et al. developed a library of biomimetic ionizable lipids derived from amino acids, peptides, and polypeptides.[518] These biomimetic lipids were synthesized via an addition reaction of amines to alkyl epoxide and acrylate esters as well as a reductive amination reaction between amines and alkyl aldehyde (Figure 62). cKK-E12 was identified as the lead lipid, which reduced FVII protein by 50% at a siRNA dose as low as 0.002 mg/kg in mice. Intravenous injection of cKK-E12 siRNA LNPs into nonhuman primates (NHP) at a siRNA dose of 0.3 mg/kg led to a reduction of TTR serum level by 95%. Structure−activity relationship analysis showed that lipids that contained a dilysine-derived diketopiperazine core and lipid tails between 12 and 14 carbon tail length were the most effective. cKK-E12, composed of a diketopiperazine core derived from lysine and four amino alcohol-based hydrophobic tails, can be synthesized via dimerization of Cbz-protected lysine, followed by removal of Cbz and epoxide ring-opening of the epoxide compound. cKK-E12 LNPs had also been used in codelivery of Cas9 mRNA and sgRNA to hepatocytes at clinical doses in mice, inducing >80% editing of PCSK9 gene in the liver.[644] In 2019, Lokugamage et al. used cKK-E12 LNPs encapsulating mRNA to study the effects of toll-like receptor 4 (TLR4) on LNPs-mediated mRNA delivery.[645]

In 2016, Fenton et al. synthesized four alkenyl α-amino alcohols (AAA)[646,647] type analogs of cKK-E12 by conjugating
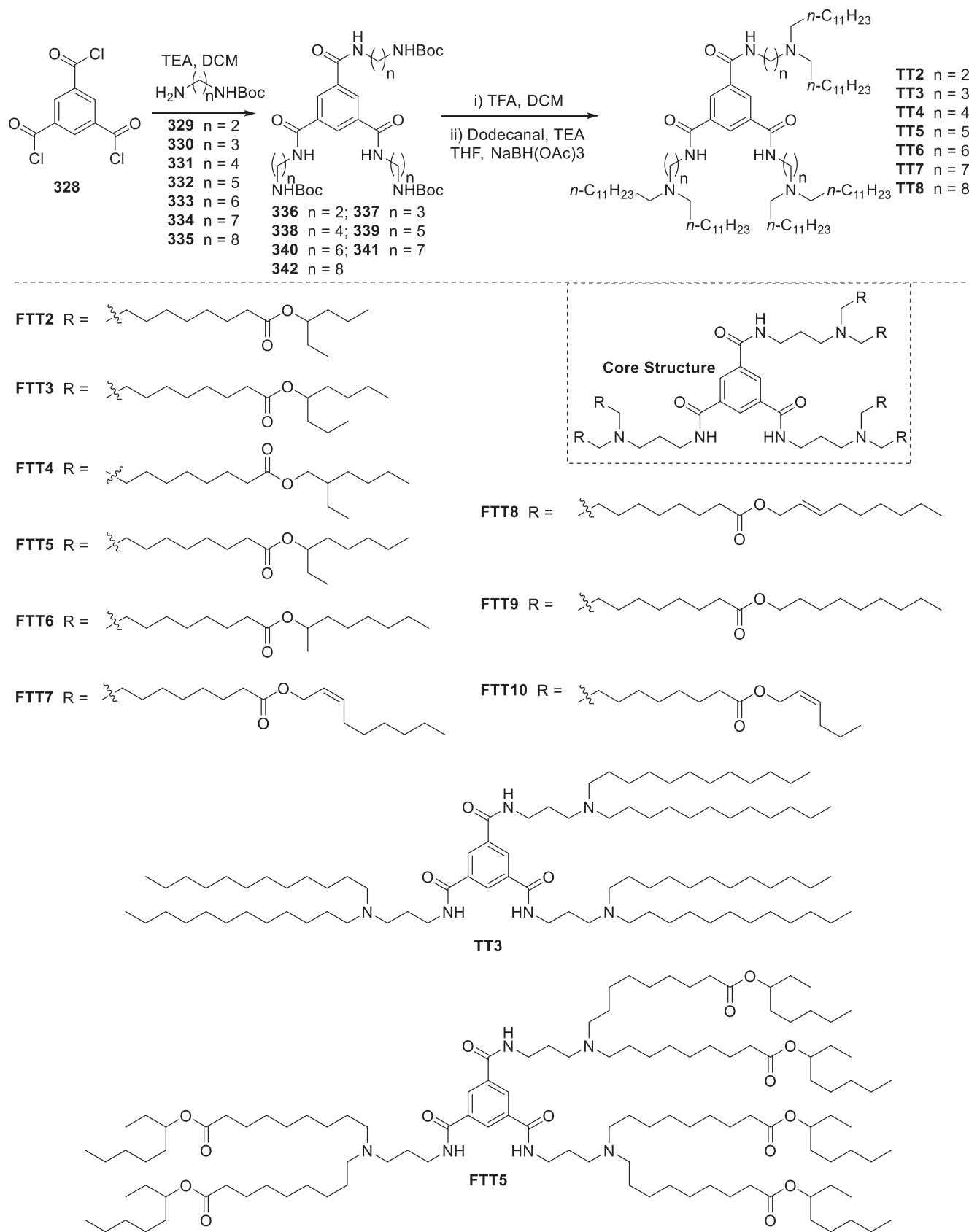
Figure 65. Synthesis of TT and FTT series of ionizable lipids.

the dilysine-derived diketopiperazine core of cKK-E12 with alkenyl epoxides derived from biologically relevant fatty acids

(Figure 63a).[648] Alkenyl epoxide precursors derived from the corresponding fatty acids were synthesized in four steps. First, a

a.

2 alkynyl carboxylic acids

343

6 alkyl thiols

C11

Thiol-yne click chemistry
UV 365 nm, 5% DMPA, THF, 1 h

344

A1 7 amines

DIC, HOBt, DCM, 16 h.

A1C11

b.

32 amines

345

1 propargyl acrylate

346

45 °C

347

1-Decanethiol

UV, DMPA
Thiol-yne click chemistry

348
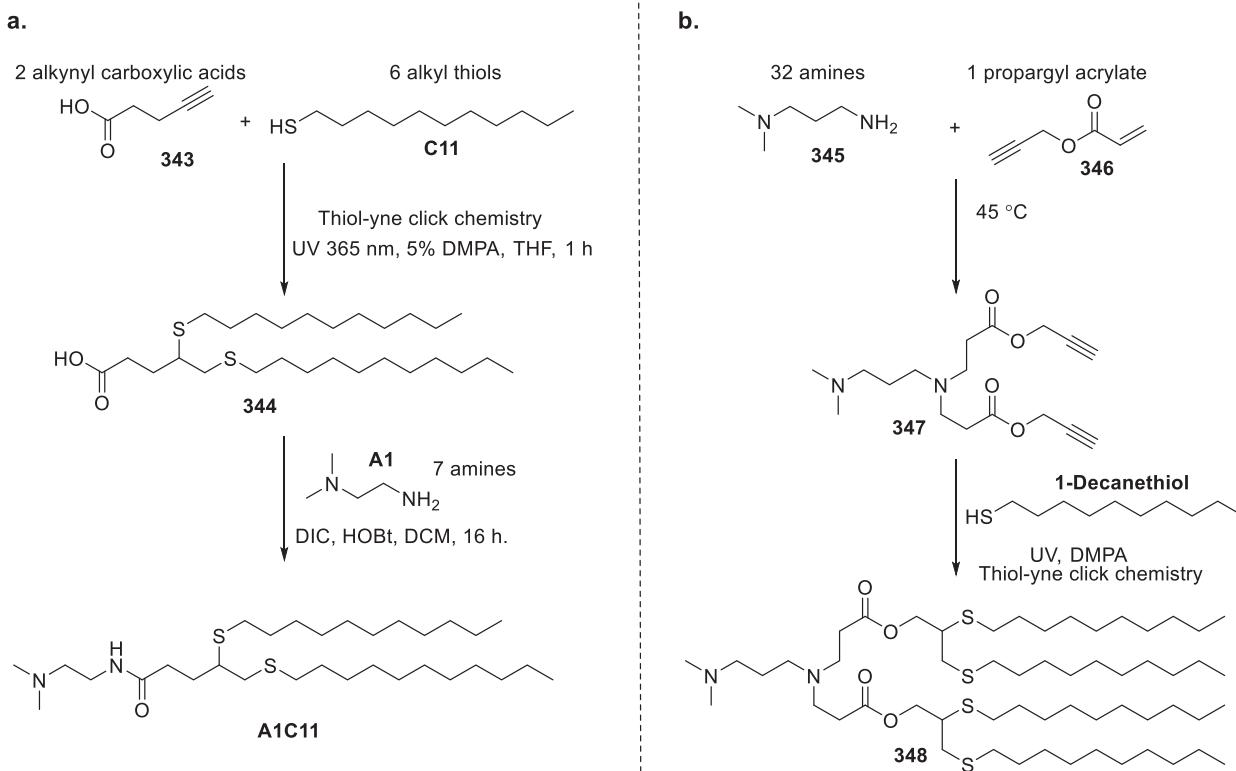
Figure 66. Synthetic routes to lipids via thiol—yne click chemistry.

lithium aluminum hydride reduction of fatty acids followed by oxidation gave aldehydes **325**. Then a one-pot α-chlorination/sodium borohydride reduction of aldehyde **325** provided the 1,2-chloroalcohols **326**.[649] The following basic dioxane promoted ring closure of these 1,2-chloroalcohols furnished the desired alkenyl epoxides AE-00 through AE-03 in 4 steps with only one silica gel chromatographic purification. Lastly, epoxide ring-opening of these alkenyl epoxides with the dilysine-derived diketopiperazine core **324** gave the desired alkenyl α-amino alcohol lipids OF-00 through OF-03. EPO mRNA-loading OF-02 LNPs outperformed EPO mRNA-loading cKK-E12 LNPs, with a 2-fold increase in EPO concentration to 14200 ± 1500 ng/mL when administrated intravenously at a dose of 0.75 mg/kg in C57BL/6 mice.[648] In another report, Fenton et al. developed an OF-Deg-Lin LNPs mRNA delivery system that was able to induce functional protein expression in mouse B lymphocytes.[650] OF-Deg-Lin is a biodegradable variant of OF-02 lipid with four degradable ester linkers attached to the diketopiperazine core and four doubly unsaturated tails; it can generate nontoxic linoleic acid upon hydrolytic cleavage (Figure 63b). The synthesis of OF-Deg-Lin started with the conjugation of the TBS-protected aldehyde **327** and the diketopiperazine core via reductive amination; then removal of the TBS protective group promoted by TBAF followed by esterification of the hydroxyl groups with linoleic acids gave OF-Deg-Lin. Much like nondegradable OF-02 Cy5 mRNA LNPs, intravenous administration of OF-Deg-Lin Cy5 mRNA LNPs in mice resulted in predominant accumulation of Cy5 mRNA in the liver. Systemic injection of nondegradable OF-02 LNPs encapsulating FLuc mRNA in mice resulted in protein expression mainly in the liver.[442,651] In 2018, Fenton et al. further modified OF-Deg-Lin by prolonging the carbon linker length from a two carbon spacer to a four carbon spacer (Figure

63b); the resulting lipid OF-C4-Deg-Lin was shown to be more potent than OF-Deg-Lin for both anti-FLuc siRNA and FLuc mRNA delivery in HeLa cells *in vitro*. OF-C4-Deg-Lin LNPs encapsulating firefly luciferase mRNA induced the majority of FLuc protein expression in the mouse spleen following an intravenous administration.[652]

In 2020, Billingsley et al. synthesized a library of 24 ionizable lipids for delivering mRNA to human T cells *ex vivo*.[653] The 24 ionizable lipids were synthesized via epoxide ring-opening of three alkyl epoxides with eight polyamines (Figure 64), and their luciferase mRNA delivery efficiency *in vitro* was evaluated. Results showed that C14-4 LNPs were the top performers, which could deliver CAR mRNA efficiently into primary human T cells *ex vivo*, generating CAR T cells with enhanced antitumor ability.[653]

2.2.3.3. Lipidoids Synthesized via Reductive Amination. In 2016, Li et al. reported a library of ionizable lipids, designated as TTs, which consist of a phenyl ring, three amide linkers, and three hydrophobic hydrocarbon tails (Figure 65).[598] TT2-TT8 lipids vary in the spacer length of the amide linker. *In vitro* screening identified TT3 as the lead lipid. PEGylated TT3 LNPs can efficiently deliver human factor IX (hFIX) mRNA in FIX-knockout mice *in vivo* after intravenous injection, resulting in the restoration of the level of hFIX.[598] In 2020, TT3 LNPs were formulated to encapsulate engineered mRNA encoding various SARS-CoV-2 antigens.[52] Vaccination of mice with this TT3 LNPs-mRNA formulation resulted in over 300-fold more specific antibody against anti-S1 as compared to that of MC3 LNPs-mRNA formulation. Additionally, antigen-specific antibodies induced by intramuscular administration of this formulation were 5-fold more than that of subcutaneous injection.[52] In 2020, Zhang et al. further developed a library of ionizable lipids designated as FTT by combining the core of
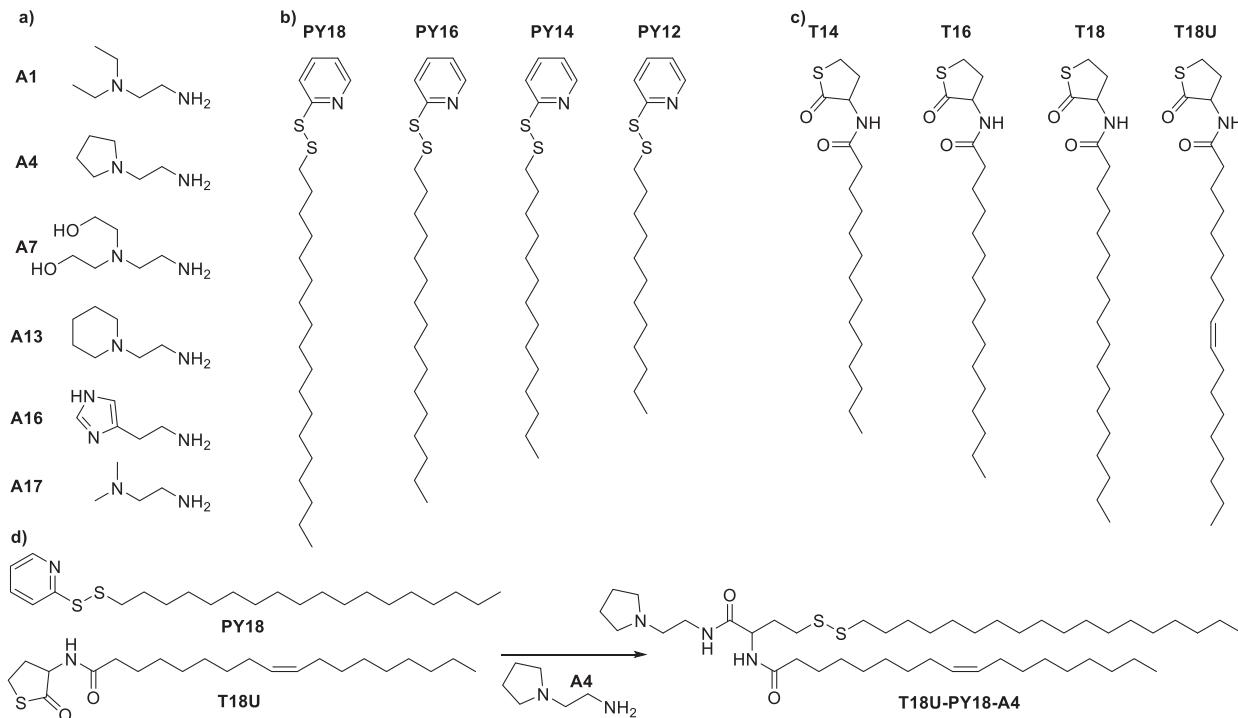
Figure 67. Synthesis of lipidoids via thiolactones ring-opening reaction followed by the thiol−disulfide exchange reaction. (a) Structures of amines. (b) Structures of pyridyl disulfide derivatives. (c) Structures of thiolactone derivatives. (d) Representative reaction scheme.

TT3 with various biodegradable tails (Figure 65).[597] Results showed that LNPs formulated with FTT lipids with branched ester tails (e.g., FTT6) mainly delivered mRNAs into the liver and spleen, and their mRNA delivery efficiencies were higher than that of FTT lipids with linear ester tails (e.g., FTT10). Among all the FTT compounds, FTT5 was the lead lipid to deliver mRNA to the liver. Images of FTT5 LNPs captured by cryo-TEM revealed the spherical morphology of FTT5 LNPs. Intravenous injection of FTT5 LNPs encapsulating FVIII mRNA in both wild type mice and hemophilia A mice resulted in potent expression of hFVIII protein. Moreover, FTT5 LNPs were able to induce dramatic base editing of PCSK9 at a dose of 0.125 mg/kg in mice.[597]

2.2.3.4. Lipidoids Synthesized via Click Chemistry. In 2012, Li et al. employed thiol−yne click chemistry[654,655] to synthesize a library of more than 100 ionizable lipids (Figure 66a).[601] These lipids were synthesized using eight alkyl thiols with varying length of the alkyl group (C6−C16), two alkynyl carboxylic acids, and seven amines. The synthesis was realized in two consecutive modular steps. First, a carboxylic acid with two hydrophobic tails was obtained via a thiol−yne click reaction between an alkyl thiol and an alkynyl carboxylic acid. Then the ionizable head group was installed through amide coupling, giving an ionizable thioether lipid (Figure 66a). Ionizable lipids with undecyl and dodecyl as hydrophobic tails showed stronger *in vitro* pDNA delivery efficiency compared to the other lipids, and A1C11 surpassed Lipo2000 in delivering siRNA into HEK293T cells *in vitro*.[601] In 2013, Alabi et al. produced a library of 32 ionizable lipids using a similar method.[600] In this study, lipids were synthesized via Michael addition between amines and propargyl acrylate followed by the thiol−yne reaction with alkyl thiols in the presence of UV and a photocatalyst (Figure 66b).[600] These lipids were then utilized to evaluate a multiparametric approach to screen LNPs. Results

showed that the pK_a of the whole LNPs, rather than the pK_a of individual lipids, was a key determinant of LNPs function *in vivo*. LNPs with above 50% silencing had pK_a values ranging from 6 to 7, while LNPs with pK_a values below 5.8 exhibited no gene knockdown activities both *in vitro* and *in vivo*.[600]

2.2.3.5. Lipidoids Synthesized via Multicomponent Reactions. Multicomponent reactions represent an efficient way to synthesize compounds with diversities.[656] In 2018, Molla et al. reported the synthesis of a library of 288 ionizable lipids via a three-component thiolactone ring-opening reaction followed by a thiol−disulfide exchange reaction.[604] Structurally, these lipids are composed of a head group with one or more ionizable tertiary amines, an amide linker, and two asymmetric alkyl tails, of which one contains a bioreducible disulfide bond (Figure 67). *In vitro* screening of these lipids identified some potent lipids for pDNA delivery in HEK-293T cells.[604] In 2020, Molla et al. further evaluated the *in vitro* anti-GFP siRNA delivery efficiency of these lipids in HeLa-GFP cells.[602] T16-PY12-A17, T18U-PY12-A17, and T18U-PY18-A4 (Figure 67) were shown to be effective in *in vitro* siRNA delivery, resulting in a reduction of GFP expression by 65%. Kdrl:EGFP Zebrafish embryos injected with T18U-PY18-A4 LNPs encapsulating anti-GFP siRNA showed a significant reduction in EGFP expression in blood vessels.[602]

In 2019, Miao et al. developed a library of 1080 ionizable lipids[603] via isocyanide-mediated three-component reaction (3-CR)[657] that simultaneously coupled primary or secondary amines,[658] ketones with different alkyl tail lengths and different degrees of saturation,[648,659] and isocyanides or isocyanide derivatives (Figure 68).[660] Results showed that lipids with longer, unsaturated tails showed higher mRNA delivery efficiency, and A2-Iso5-2DC18 (A2) and A12-Iso5−2DC18 (A12) were the top-performing lipids for mRNA delivery in bone marrow-derived dendritic cells (BMDCs), bone marrow-
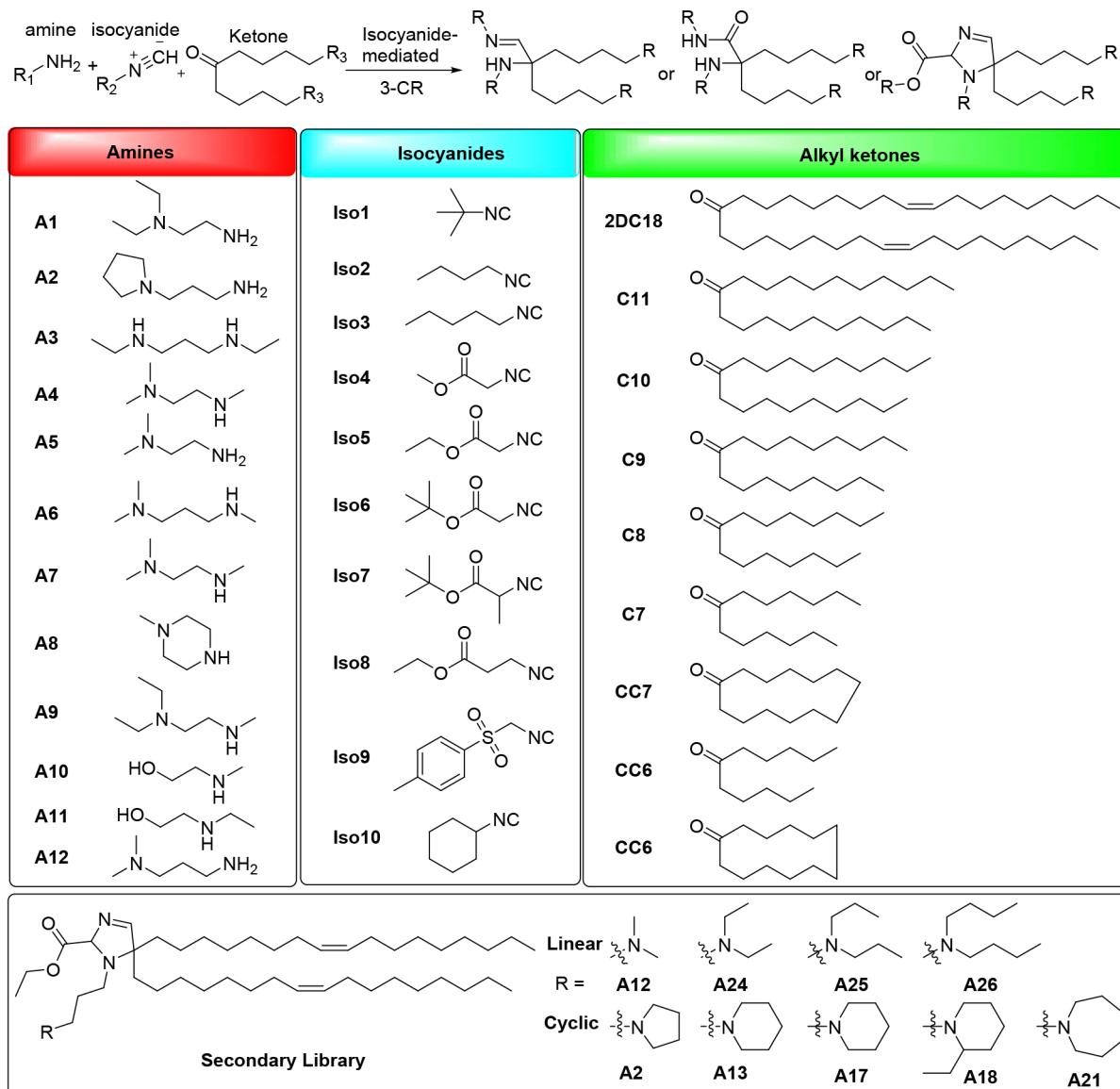
Figure 68. Isocyanide-mediated three-component reactions for the synthesis of lipidoids.
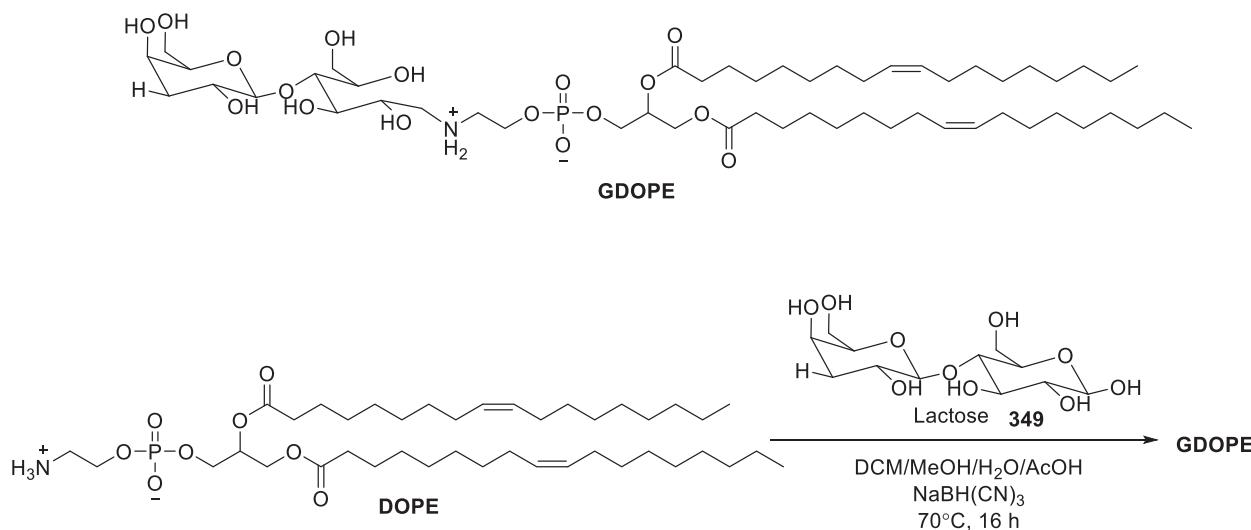
Figure 69. Chemical structure of zwitterionic lipid GDOPE.
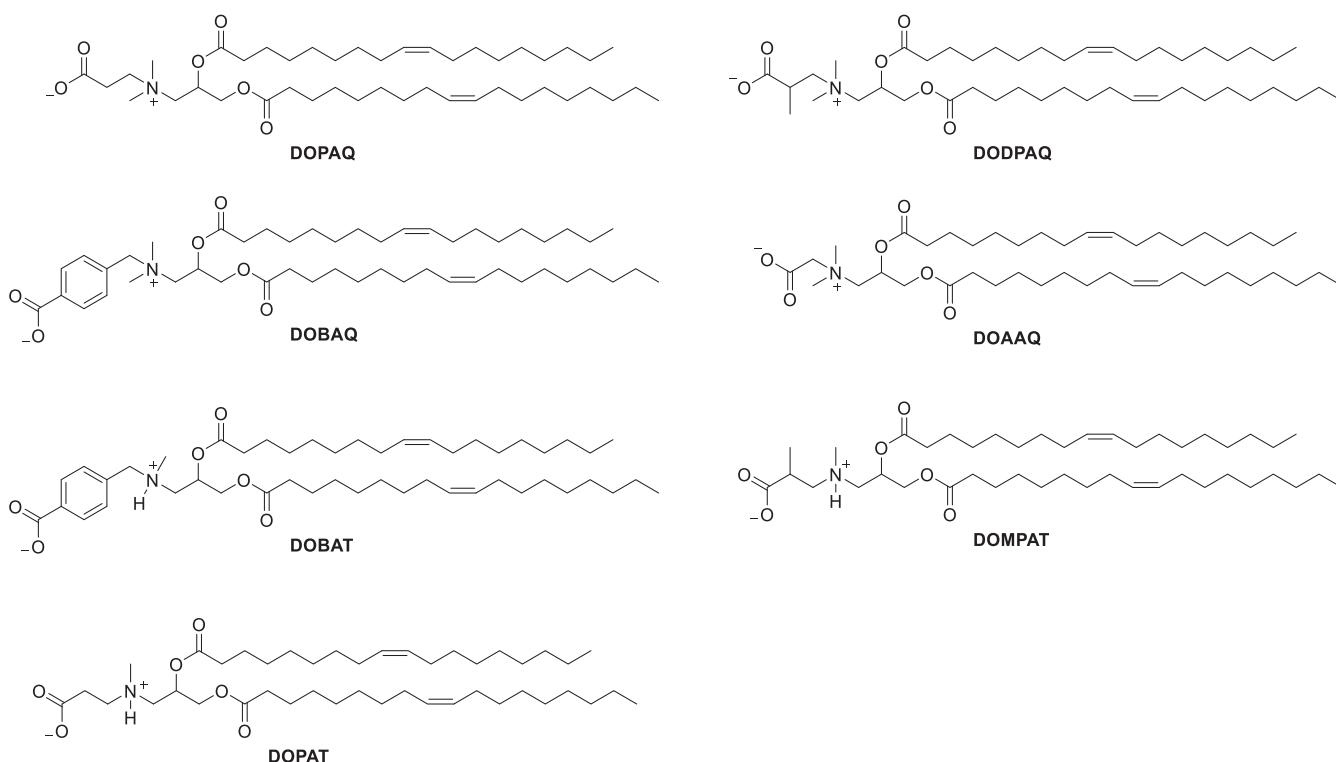
Figure 70. Chemical structures of zwitterionic lipids.
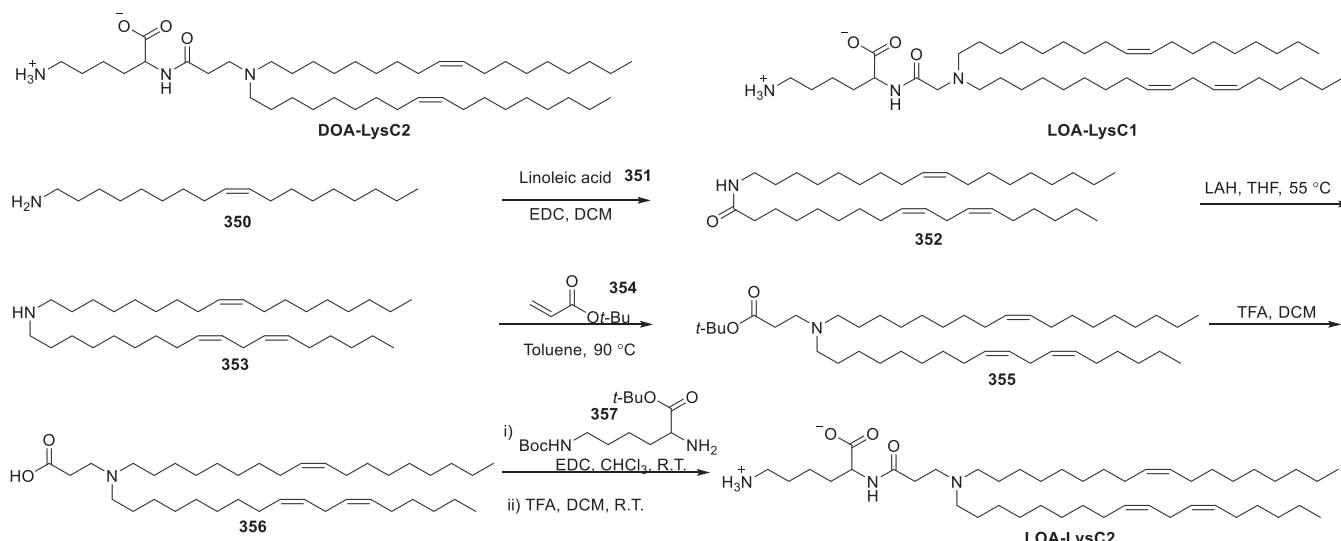
Figure 71. Chemical structures of lysine-derived zwitterionic lipids.

derived macrophages (BMDMs), and Hela cells. Both A2 and A12 contain two amines that are spaced three carbons apart and an ester group. A2 LNPs and A12 LNPs encapsulating Cre mRNA led to comparable levels of protein expression and could deliver mRNA into central APCs including CD11b⁺ and CD11c⁺ in the Ai14D reporter mouse model. A2 LNPs encapsulating mRNA encoding OVA induced much higher adaptive immune response and antitumor efficacy in the Ovalbumin (OVA)-expressing B16F10 mouse compared to A12 LNPs encapsulating mRNA encoding OVA. Rechallenge experiments indicated that only the A2 LNPs were able to induce strong antitumor immunity. Structurally, the main difference between A2 and A12 lies in their head groups: A2

has a heterocyclic amine head group whereas A12 contains a linear amine head group. They further developed a secondary library of lipids with different head groups to investigate the relationships between the head group (cyclic versus linear) and their immunogenicity (Figure 68). Lipids containing hetero-cyclic amine head groups induced higher expression of IFN-γ as compared to lipids with linear amine head groups after mRNA encoding OVA vaccination. In particular, A18 LNPs showed much higher IFN-γ-positive secretion than the other lipids, and lipid A18 also had intrinsic stimulatory effects. A18 was finally identified as the lead cyclic lipid candidate, which facilitated the mRNA delivery and induces a strong immune response partially
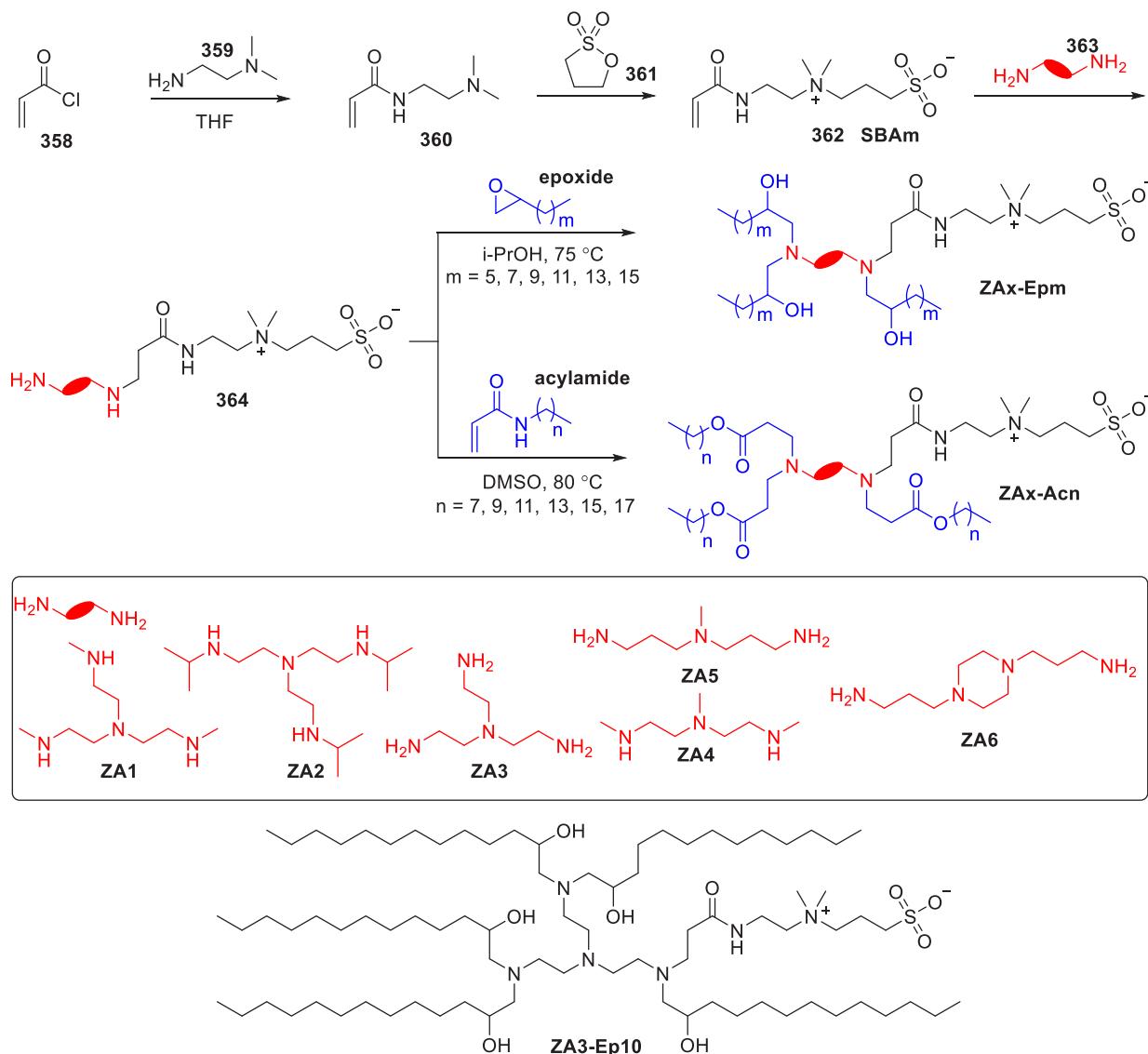
Figure 72. Chemical structures and the synthesis of zwitterionic lipid ZA3-Ep10.

mediated by the stimulator of the interferon gene (STING) pathway.

2.3. Zwitterionic Lipids

Zwitterionic lipids are lipids that contain covalently bonded cationic groups and anionic groups. In 2011, Sonoke et al. synthesized a galactose-modified zwitterionic lipid GDOPE by conjugating lactose with DOPE via reductive amination reactions (Figure 69).[661] Compared to nongalactosylated LNPs, GDOPE-based LNPs showed enhanced delivery of siRNA to the liver in mice.

In 2012, the Szoka group synthesized a class of zwitterionic lipids with head groups containing a tertiary amine or quaternary ammonium head group and carboxylate linked by various carbon spacers (Figure 70).[662] LNPs containing these zwitterionic lipids showed efficient siRNA encapsulation when ionized.[662] DOBAQ LNPs was used for the delivery of Cas9 mRNA to the back of the eye in mice.[663]

In 2013, Walsh et al. developed a series of lysine-based zwitterionic lipids termed ILL, which contained a zwitterionic lysine head group linked to dialkyl amines through an amide linker at the lysine α-amine (Figure 71).[664] As shown in Figure

71, synthesis of LOA-LysC2 began with the coupling of linoleic acid **351** with oleylamine **350** promoted by EDC; the resulting amide **352** was treated with lithium aluminum hydride to give dialkylamine **353**. The dialkylamine **353** was then functionalized via Michael addition reaction with *tert*-butyl acrylate **354** followed by deprotection of the resulting *tert*-butyl ester **355**, affording β-amino acid **356**. Compound **356** was coupled with protected lysine **357** via amide bond formation; the following global deprotection of the resulting compound yielded LOA-LysC2. Containing a primary amine, a tertiary amine, and a carboxylate, these ILLs exhibited a pH-dependent ionization property that varied with their chemical structures.[664] ILLs form small-diameter LNPs that could efficiently entrap anti-Luc siRNA and deliver anti-Luc siRNA in HeLa cells, resulting in potent luciferase gene silencing.[664]

In 2017, Miller et al. developed a library of zwitterionic amino lipids (ZALs) that contained a zwitterionic sulfobetaine head group and an amine-rich core that were attached to various hydrophobic tails via Michael addition or epoxide ring-opening (Figure 72).[665] Acrylation of *N,N¹*-dimethylethane-1,2-diamine **359** gave 2-(dimethylamino) ethyl acrylamide **360**, which underwent ring-opening reaction with 1,3-propane sultone **361**
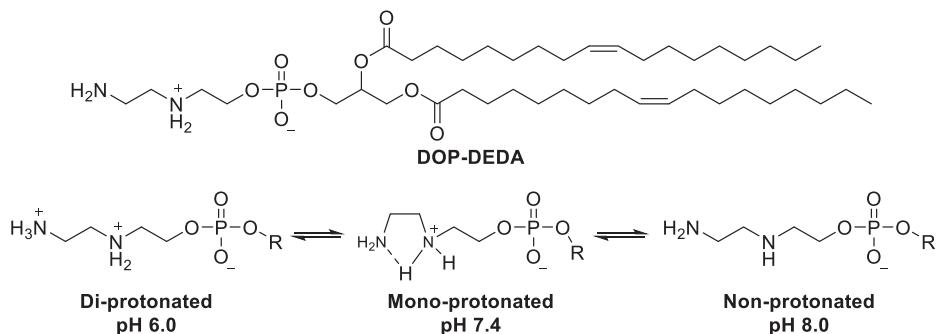
Figure 73. Chemical structure of DOP-DEDA and schematic illustration of the pH-responsive ability of DOP-DEDA.
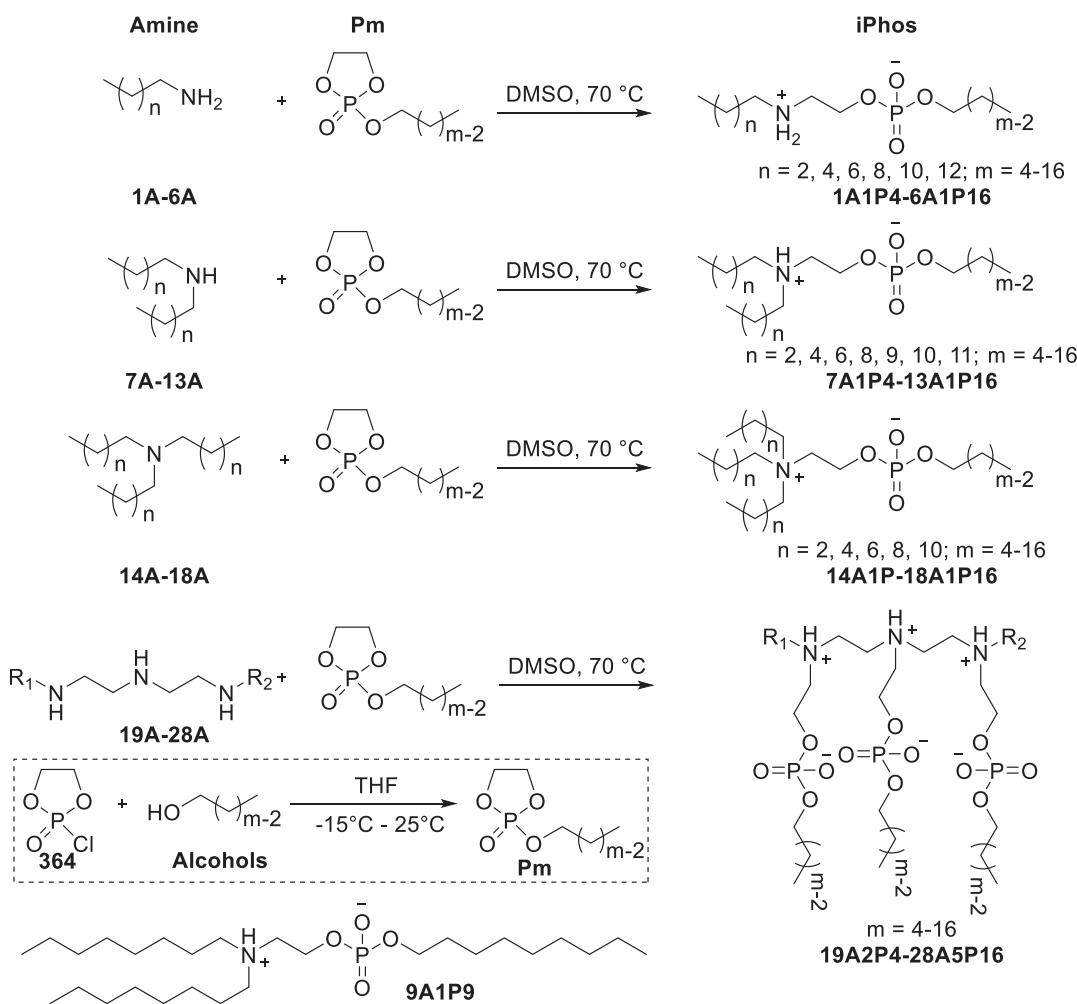
Figure 74. Chemical structures and synthesis of representative iPhos series lipids.

to afford the electrophilic precursor **362** (SBAm). Then a series of zwitterionic amines were prepared by conjugate addition of a library of polyamines to SBAm. The free amines of the resulting zwitterionic intermediate **364** were reacted with alkyl epoxides and alkyl acrylates to install hydrophobic tails and alcohol or ester groups, resulting in a 72-member ZALs library (Figure 72). Epoxide-based ZALs (ZAx-Epm) were more efficient than acrylate-based ZALs (ZAx-Acn; Figure 72) in siRNA delivery into HeLa cells. Intravenous administration of ZA3-EP10 LNPs containing Cas9 mRNA and LoxP sgRNA induced floxed tdTomato expression and enabled elimination of the STOP cassette in the liver, lungs, and kidneys of engineered mice.[665]

In 2020, Hirai et al. synthesized a DOPE-derived charge-reversible lipid, dioleoylglycerophosphate−diethylenediamine conjugate (DOP-DEDA), which was composed of a negatively charged phosphoric acid, two ionizable amino groups, and two oleyl hydrocarbon tails.[666] LNPs composed of DOP-DEDA/ DPPC/cholesterol (DOP-DEDA LNPs) were stable in the physiological medium in the absence of PEG-conjugated lipid. The charge property of DOP-DEDA LNPs depends on pH: at pH 6.0 the DOP-DEDA LNP is protonated and positively charged, at physical pH (pH 7.4) it is neutral, and at pH 8.0 it is negatively charged (Figure 73). DOP-DEDA LNPs encapsulating polo-like kinase 1 (PLK1) siRNA-induced suppression on
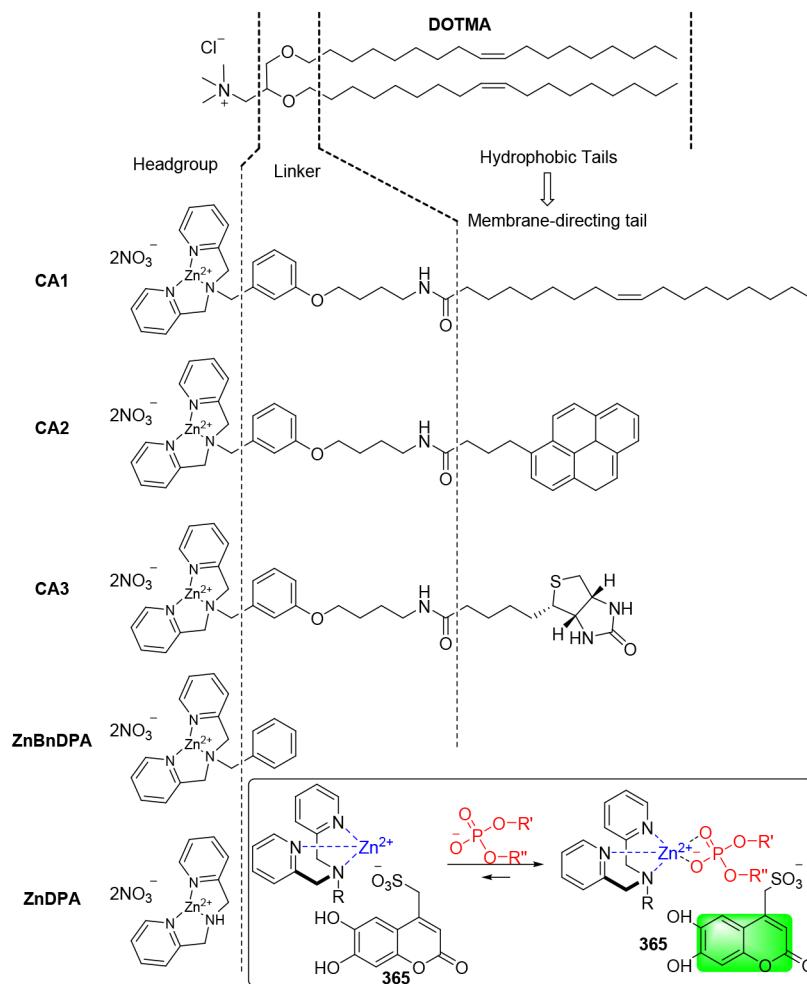
Figure 75. Schematic illustration of the interactions between Zn/DPA, sulfonic acid **365**, and phosphate.
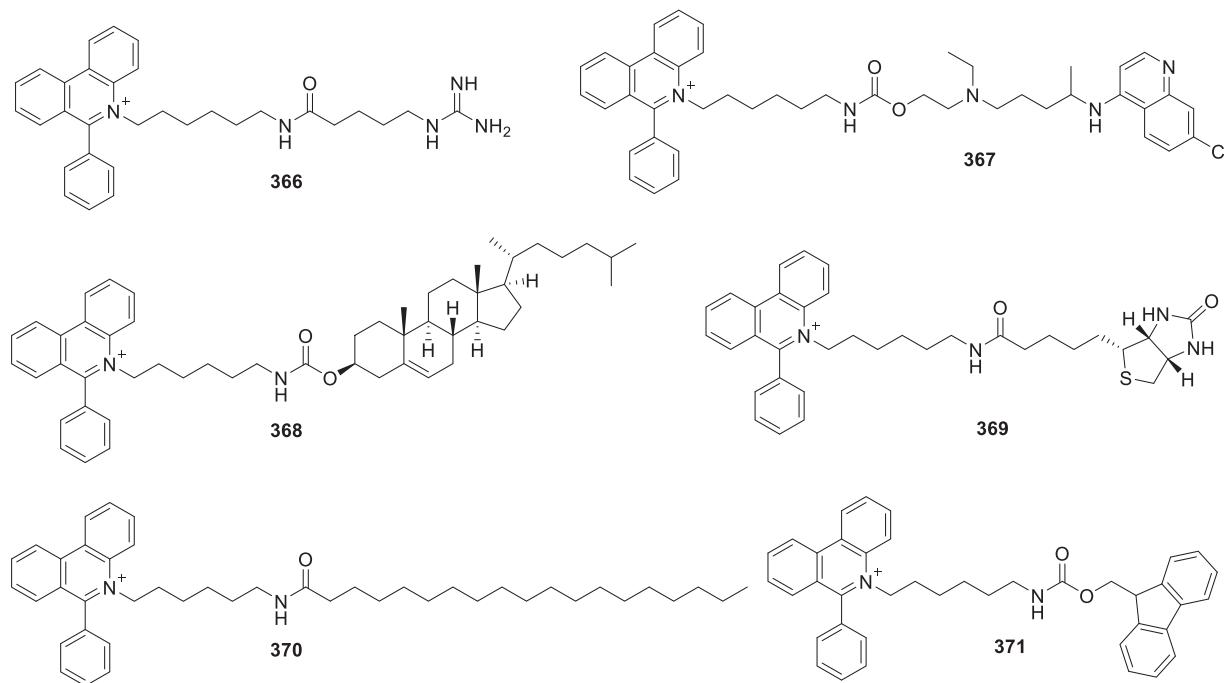
Figure 76. Chemical structures of representative coordinative amphiphiles (CAs).

	Headgroup X	Phospholipid	Abbreviation
	H	Phosphodatic Acid	PA
		Phosphatidylcholine	PC
		Phosphatidylethanolamine	PE
		Phosphatidylserine	PS
		Phosphatidylglycerol	PG
		Phosphatidylinositol	PI

Acyl chains with different length (C8–C24) and different unsaturation Degrees
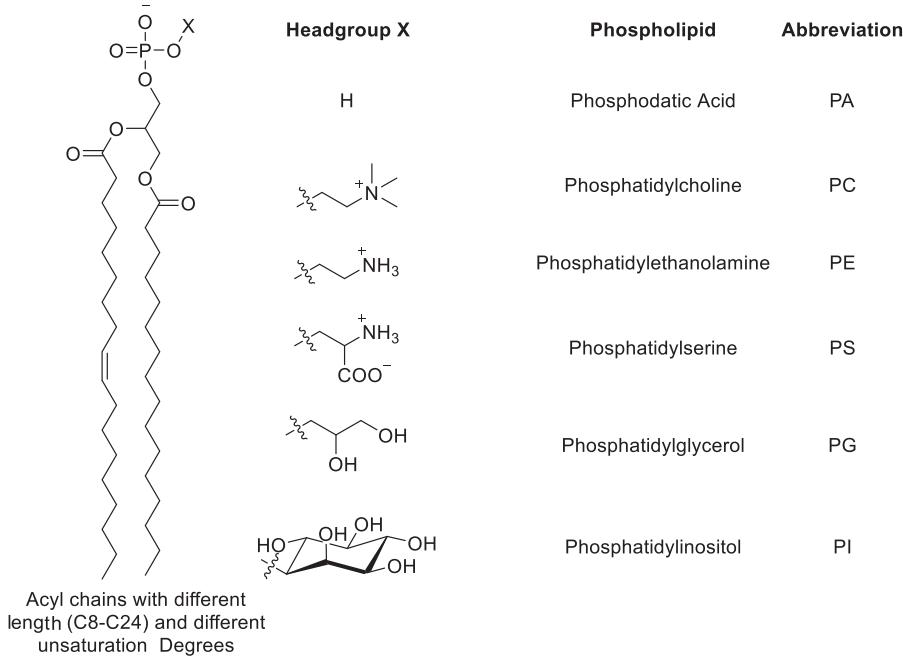
Figure 77. General chemical structure of phospholipids.

the PLK1 expression in a dose-dependent manner in MDA-MB-231 cells.[666]

To improve the endosomal escape of LNPs, Liu et al. synthesized a library of zwitterionic phospholipids (iPhos) that mimic natural phospholipids (e.g., DOPC and DOPE) through combinatorial chemistry in 2021.[667] As shown in Figure 74, phosphorylation of variant aliphatic alcohols with different alkyl tail lengths gave the key intermediate alkylated dioxaphospholane oxide (Pm), which underwent ring-opening reaction[668,669] when treated with variant amines (1A-28A), generating the library of 572 zwitterionic phospholipids (iPhos). Results of initial screening experiments for mRNA delivery in ovarian cancer cells showed that iPhos 7A1P4—13A1P16, containing an ionizable tertiary amine, a negatively charged phosphoric acid, and three hydrophobic hydrocarbon tails, were the top-performing phospholipids for mRNA delivery. 7A1P4—13A1P16, composed of the small head groups and large tails body, tend to adopt the inverted hexagonal (H_{II}) phase, thus promoting membrane fusion, destabilizing the endosomal membrane, and allowing the endosomal escape of RNA molecules. iPhos 9A1P9 was shown to be the most active component of iPhos LNPs (iPLNPs); it exhibited 40 and 965 times higher *in vivo* mRNA delivery efficiency than DOPE and DSPC, respectively. Optimization of formulation of 9A1P9 LNPs was then carried out by introducing a supplemental selective organ-targeting (SORT)[670,671] lipid. 9A1P9-5A2-SC8 LNPs mainly delivered Cre mRNA in the liver, while 9A1P9-DDAB LNPs mediated high accumulation of Cre mRNA in the lung. 9A1P9-5A2-SC8 LNPs and 9A1P9-DDAB LNPs were used for codelivering Cas9 mRNA and Tom1 sgRNA into Ai9 mice via intravenous administration at an mRNA dose of 0.75 mg/kg, resulting in specific Tom1 gene editing in the liver and the lung, respectively.[667]

2.4. Other Lipids

In 2016, Kim et al. developed a class of coordinative amphiphiles (CAs) as transporters for siRNA delivery.[672] These amphiphiles mimic cationic lipids, in which the cationic head group was

replaced by zinc(II)—dipicolylamine complex (Zn/DPA) as an RNA phosphate backbone coordinating group, and a variety of membrane-directing groups were incorporated as substitutes of the hydrophobic tails (Figure 75). The CAs aggregate in aqueous solutions and the Zn/DPA head group coordinate with the negatively charged phosphate backbones of siRNAs, protecting siRNAs from degradation by RNase. The induction of different membrane-directing groups is necessary for enhanced siRNA delivery, as the Zn/DPA head group alone exhibits only moderate delivery efficiency.[672]

In 2018, Tai et al. developed a class of bifunctional chemical tags (**366−371**) that were capable of noncovalently binding and delivering siRNA into the cytosol directly (Figure 76).[673] The bifunctional tags are composed of a siRNA-binding domain and a steroid region that can readily fuse with cell membrane. Compared to the conventional covalent siRNA—steroid conjugates, the noncovalently tagged siRNA is cell membrane-permeant and cytosol targeting, thus enabling effective siRNA delivery directly into PC-3 cells without involving the endocytic pathway.[673]

3. HELPER LIPIDS

To stabilize the lipid-based RNA delivery system, many helper lipids, such as cholesterol, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (DSPC), or 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE),[288] have been included as the formulation components. These helper lipids may not only stabilize the particles but also enhance RNA delivery efficiency.[194]

3.1. Phospholipids

A typical phospholipid is composed of glycerol, two hydrophobic fatty acid tails, and a phosphate-linked head group (Figure 77). Due to their amphiphilic characteristic, they can form lipid bilayers as the main components of the cell membrane. Modifications of the phosphate group with the simple organic molecules choline, ethanolamine, or serine can give the corresponding phosphatidylcholine (PC), phosphati-
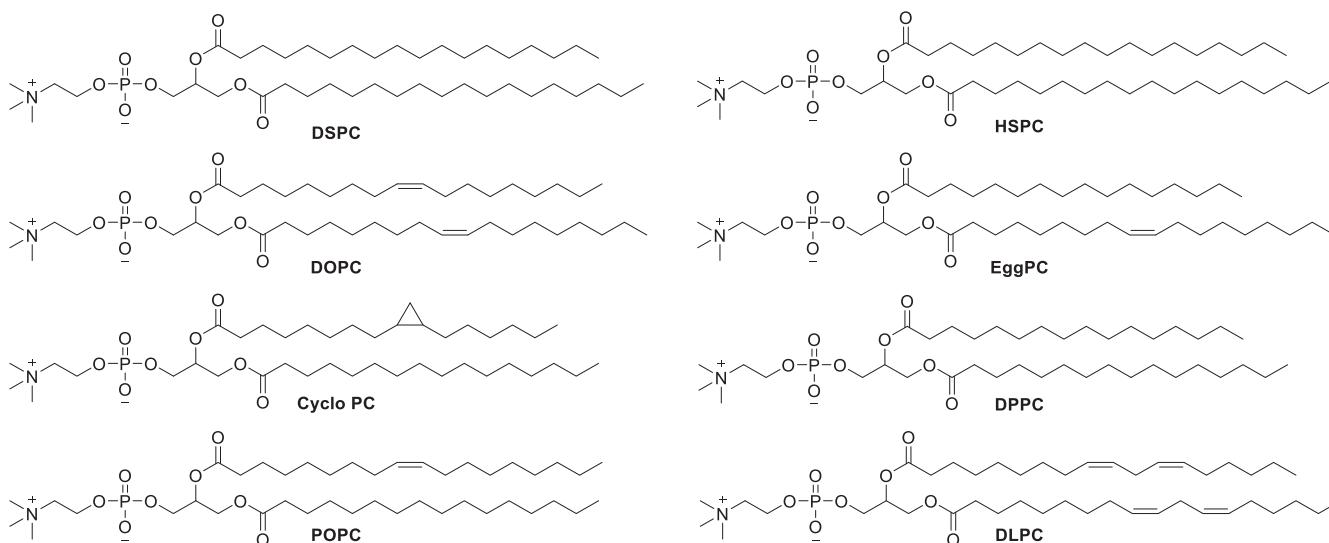
Figure 78. Chemical structures of representative phosphatidylcholines.
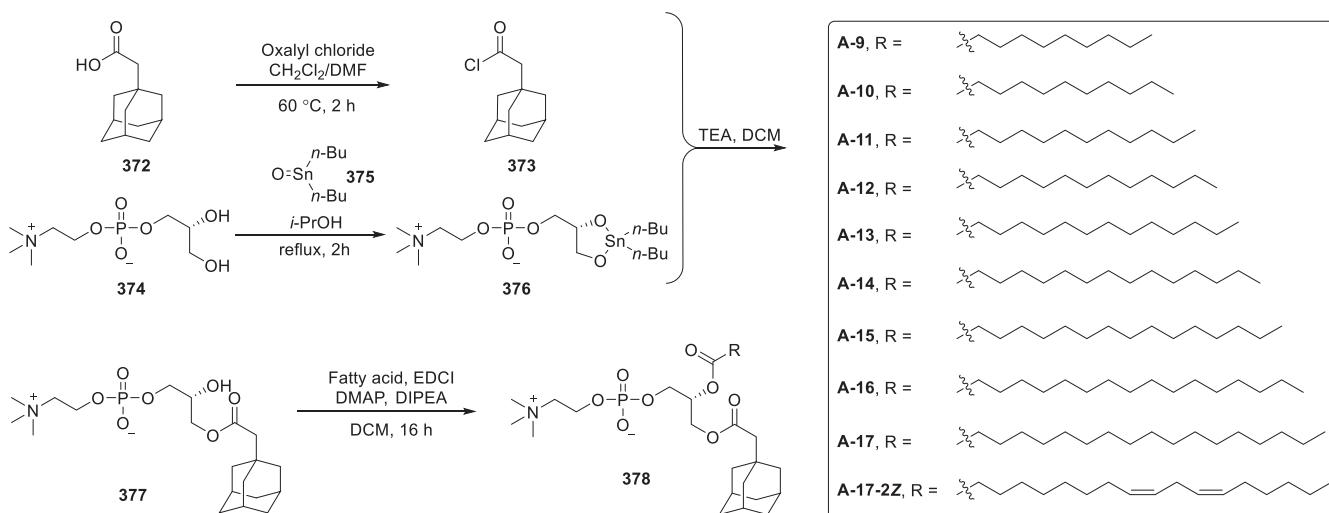
Figure 79. Synthesis of phosphatidylcholines incorporating adamantyl groups.

dylethanolamine (PE), and phosphatidylserine (PS). Phospholipids provide important structural components for the LNPs and may also aid the process of endosomal escape.[107] Both synthetic and natural phospholipids can be used in the formulation of LNPs for RNA delivery.

3.1.1. Phosphatidylcholines. Phosphatidylcholines (PCs) are one of the major components (50%) of cell membranes. Due to the cylindrical geometry of PCs, they tend to adopt a bilayer phase (Figure 78),[674] thus improving the stability of LNPs.[675] PCs with saturated tails, such as HSPC (hydrogenated soybean PC) and DSPC (distearoylphosphatidylcholine), have high melting temperatures and are generally used to prepare highly stable LNPs. It is worth mentioning that DSPC is used as a helper lipid in patisiran, mRNA-1273, and BNT162b2.[530] DOPC liposomes encapsulating EphA2 siRNA were shown to induce EphA2 gene silencing and repression of tumor growth in mice following either intravenous or intraperitoneal administration.[676,677] Cyclo PC is found in the membrane of *Escherichia coli*.[678] In 2019, the Cullis group studied the role of DSPC-cholesterol in LNPs formulation of siRNA and found that in empty LNP systems, DSPC-cholesterol resides in the outer layers of LNPs, whereas in siRNA-loaded LNPs, DSPC−cholesterol was partially internalized together with siRNA.[195] DSPC can enhance the encapsulation of siRNA in the LNP−siRNA system by participating in the formation of siRNA−lipid complexes.[195] In 2020, the Sahay group formulated LNPs with Cyclo PC instead of DSPC; the resulting Cyclo PC-LNPs showed enhanced delivery of mRNA into the cells compared to DSPC LNPs.[679] They found that the structural differences between Cyclo PC and DSPC were not significant enough to influence *in vitro* delivery.[679] This can be further supported by the report that alteration of symmetrical hydrophobic tails to asymmetrical hydrophobic tails did not significantly change the siRNA delivery efficiency of LNPs.[568] DOPC has also been used in the formulation of neutral lipid emulsions (NLEs)[288] along with Tween 20/squalene; the resulting NLEs selectively delivered R-34a miRNA to lung tumors, resulting in a 60% reduction of tumor area in mice after intravenous administration.[680]

Adamantane is a diamondoid hydrocarbon that consists of three linked cyclohexane rings. When the adamantyl group is incorporated in small molecular drugs, not only can it influence
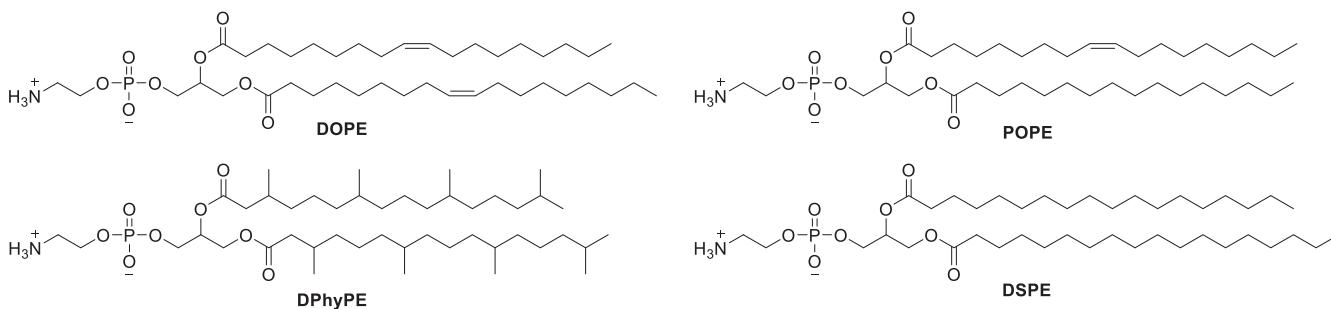
Figure 80. Chemical structures of representative phosphatidylethanolamines.
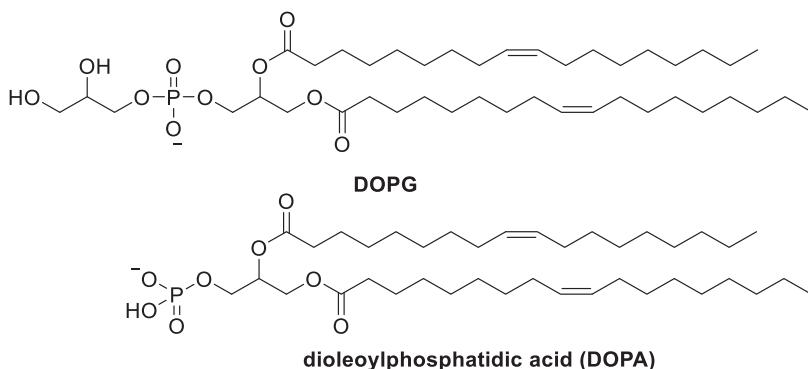
Figure 81. Chemical structures of representative phosphatidylglycerol.

the interactions between the small molecules and cell membrane,[681] but it also can improve the pharmacokinetics of small molecule drugs.[682] Besides, the constrained adamantyl group improves drug metabolic stability.[683] In 2019, the Dahlman group reported that LNPs formulated with ionizable lipids that contain an adamantly group could deliver siRNA to splenic T cells without targeting ligands (Figure 79).[546] Generally, PCs that are incorporated in LNPs contain unconstrained hydrocarbon tails. Based on the unique properties of constrained adamantyl groups, they developed a library of phosphatidylcholines containing an adamantyl group and further evaluated their mRNA delivery *in vivo*.[684] They synthesized a series of constrained phosphatidylcholines, each of which contained a quaternary ammonium head group, a phosphodiester linkage, a constrained adamantly group, and an unconstrained hydrocarbon chain with varied length and saturation degree. The synthesis of these phosphatidylcholines started with the dibutyltin oxide-mediated chemoselective monoacylation of L-α-glyceryl phosphorylcholine **374** with adamantyl chloride **373**.[685] Then the hydrocarbon tail was attached to the remaining hydroxyl group via Steglich esterification,[686] affording desired lipids A-10 through A-17-2Z (Figure 79). Then they utilized Fast Identification of Nanoparticle Delivery (FIND)[687] to quantify mRNA delivery mediated by the 109 LNPs formulated with the constrained phosphatidylcholines in a single Ai14 mouse. Intravenous administration of A-11 LNPs encapsulating Cre mRNA in Ai14 mice at a dose of 0.5 mg/kg resulted in Cre mRNA accumulation in the liver Kupffer cells preferentially.[685]

3.1.2. Phosphatidylethanolamines. Previously, DOPE (Figure 80) was frequently used as a helper lipid in the formulation of cationic liposomes for DNA delivery for gene therapy.[688] DOPE is composed of a primary amino head group, phosphoethanolamine, and two unsaturated oleoyl tails. With

two unsaturated chains, DOPE has low melting temperatures (30 °C). In the physiological temperature, DOPE can induce the inverted hexagonal (H_{II}) phase, facilitating membrane fusion and/or bilayer disruption.[689]

Phosphatidylcholines (PCs), containing a quaternary amine head group and two saturated hydrocarbon tails, have a *P* value less than 1, so they tend to aggregate into a lamellar structure. Phosphatidylethanolamine (PE), which contains a bit smaller primary amine head group than that of PCs and a *P* value > 1, favors inverted micelles or an inverted hexagonal (H_{II}) phase.[690] Kauffman et al. designed a series of experiments to determine the importance of LNP formulation on delivery efficacy and found that the use of the phospholipid DOPE instead of DSPC enhanced RNA delivery efficiency.[629] In several studies, DOPE-formulated LNPs are more efficient than DSPC-formulated LNPs for mRNA delivery.[598,611,627,629] The inclusion of DOPE during the formulation of LNPs may reduce membrane stability, thus facilitating endosomal escape.[691,692] Another possible reason may be that the stronger complexation of mRNA to lipid in LNPs containing DSPC may hinder the decomplexation of mRNA from lipids in the cytosol, thus inhibiting the release and translation of mRNA payload. DSPC usually inhibits membrane fusion with the endosomal membrane, thus inhibiting endosomal escape, whereas DOPE, with unsaturated hydrophobic tails, can undergo a phase transition to an inverted hexagonal (H_{II}) phase, thus facilitating membrane fusion-mediated endosomal escape.[693,694] In 2006, Santel et al. prepared siRNA-lipoplex by formulating ATUFect01 with DPhyPE, a diphytanoyl zwitterionic phospholipid. Systemic administration of this siRNA-lipoplex led to downregulation of the corresponding mRNA and protein *in vivo*.[391] In 2021, Zhang et al. investigated the influence of DOPE and DSPC on the interactions between LNPs and ApoE.[695] Results of high-throughput *in vivo* screening of 96 LNPs showed that several
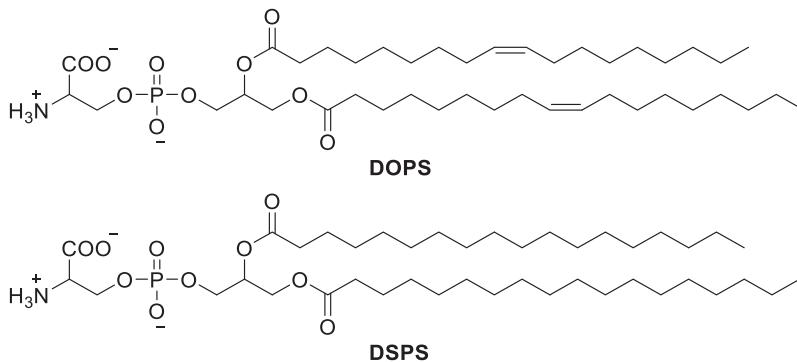
Figure 82. Chemical structures of representative phosphatidylserine.
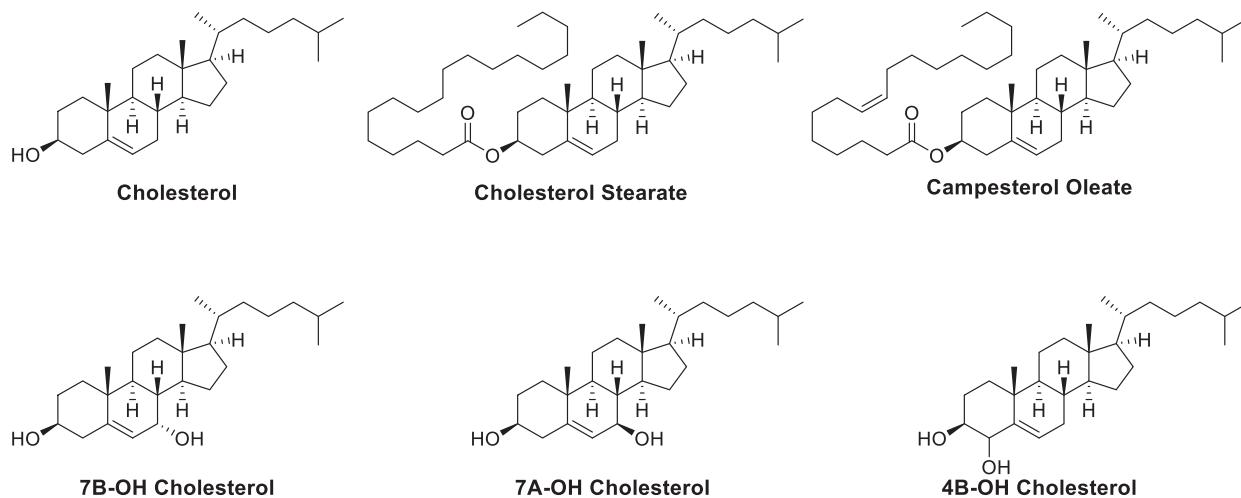
Figure 83. Chemical structures of cholesterol variants formulated in LNPs.

LNPs incorporating DOPE (e.g., LNP 42) tended to accumulate in the liver, whereas LNPs formulated with DSPC (LNP 90) preferentially delivered RNA in the spleen. Results of QCM-D experiments showed that LNP containing DOPE (e.g., LNP 42) had stronger interactions with ApoE than LNP 90.[695]

3.1.3. Phosphatidylglycerols. Phosphatidylglycerol (PG) is a non-pH sensitive anionic lipid that can provide balancing charges to an ionizable lipid (Figure 81). In 2012, Kapoor et al. developed effective DOPG-based anti-eGFP siRNA lipoplexes.[696] In this work, anionic lipoplexes were prepared by complexing anionic liposomes formulated with DOPG and DOPE and siRNA using calcium ion bridges. The silencing activity of the anionic lipoplex composed of DOPG/DOPE was similar to that of Lipofectamine 2000. To inhibit the rapid growth of calcium phosphate (CaP) particles[697] that encapsulate RNA, Zhang et al. developed a lipid-coated calcium phosphate (LCP) nanoparticle encapsulating cMyc siRNA, wherein the CaP core is coated with DOPA and DOTAP.[698] The combination of chemo- and gene-therapeutics resulted in a dramatic suppression of tumor growth in H460 tumor-bearing mice following intravenous administration.[698]

3.1.4. Phosphatidylserines. In 1978, Giorgos J. Dimitriadis prepared phosphatidylserine-based unilamellar liposomes to deliver rabbit globin mRNA into mouse lymphocytes *ex vivo*, resulting in functional protein expression.[189] It was reported that anionic phosphatidylserine in the endosomal membrane could displace plasmids from the plasmids lipoplex, thus assisting in the release of plasmid after cell uptake of

nanoparticles.[699−701] In 2001, Hafez et al. found that coformulation of anionic phosphatidylserines (Figure 82) and cationic lipids preferentially adopts the inverted hexagonal (H_{II}) phase, thus facilitating the release of nucleic acid payload into the cytoplasm.[292] Besides, replacing 1,2-distearoyl-*sn*-glycero-3-phospho-L-serine (DSPS) with 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) resulted in a clear inverted hexagonal (H_{II}) phase when fusogenic cationic lipids were incorporated.[292]

3.2. Sterols

Steroids are a family of terpenoid lipids that contain four fused cyclic carbon rings, and sterols are steroids that contain a hydroxyl group, such as cholesterol.[702] Cholesterol and related sterols are precursors for the synthesis of many vital steroids; they are naturally occurring lipids present in animal cell membranes. In the formulation of lipid-based nanoparticles, cholesterol acts as a helper lipid that can enhance nanoparticle stability and promotes the fusion of the nanoparticles with the cell membrane.[176,703,704] Lipid nanoparticles without cholesterol may result in cholesterol shuttling from serum components to nanoparticles.[705] Besides, cholesterol might reside on the surface of lipid nanoparticles in a crystalline form.[706,707] It is reported that a cholesterol level increase by 7% improved the release of drug from 5% to 90%,[708] which proves the essentiality of cholesterol in the delivery of siRNA.

Cholesterol variants derived from natural esterification and oxidation of cholesterol are differentially trafficked via lipoproteins to cells including endothelial cells, hepatocytes, and
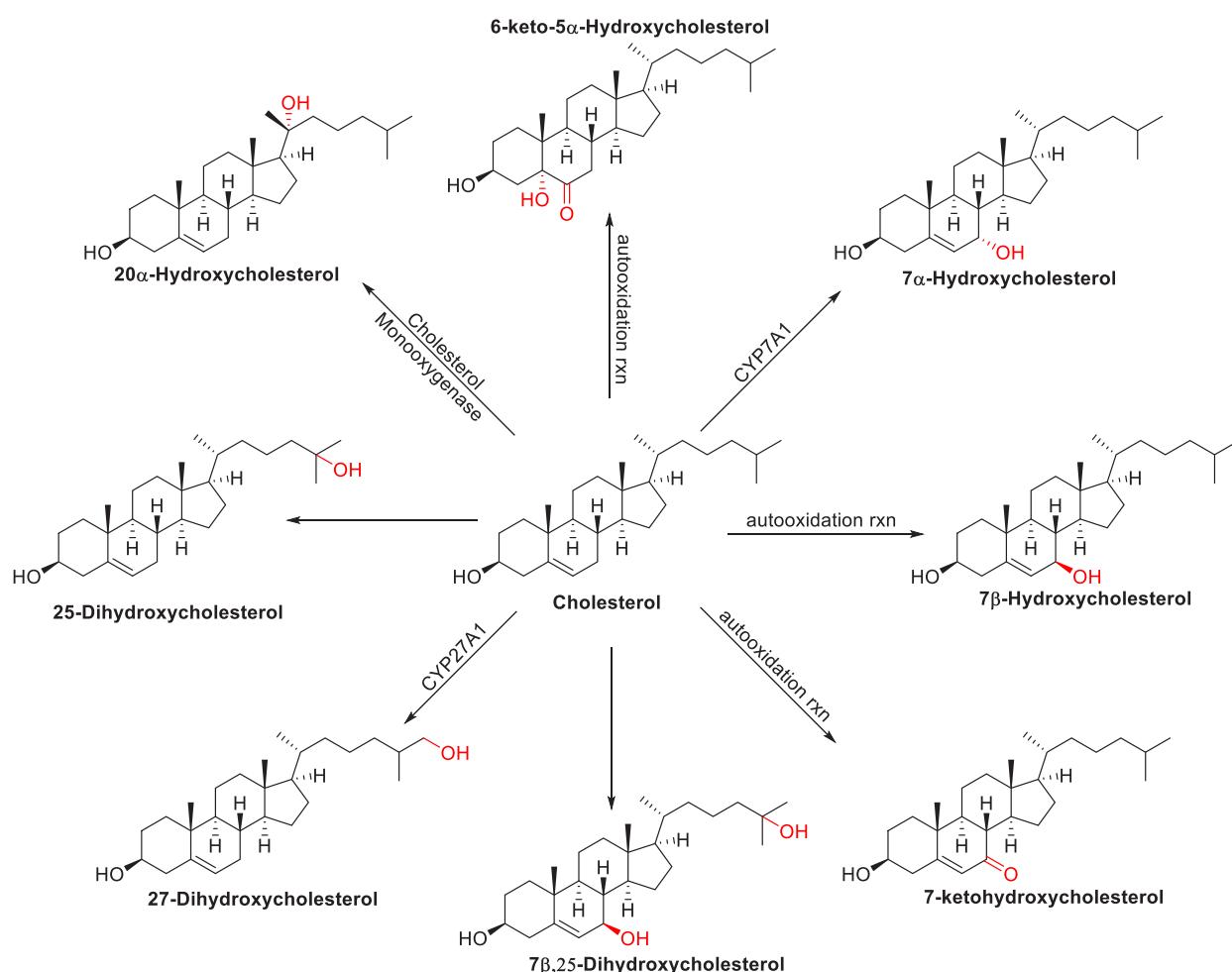
Figure 84. Chemical structures of sterol variants modified from cholesterol.

macrophage.[709] In 2018, Paunovska et al. evaluated whether incorporation of different cholesterol variants (Figure 83) in LNPs would lead to different nanoparticle targeting *in vivo*.[710] *In vivo* RNA delivery data points showed that modified cholesterols could affect the targeting ability of nanoparticles. Additionally, LNPs formulated with esterified cholesterol (e.g., cholesterol stearate) showed higher RNA delivery efficiency compared to LNPs containing regular or oxidized cholesterol (e.g., 7B-OH cholesterol) in mice. They also identified LNPs containing cholesteryl oleate as efficient nanocarriers for delivery of siRNA and sgRNA to liver endothelial cells in mice.[710] This work also showed the possibility that rational design of the cholesterol analogs that closely mimic natural lipoproteins or interact with natural cholesterol trafficking pathways may enhance LNPs delivery efficiency.[711]

In 2019, a library of nine cholesterol analogs was studied to evaluate the influence of chemical structures of sterol variants on mRNA delivery efficiency of LNPs.[712] These side-chain or ring-oxidized cholesterol analogs were obtained via treating cholesterol with enzymes (Figure 84). 125 FIND[545,713] LNPs were formulated with the ionizable lipid cKK-E12,[714] two PEG-lipids, the DOPE, and one of nine cholesterol variants. They observed that systematically oxidative modifications on the hydrocarbon tail (e.g., 20-hydroxycholesterol) were more potent than those with an oxidized B ring (e.g., 7-ketohydroxycholesterol). LNPs formulated with 20-hydroxycholesterol preferentially delivered Cre mRNA to hepatic cells

and Kuffper cells rather than hepatocytes at a dose of 0.5 mg/kg after systemic administration in Ai14 mice. Oxidation of the tail attached to the sterol ring D may alter the interaction between LNPs and serum proteins, resulting in the biodistribution of LNPs in preference to the liver endothelial cells and Kupffer cells.[712]

The Sahay group improved RNA delivery by using plant-based analogs of cholesterol instead.[715−717] In 2020, Eygeris et al. found that when C-24-alkylated derivatives of cholesterol (e.g., β-sitosterol) were included in the mRNA-loading LNPs, enhanced mRNA delivery was observed.[715] To further evaluate the effect of cholesterol analogs on RNA delivery efficacy, three groups of naturally occurring cholesterol analogs were selected based on structural resemblances (Figure 85).[716] Group I contained vitamin D_1, vitamin D_2, and vitamin D_3, group II was composed of C-24 α-alkyl sterols (e.g., β-sitosterol), and group III consisted of pentacyclic terpenoids (e.g., betulin). The screening result showed that group I analogs showed low mRNA delivery efficiency. Incorporation of the group II analog β-sitosterol in LNPs could result in mRNA translation efficiency improvement by 48-fold in cancer cells, whereas the inclusion of group III analogues led to ≥50% decrease in mRNA encapsulation efficiency and increased size of LNPs, resulting in poor mRNA delivery efficiency.[718,719]

The C-24 alkyl group of phytosterols induces crystal defects that are in proportion to the length of the C-24 alkyl group (cholesterol < campesterol < β-sitosterol).[720] Three additional
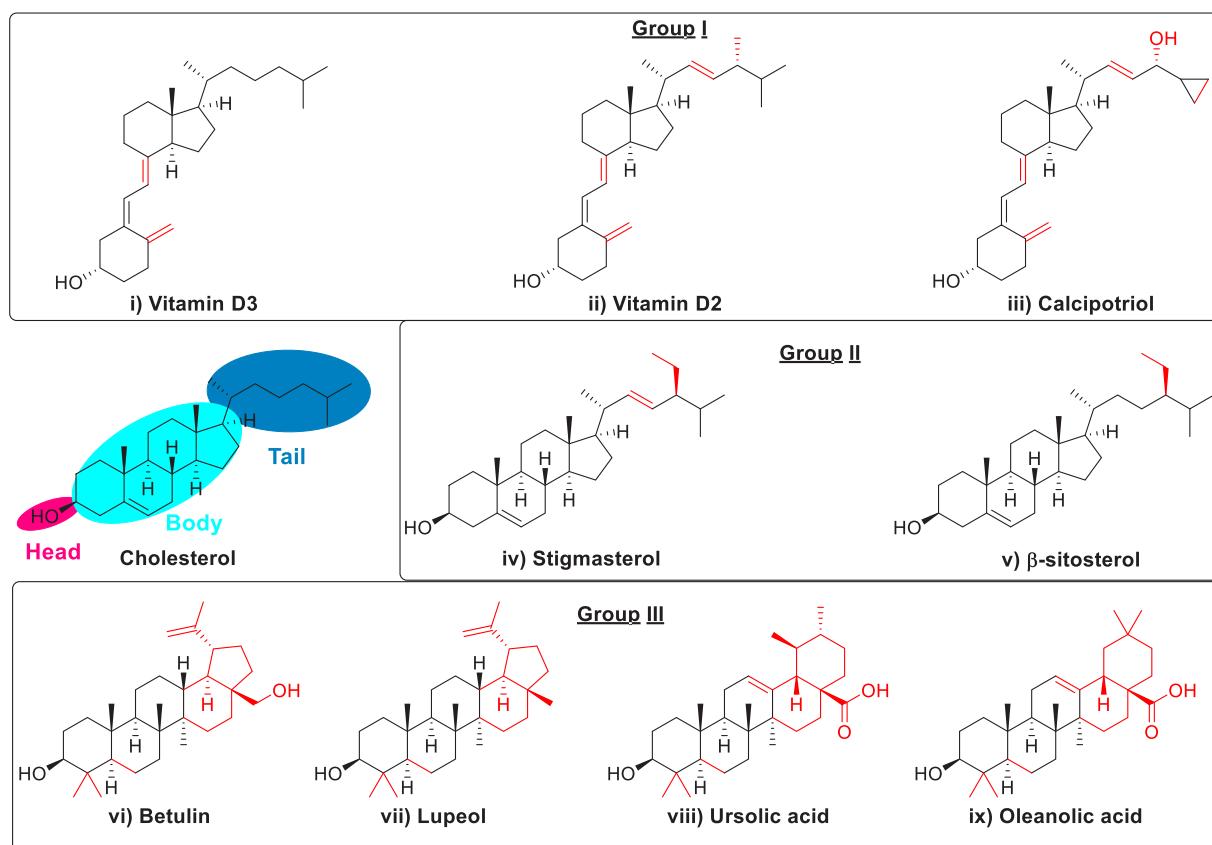
Figure 85. Chemical structures of three groups of cholesterol analogs.
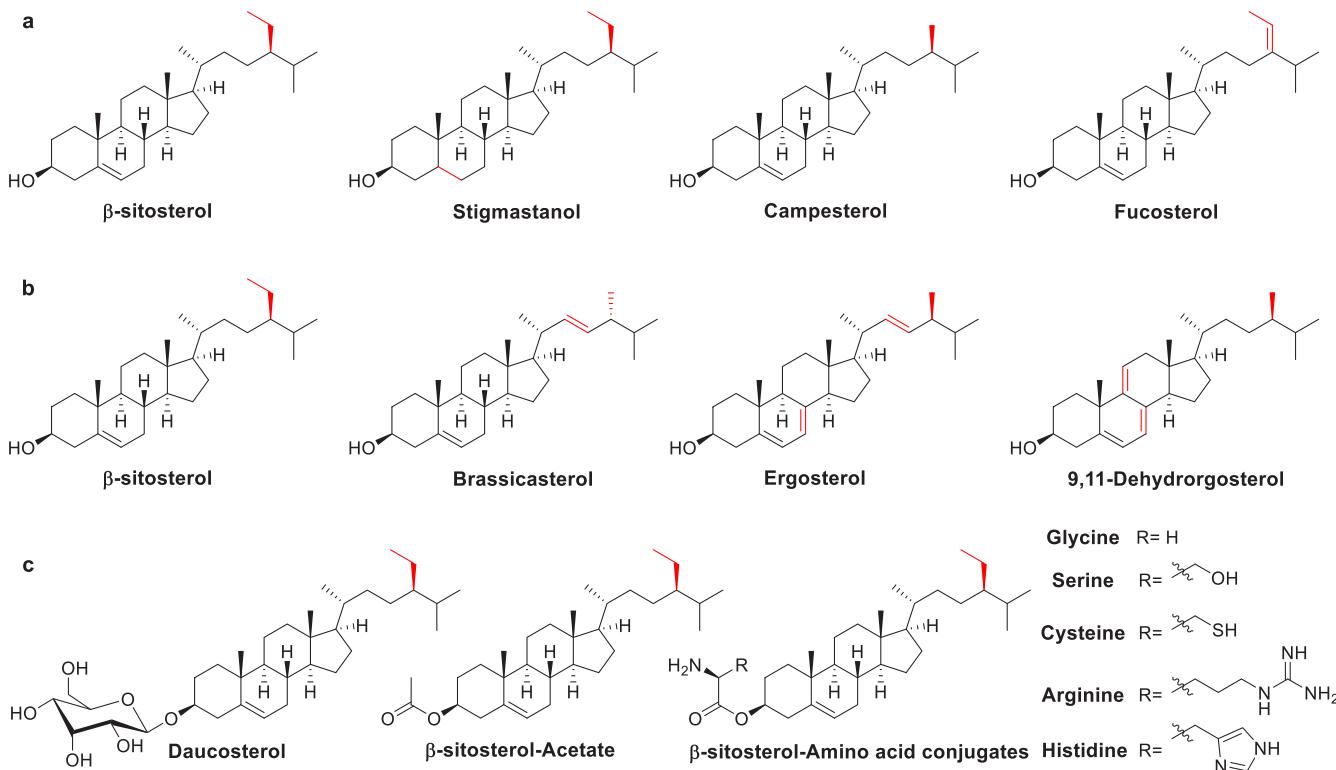
Figure 86. Structural features of C-24 alkyl derivatives of cholesterol.

phytosterols, namely stigmastanol, fucosterol, and campesterol, were selected and formulated (Figure 86). The resulting LNPs exhibited high encapsulation (>90%) and an 11- to 211-fold improvement in mRNA delivery efficiency than cholesterol-

based LNPs. Results of brassicasterol, ergosterol, and 9,11-dehydroergosterol (Figure 86) showed that reduced flexibility in the body and tail domain of cholesterol variants could inhibit RNA delivery. β-Sitosterol amino acid conjugates (polar) and β-sitosterol acetate (nonpolar) were also evaluated and showed that shielding the hydroxy group on ring A resulted in low or no RNA delivery efficiency. Lysosomal transporters recognize the hydroxyl group on cholesterol ring A and deliver cholesterol to the endoplasmic reticulum.[721] The structure−activity relationship analysis of cholesterol analogs revealed that the high mRNA delivery efficiency was closely related to the flexibility of the sterol ring, the alkyl tail length, and the polarity associated with the hydroxyl group. The enhanced mRNA delivery efficacy may have been caused by morphological changes in the internal and external structure of mRNA-loading LNP.[716,722] The structural analysis revealed that LNPs formulated with phytosterols had a polymorphic shape and exhibited different degrees of multi-lamellarity and rigidity. Unlike the smooth surface of LNPs containing cholesterol, LNPs containing β-sitosterol had a multifaceted surface, which might be caused by the changes in the surface lipid composition[723] and could enhance the fusion of LNPs with membranes,[724] thus leading to higher mRNA delivery efficiency. LNPs formulated with campesterol, stigmasterol, or β-sitosterol showed higher lamellarity and few internal defects as compared to LNPs containing cholesterol. The increased lamellarity may promote the fusion of LNPs with the endosomal membrane, while the internal structure of the LNPs may not be critical for the improved mRNA delivery efficiency of LNPs.[715] They further found that LNPs formulated with vitamin D2 cannot cross the cell membrane due to the high fragility of their fluid lipid membrane, while the fucosterol-containing LNPs fail to deliver the mRNA payload due to their strong stability caused by the excessively rigid membrane. Moreover, live-cell imaging showed that LNP containing β-sitosterol showed extended retention in the endocytic vesicles, thus boosting the endosome escape of LNPs.[717]

3.3. Fatty Acids

When anionic lipids (Figure 87) are formulated into LNPs, they can interact with cationic lipids to form ion pairs, which can improve the pH-sensitivity of the LNPs and promote endosomal membrane destabilization, thus increasing nucleic acids delivery efficiency.[725,726] Anionic lipids, such as fatty acids, can be protonated in the acidic endosome following cellular uptake.
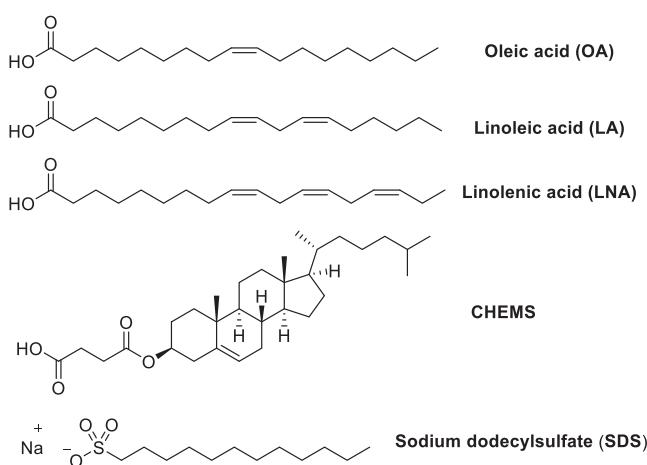
Figure 87. Chemical structures of anionic lipids.

Protonation of the anionic lipids can cause the LNPs to be surface positively charged and/or induce lipid phase transition. Previous studies showed that if unsaturated fatty acids such as oleic acid (OA), linoleic acid (LA), or linolenic acid (LNA) are incorporated in LNPs, in place of PCs, the delivery efficiency of siRNA or miRNA may be significantly enhanced.[194,727,728] DOTMA-based LNPs containing oleic acid (OA) were shown to be more efficient in microRNA-122 delivery than the commercially available Lipofectamine 2000.[729] As linoleic acid (LA) is an essential fatty acid, which can participate in fatty acid metabolism of liver cells through plasma membrane fatty acid binding protein (FABPpm),[730−733] the inclusion of LA can enhance hepatocyte uptake of LNPs. Yu et al. reported that their TRENL3-based LNPs formulated with anionic fatty acids had a much lower surface charge, thus leading to the improvement of the biodistribution of LNPs and enhanced uptake into hepatocytes in mice following intravenous administration. Besides, incorporating unsaturated fatty acids in LNPs led to a slight reduction of the mean diameter of LNPs.[734] In the formulation of Smarticles LNPs, carboxylate-based anionic lipids (e.g., CHEMS) were incorporated to achieve pH-responsiveness. Smarticles LNPs have been used in the delivery of small activating RNA (saRNA) into HepG2 human hepatocellular carcinoma cells *in vitro*, resulting in activation of CEBPA mRNA and growth inhibition of liver cancer cell.[735] Sodium dodecyl sulfate (SDS) is an anionic lipid that is not commonly used in the preparation of RNA delivery systems. In 2013, SDS-CTAB vesicles were developed by Russo et al., which could efficiently deliver CAT-A98 mRNA into the HEK-293 cells.[736]

He et al. synthesized an anionic helper lipid named DC and developed DC-based LNPs, termed nano-Transformers.[737] Synthesis of DC began with the coupling of (9E)-octadec-9-enoic acid **379** with diol **380**; the resulting ester **381** was treated with TFA to remove the Boc protecting group, giving amine **382**. Ring-opening reaction of citraconic anhydride **383** with the primary amine group of **382** afforded DC (Figure 88). DC LNPs were negatively charged in physiological pH (pH = 7.4) and nearly neutral in endosome. Upon protonation at acidic pH, the positive surface charge of DC LNPs induces endosomal membrane fusion, thus facilitating the release of siRNA into the cytosol. DC LNPs delivered cyclin-dependent kinase 1 (CDK1)-siRNA efficiently, leading to up to 95% reduction of CDK1 mRNA in HepG2 cells *in vitro*, and significantly suppressed the HepG2 tumor growth in nude mice.[737]

3.4. Fatty Acid Esters

Both Span80[738] and Tween80[739] (Figure 89) have been used as surfactants in the formulation of cationic noisome for siRNA delivery. MOG can act as a helper lipid in LNPs-siRNA-mediated gene silencing because it can form gyroid inverted cubic structures that favor membrane fusion.[347,437,740−744] DODAB/MOG (2:1) liposomes showed an efficient siRNA delivery.[743,745,746] Cetyl palmitate is used as a component of some LNPs for RNA delivery, where the inclusion of cetyl palmitate can decrease the diameter of LNPs.[747] Compritol 888 ATO is a mixture of glyceryl monobehenate (12−18% w/w), glyceryl dibehenate (45−54% w/w), and glyceryl tribehenate (28−32% w/w). Montana et al. reported delivery of siRNA via LNPs formulated with Compritol ATO 888 as matrix lipid.[748]

3.5. Other Helper Lipids

LNPs can be modified by incorporating lipids that contain various ligands to control their biological properties such as
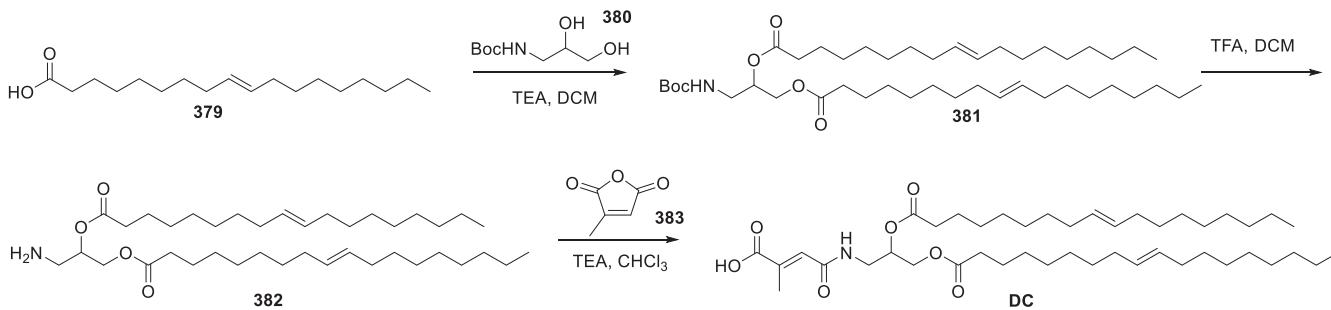
Figure 88. Synthesis procedures of DC.
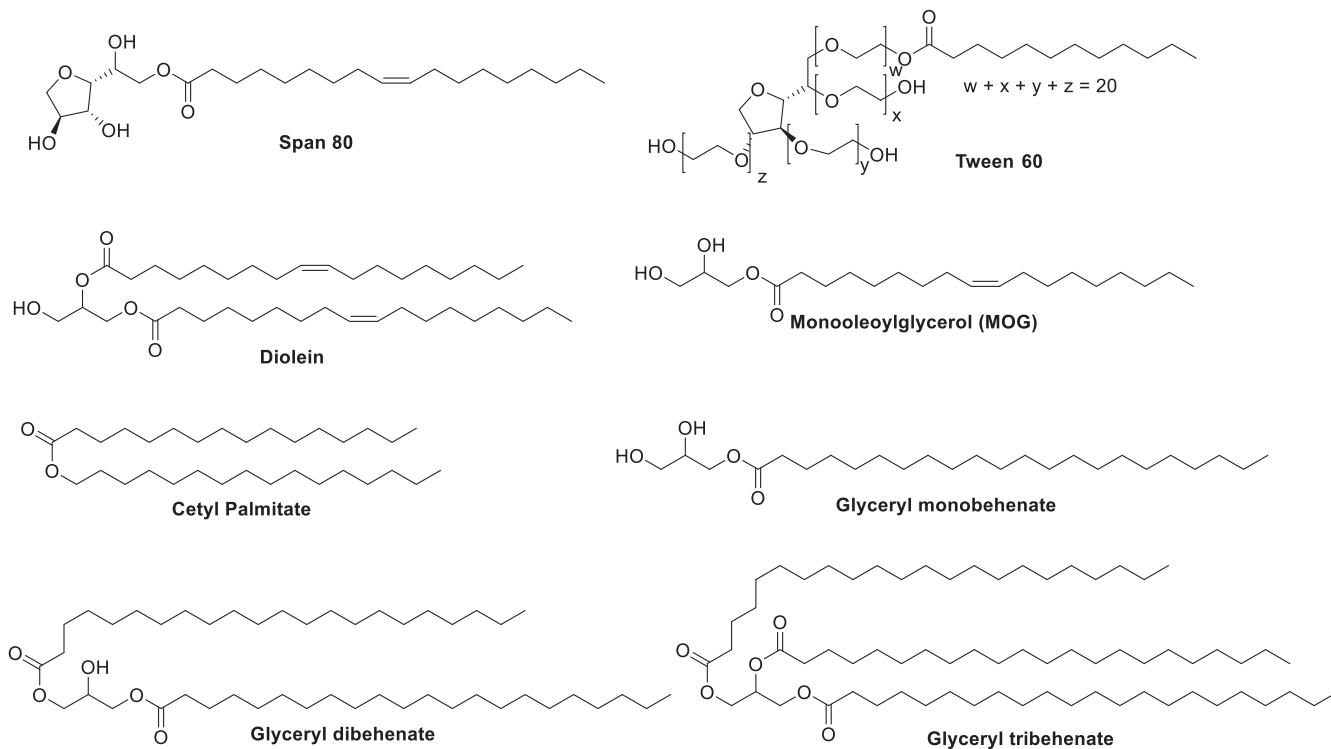
Figure 89. Chemical structures of fatty acid esters for RNA delivery.

circulation stability and targeting ability.[749] Antigen-presenting cells (APCs), such as dendritic cells and macrophages, express abundant mannose receptors on their cell surfaces; these mannose receptors can bind carbohydrates and their conjugates.[750−753] To achieve site-specific targeting and enhance uptake of LNPs, mannose has been extensively investigated for modification of LNPs.[575] Mannosylated lipid conjugate **384** (Figure 90) contains a D-mannose residue that is connected with dialkylglycerol via succinyl. The mannosylated LNPs incorporating mannosylated lipid conjugate **384** were used to deliver melanoma total RNA into DCs both *ex vivo* and *in vivo* and was shown to be more efficient in inducing CTL response compared to the control group.[754] Mannosylated LNPs formulated with lipoconjugate **384** or **385** were efficient in delivering melanoma B16 RNA *in vivo*, inducing the generation of the melanoma B16-specific T-lymphocytes in mice and B16 cell apoptosis.[755] Manchol, a mannose−cholesteryl amine conjugate, has also been used in the formulation of mannosylated LNPs loading self-amplifying mRNA (SAM) encoding hemagglutinin.[756] Compared to the unglycosylated LNPs, mannosylated LNPs showed enhanced *in vitro* cellular uptake in BMDCs and induced a faster

antibody response in mice, independent of the administration route.[756] DGTS (Figure 90), a plant-derived structural lipid, is associated with lipid metabolism[679] and cell survival in stress conditions.[757,758] Kim et al. substituted DSPC in an LNP formulation, to a series of naturally occurring membrane lipids, especially DGTS.[679] DGTS LNPs induced 20-fold lower luciferase expression compared to DSPC LNPs. However, DGTS led to an enhanced liver delivery of mRNA. This discrepancy may be due to the DGTS-induced LNP morphology modification, which changes both the stability and tolerance of LNPs.[759] Both squalene[760−762] and lycopene[763,764] (Figure 90) can be used as neutral helper lipids in the preparation of noisome for RNA delivery.

4. LIPID-DERIVED MACROMOLECULES

In addition to small molecular lipids and lipid derivatives, lipid-derived macromolecules have also been extensively explored as RNA delivery materials.[722,765] The composition, structure type, and charge of lipid macromolecules have an important impact on the delivery efficiency of RNA.[183] The lipid-derived macromolecules used for RNA delivery mainly include lip-
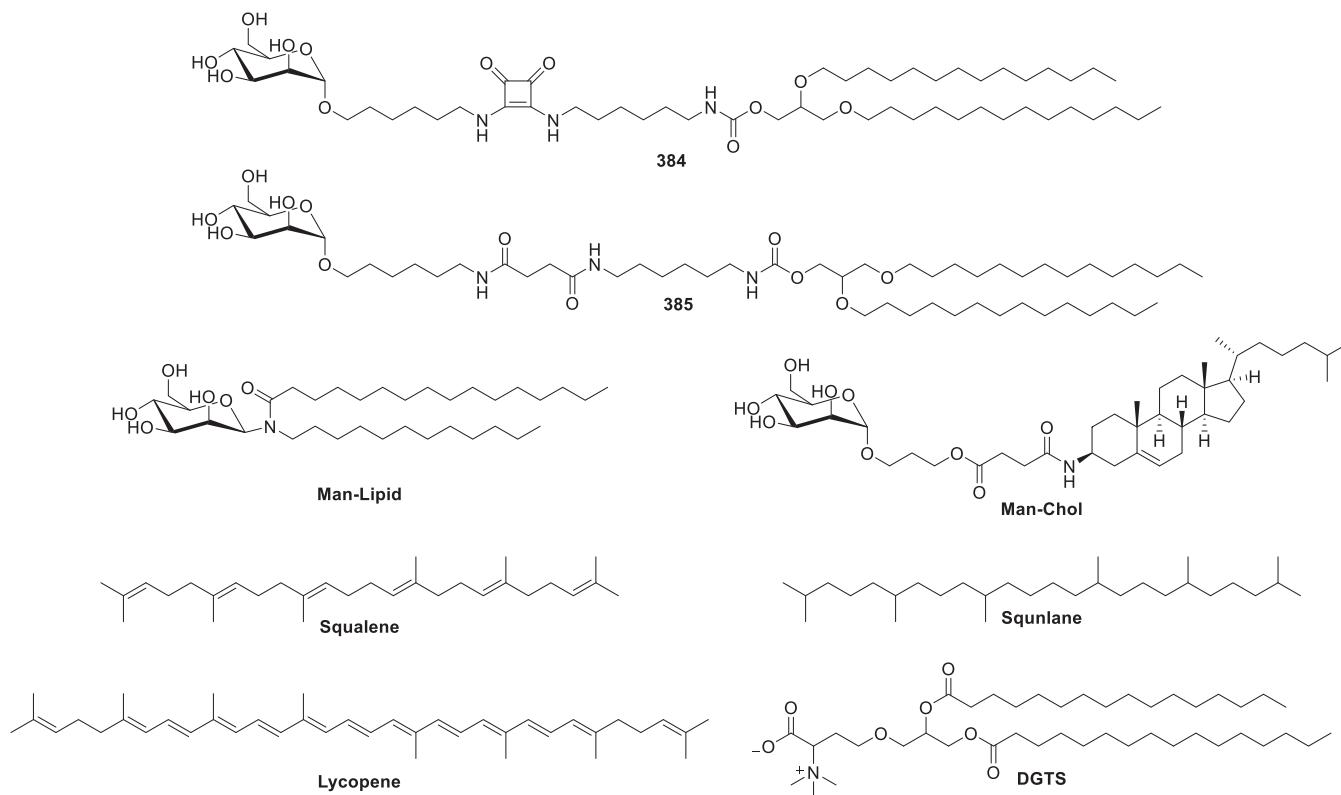
Figure 90. Chemical structures of other helper lipids for RNA delivery.

opolymers,[766] lipopeptides,[767] and lipoproteins.[768] Since each type of macromolecule contains a large number of compounds, we generally highlight these lipid-derived macromolecules with an emphasis on their diverse chemical structures.

4.1. Lipopolymers

Lipopolymers have been widely used as a class of RNA delivery materials for decades.[769,770] Lipopolymers are usually included in lipid nanoparticle formulations as auxiliary lipids or directly complexed with RNA molecules.[771−773] They generally have the functions of enhancing the stability of lipid nanoparticles, promoting cellular uptake, and targeting diseased sites, thereby improving the efficiency of RNA delivery.[774] From the perspective of chemical structures, lipopolymers are mainly composed of two parts: lipid fragments and polymer backbones. In this section, we mainly classify lipopolymers according to the types of polymer backbones including PEG-lipids, PEI-lipids, dendrimer-lipids, and other types of lipopolymers.

4.1.1. PEG-lipids. PEG-lipids are important components usually incorporated on the surface of LNPs.[195] PEG-lipids are composed of hydrophilic PEG conjugated to a hydrophobic alkyl chain through phosphate, glycerol, or other linkers. The PEG component can increase the stability of RNA-loaded LNPs and prolong their circulation time in the blood, thereby promoting the distribution and accumulation of nanoparticles in the diseased site.[775] In this part, PEG-lipids are divided into two major types: general PEG-lipids and functionalized PEG-lipids.

4.1.1.1. General PEG-lipids. PEG-lipids have been commonly applied in the construction of LNPs for drug delivery, such as DSPE-PEG$_{2000}$ as one of the important components of Doxil LNPs.[776,777] Later on, PEG-lipids are also used as an important ingredient of LNPs for RNA delivery.[778] PEG$_{2000}$-C-DMG is used as the PEG-lipid anchor in the Onpattro (patisiran) LNP

formulation, which is the first FDA approved LNP-based siRNA drug for the treatment of hereditary transthyretin (hTTR) amyloidosis.[779] Recently, DMG-PEG$_{2000}$ and ALC-0159 are respectively used as one of the components of the mRNA-1273 and BNT162b2 vaccines against the COVID-19 pandemic.[524] The PEG-lipids in the LNPs formulations of these three clinically used RNA drugs/vaccines have similar characteristics. These three PEG-lipids possess an mPEG$_{2000}$ component as the hydrophilic chains and two alkyl chains with 14 carbon chains in length.

Since PEG-lipids have a great influence on the properties of LNPs, many studies have been conducted to investigate the effects of PEG-lipid on RNA LNPs.[780,781] In 2008, Sonoke et al. synthesized several PEG-lipids containing acyl chains with 12 to 18 carbon atoms, which were used to prepare siRNA LNPs to investigate the influence of the alkyl chains length on LNPs.[782] Each PEG-lipid/siRNA complex was intravenously injected in mice. Four hours post administration, the plasma concentration of ^{3}H-labeled siRNA was over 10-fold higher in the group of C18-PEG$_{2000}$ than that of the PEG-lipid/siRNA complex formed by shorter acyl chains (e.g., C-12 to C-16). In 2013, Mui et al. found that PEG-lipids with long alkyl chains (e.g., C-18) had a longer time associated with the LNPs than PEG-lipids with short alkyl chains (e.g., C-14).[783] The dissociation of C-18 PEG-lipids from LNPs is approximately 0.2%/h in mice. Moreover, the molar ratio of PEG-lipid is another important factor.

In 2017, Oberli et al. found that the molecular weight of PEG (e.g., Mw: 350, 1000, 2000, 3000) and the length of the anchoring lipid (e.g., C-14, C-18) affected the particle size of LNPs (DMPE-PEG$_{2000}$: ~67 nm).[784] They applied DMPE-PEG$_{2000}$ to prepare LNPs loaded with mRNA encoding various antigens as potential cancer vaccines. In the same year, Zhu et al.
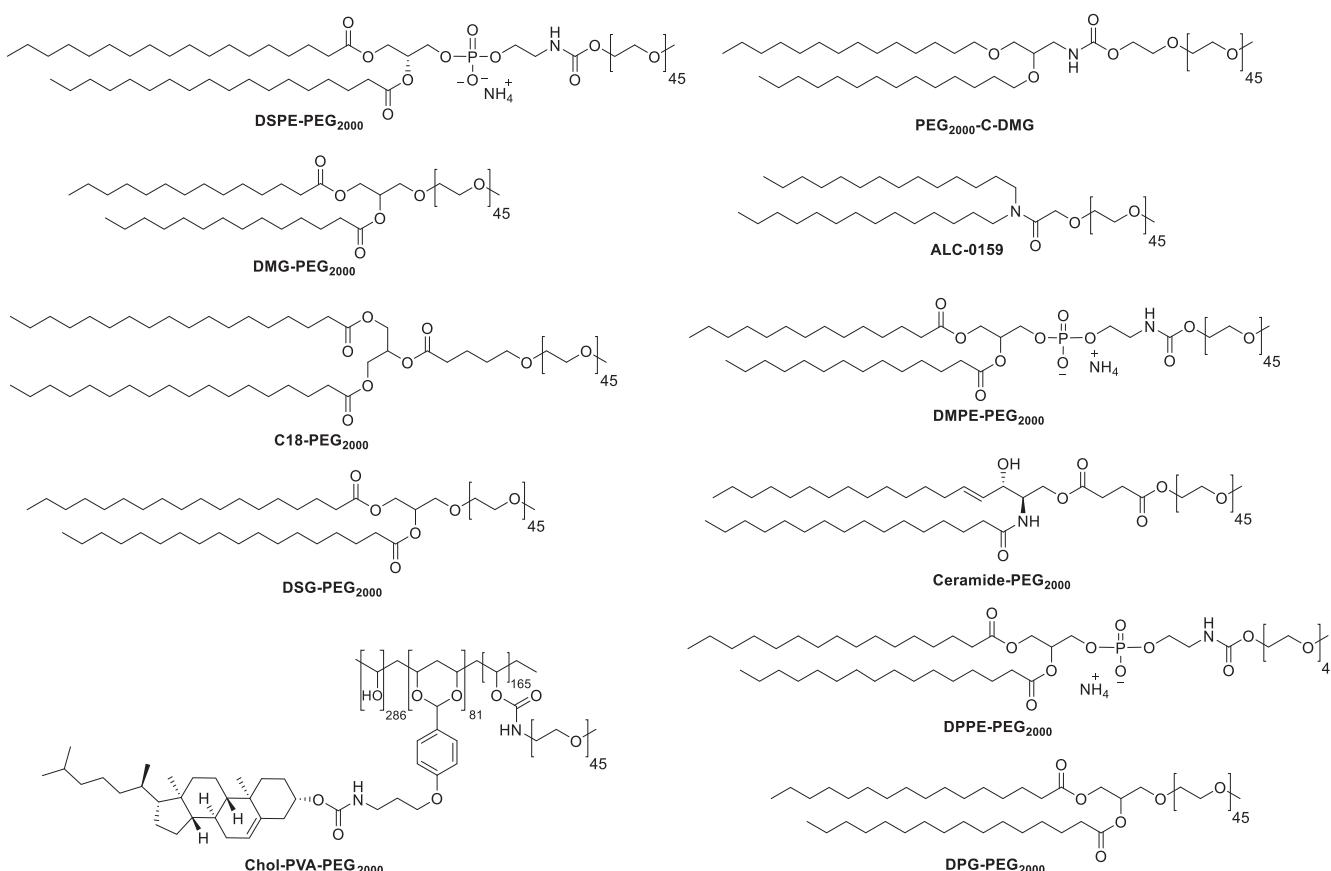
Figure 91. Chemical structures of PEG-lipids.

used the hybrid nanoparticle platform of a lipid-PEG shell and a PLGA/G0-C14 solid core to effectively achieve dePEGylation and control the delivery of siRNA.[785] They found that the length of the PEG-lipids alkyl chains has a great influence on the dissociation rate of PEG from LNPs, and the dissociation rate of PEG on long alkyl chain lipids (e.g., DSG-PEG and DSPE-PEG) is slower than that on short alkyl chains (e.g., DMG-PEG and DMPE-PEG). Meanwhile, the unsaturated alkyl chain accelerates the dissociation of PEG (ceramide-PEG vs DPG-PEG), while the neutral and anionic PEG-lipids (DPG-PEG vs DPPE-PEG; DSG-PEG vs DSPE-PEG) with the same alkyl chain showed similar dissociation kinetics.[785] This lipid−polymer hybrid RNA delivery system has also been used in delivering phosphatase and tensin homologue (PTEN) mRNA to restore tumor-growth suppression in mice.[786]

The preparation of acid-sensitive PEG-lipids is a method to improve the performance of LNPs. In 2012, Kulkarni et al. used the acid-sensitive benzylidene acetal bond to make Chol-PVA-PEG (Figure 91).[787] The formulated Chol-PVA-PEG/siRNA nanoparticles showed a gene silencing activity comparable to bPEI and Lipofectamine 2000.

4.1.1.2. Functionalized PEG-lipids. PEG can also be functionalized to incorporate specific functions such as cell targeting.[788,789] In 2008, Li et al. linked anisamide to DSPE-PEG and prepared DSPE-PEG-anisamide that can target the sigma receptor on the B16F10 cancer cells.[790] Then, DSPE-PEG-anisamide was used as a PEG-lipid to form a targeted LNPs-siRNA formulation. Compared to nontargeted LNPs, the targeted LNPs showed a 4-fold increase in cellular uptake in B16F10 cells. In 2010, Akinc et al. used targeting ligands

containing multivalent *N*-acetylgalactosamine (GalNAc) clusters and linked them with DSG-PEG to prepare DSG-PEG-GalNAc that can be targeted to the liver hepatocytes.[791] DSG-PEG-GalNAc, factor VII-targeting siRNA, and other components were formulated to LNPs, which were intravenously administered in mice at the siRNA doses of 0.022, 0.067, and 0.2 mg/kg. The LNPs-mediated factor VII in mice exhibited dose-dependent silencing. In 2016, Zang et al. conjugated anti-EphA10 antibody with Chol-SIB-PEG to prepare tumor-targeting Chol-SIB-PEG-Eph.[792] In MCF-7/ADR cells, MDR1 siRNA LNPs prepared with Chol-SIB-PEG-Eph reduced the expression level of MDR1 protein. Furthermore, the LNPs containing the fluorescent molecule DIR were injected into the MCF-7/ADR xenograft tumor mouse model for fluorescence imaging. After 24 h of injection, the Chol-SIB-PEG-Eph-formulated LNPs displayed the highest accumulation in the tumor compared with the control group.[792]

In 2017, Krzysztoń et al. applied DSPE-PEG-FA as a folate-targeted PEG-lipid to form LNPs for siRNA delivery.[793] These LNPs could significantly down-regulate luciferase activity compared with LNPs without a folate targeting group. In the same year, Santiwarangkool et al. synthesized DSG-PEG-GALA containing a polypeptide GALA that could target the lung endothelium for RNA delivery.[794] Compared with LNPs composed of DSG-PEG, LNPs composed of DSG-PEG-GALA showed higher silencing activity in the mouse lungs, with an ED_{50} value of 0.21 mg/kg. In 2020, Qiao et al. synthesized vitamin A-modified PEG-lipid Chol-PEG-VA for RNA delivery (Figure 92).[795] The cell uptake of Chol-PEG-VA containing LNPs was over 80% in HSC-T6. Compared with cells
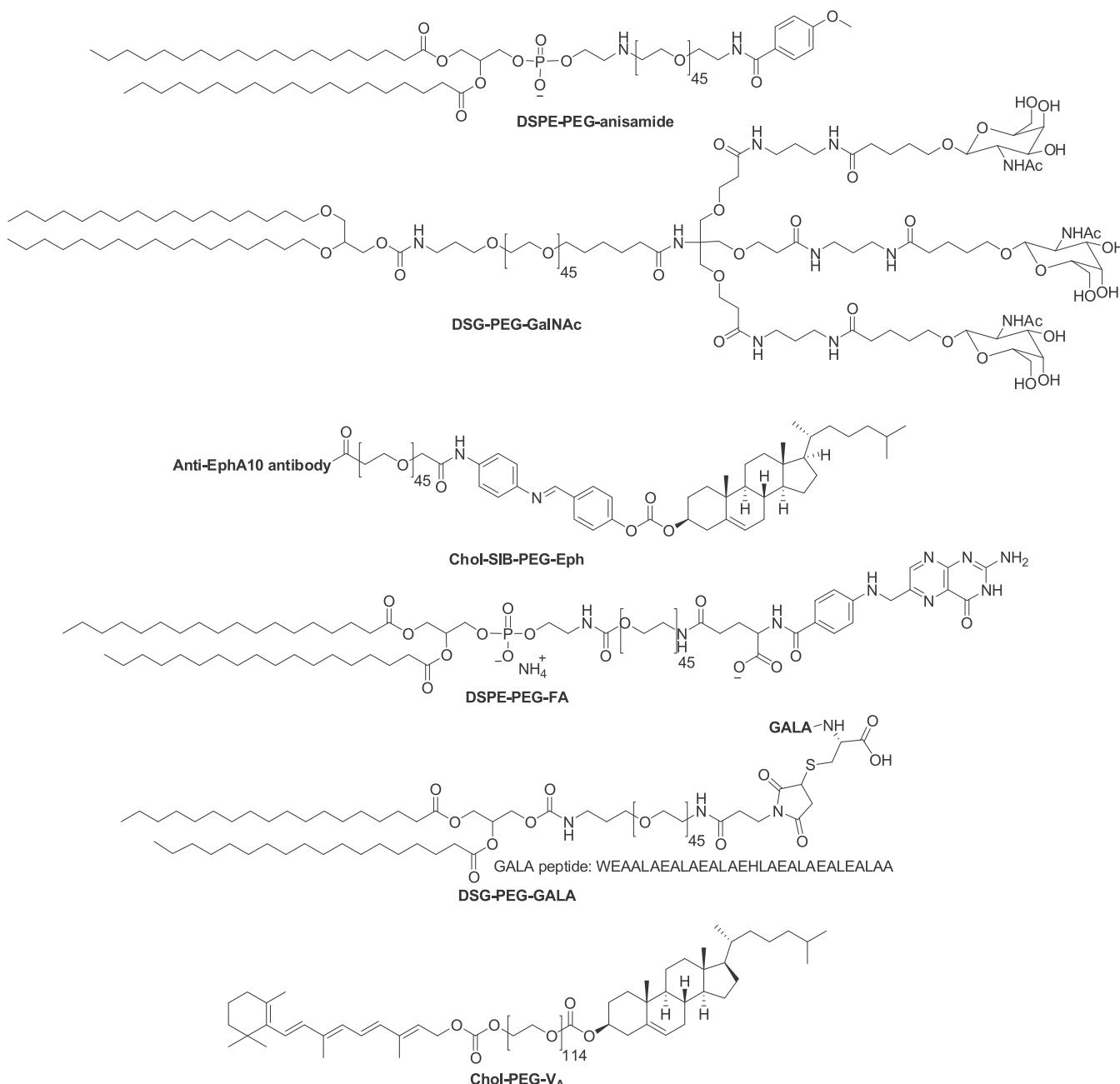
Figure 92. Chemical structures of functionalized PEG-lipids.

treated with single Col1α1 siRNA LNPs or TIMP-1 siRNA LNPs, double-siRNA nanoparticles Col1α1/TIMP-1 siRNA LNPs showed a higher inhibitory effect on collagen I accumulation. Among the various siRNA LNPs treatment groups, Col1α1/TIMP-1 siRNA LNPs treated mice led to the lowest collagen accumulation, which is similar to the level in normal mice.[795]

4.1.2. PEI-lipids. PEI is another class of polymers used in RNA delivery.[796,797] To improve the pharmaceutical properties of PEI, various lipids are conjugated with PEI.[798,799] In 2014, Dahlman et al. synthesized a library of PEI analogs with diverse structures by conjugating small polyamines with alkyl tails of different lengths in different molar ratios.[800] PEI-lipid **386** (Figure 93) was obtained by the epoxy-opening reaction between C15 epoxy-terminated lipid chains and PEI$_{600}$ (14:1 molar ratio) and screened from the *in vitro* cell assays after

formulation with PEG-lipid and siRNA. The lead material was able to silence multiple endothelial genes in mice.[800] In the same year, Navarro et al. combined DOPE with PEI to obtain the lipopolymer **387**.[801] The DOPE-PEI effectively delivered GFP siRNA in c166 cells stably expressing GFP.[801] In 2020, Fan et al. synthesized the Triton X-100-modified PEI (low molecular weight), which was then attached to dopamine grafted vitamin E (VEDA) and 4-carboxyphenylboronic acid (PBA) to obtain lipopolymer **388** (Figure 93).[802] 388-siEGFR complexes suppressed the protein expression of EGFR in A375 cells to 43.1% and reduced its EGFR mRNA level to 34.9% (Figure 93).

4.1.3. Dendrimer-lipids. Dendrimers consist of three main parts: a central core, a growing branch unit (called generation (G)), and a large number of terminal functional groups (usually amino groups) on the surface.[803] The terminal groups of the dendrimer can be lipid-modified to obtain the dendrimer-
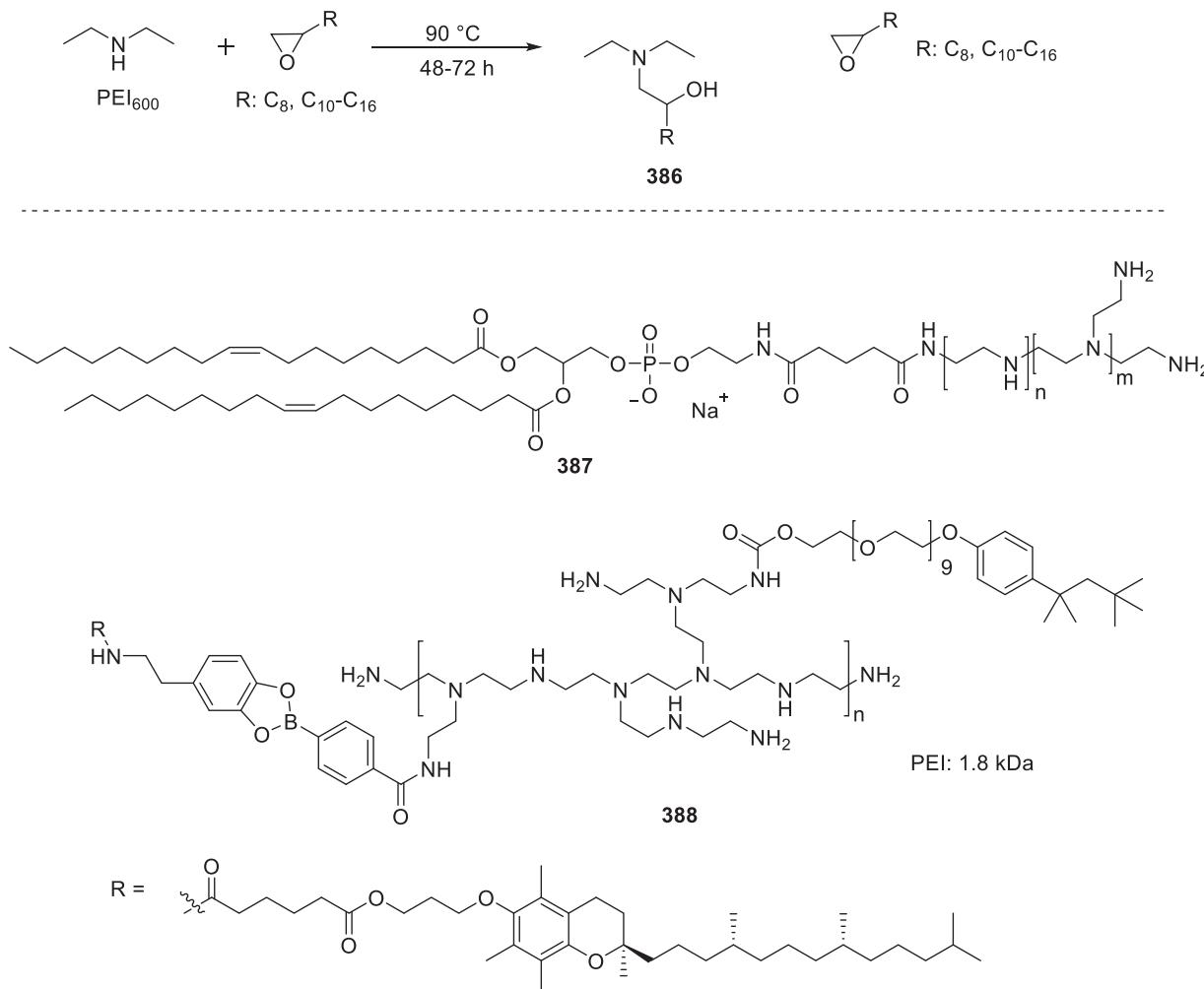
Figure 93. Chemical structures of PEI-lipids.

lipids.[804−806] In 2012, Yu et al. synthesized a class of dendrimer-lipids with different alkyl chain lengths and dendrimer generations through click chemistry.[807] The long alkyl chain of **389** (Figure 94) promotes the assembly of siRNA/carrier, can form a stable self-assembled complex with siRNA, and increases the stability of the complex through hydrophobic interactions. Moreover, the dendrimer-lipid **389**/siRNA complex significantly downregulated Hsp27 and induced in a mouse prostate cancer model.[807] In 2014, Khan et al. prepared LNPs with dendrimer-lipids **390** and **391** to deliver siRNA to the liver endothelium *in vivo* (Figure 94).[808] In a hepatocellular tumor model, the formulated **390** and **391** showed 51% and 92% knockdown of Alpha-fetoprotein (AFP), respectively (siRNA dose: 1 mg/kg). In 2015, they used terminal epoxides with different alkyl chain lengths to react with free amines in poly(propylenimine) and poly(amidoamine) dendrimers to obtain dendrimer-lipids **390−394** (Figure 94).[809] The nanoparticles of **393** loaded with Cy5.5-labeled siRNA showed greater fluorescence than other dendrimer-lipids in mouse lungs.

In 2016, Chahal et al. synthesized the modified dendrimer **395** and formulated it with PEG-lipids and self-amplifying RNA to form the nanoparticles (Figure 94).[773] These nanoparticles were able to deliver many types of self-amplifying RNAs encoding viral antigens such as Ebola virus, *Toxoplasma gondii*, and H1N1 influenza. After a single-dose vaccination, the nanoparticles-treated mice developed effective CD8 T cells and antibody responses against specific pathogens.[773]

In 2020, Xiong et al. synthesized six low-molecular-weight epoxy-derived fluorine-substituted dendrimers (**396−401**) by reacting fluorine-containing alkyl propylene oxide with PAMAM-G0 (Figure 94).[810] The formulation of dendrimer lipid **400** with Luc siRNA decreased over 50% of the luminescence signal at a siRNA concentration of 20 nM in HeLa-Luc cells. The siRNA targeting PHB1 (PHB1 siRNA) was also complexed with dendrimer lipid **400** to prepare the nanoparticles, which resulted in increased cell apoptosis rate in A549 lung carcinoma cells after treatment with this formulation.[810]

4.1.4. Other Types of Lipopolymers. There are many other types of lipopolymers such as lipocationic polyesters, brush-like lipopolymers with poly(glycoamidoamine) (PGAAs) as a backbone, and charge-altering releasable transporters (CARTs) lipopolymers.[811,812] In 2015, Hao et al. synthesized over one hundred lipocationic polyesters (**404**) using amino- or alkyl- valerolactone monomers (Figure 95).[813] Six of these lipocationic polyester-formulated nanoparticles enabled over 90% silencing at a siRNA concentration of 38.4 nM *in vitro*. One lead material showed effective siRNA delivery in a MDA-MB-231-Luc xenograft mouse tumor model.[813] In 2016, Dong et al. synthesized a series of lipopolymers (e.g., TarN3C10) through the reactions of poly(glycoamidoamine) (PGAAs) and epoxides
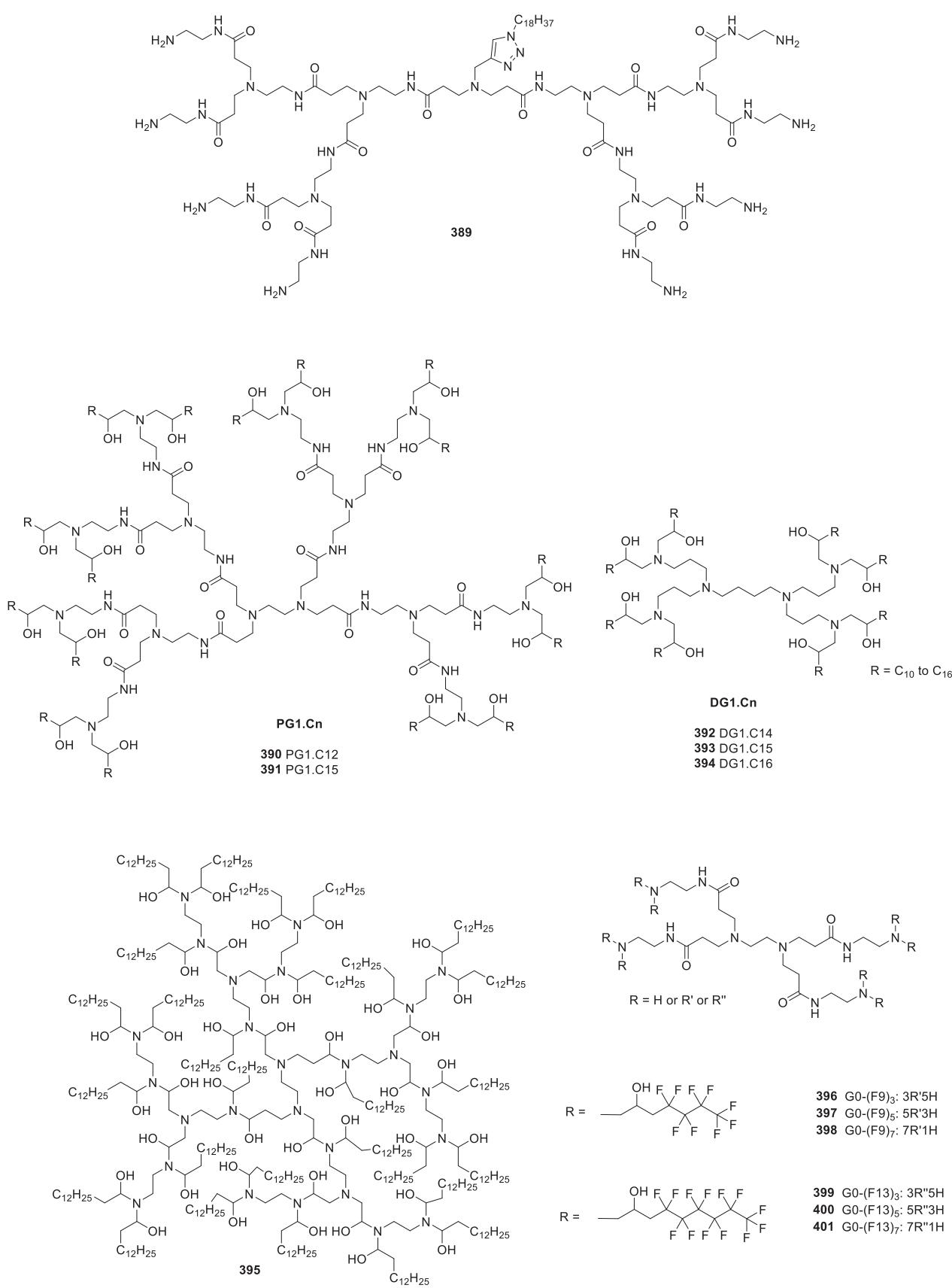
Figure 94. Chemical structures of dendrimer-lipids.

(Figure 95).[814] A number of the formulated PGAAs-lipids silenced over 50% of the FVII production.[814] In 2017, Luo et al.

continued to improve the brush lipopolymer based on the PGAA backbone and synthesized three additional PGAA lipid

Lipocationic Polyesters
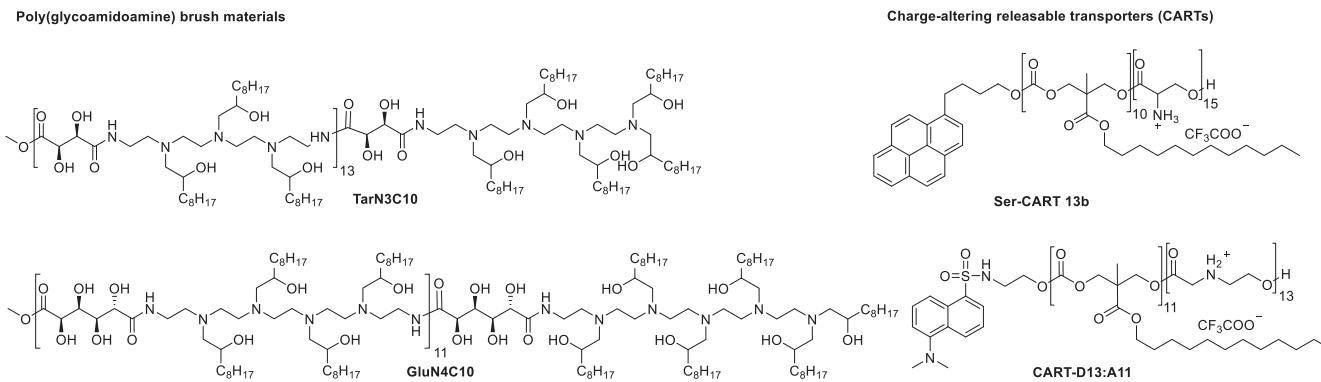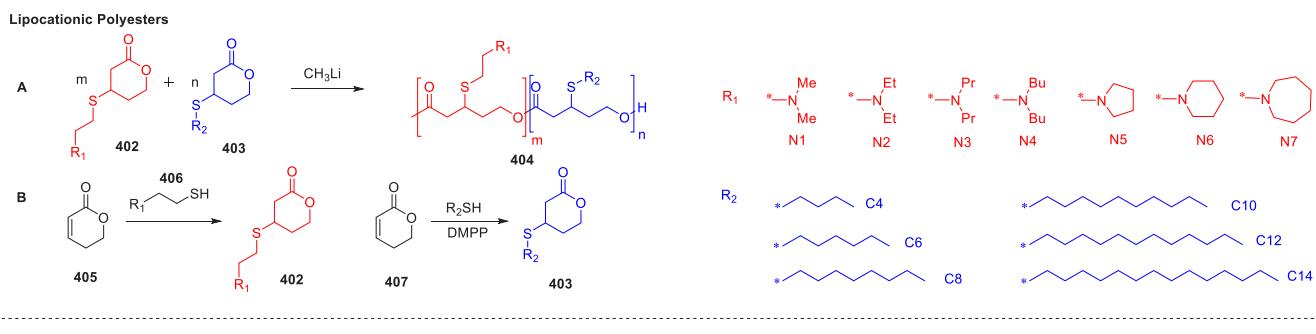
Figure 95. Chemical structures of other types of lipopolymers.
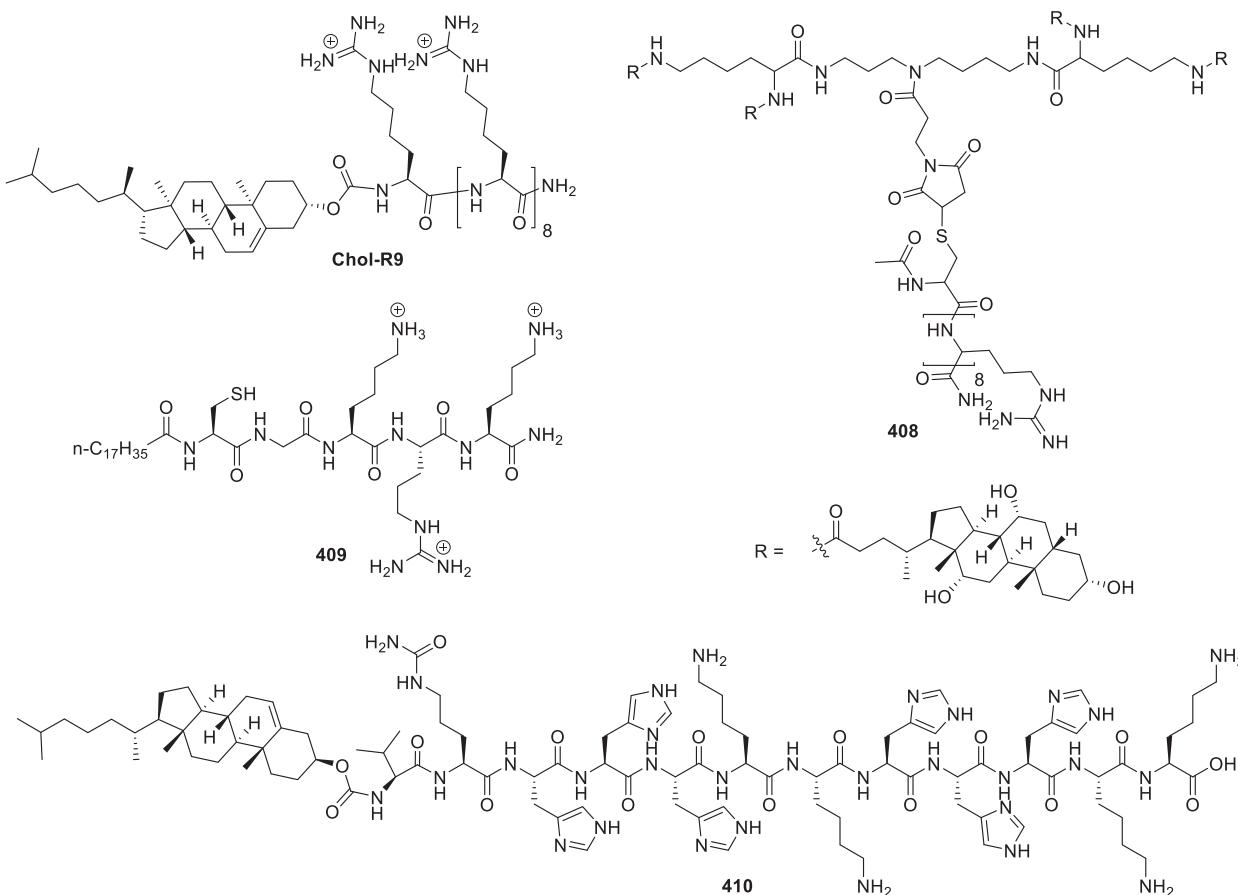
Figure 96. Chemical structures of lipopeptides.

derivatives.[815] The GluN4C10 nanoparticles showed more effective gene silencing of FVII compared to the TarN3C10 nanoparticles. In the same year, McKinlay et al. synthesized

several amphiphilic charge-altering releasable transporters (CARTs) for RNA delivery (Figure 95).[816] Among the different CARTs, CART-D13:A11 showed effective mRNA delivery in

BALB/c mice. Later on, Benner et al. further developed Ser-CART 13b and evaluated its mRNA delivery via intramuscular or intravenous (*iv*) injection in mice.[811]

4.2. Lipopeptides

Lipopeptides possess the properties of both lipids and peptides.[817−819] Lipopeptides not only have biological functions such as targeting effects but also take advantage of lipid features such as hydrophobic interactions.[820,821] In 2006, Kim et al. conjugated oligoarginine with cholesteryl chloroformate to produce the oligomer Chol-R9 (Figure 96).[822] The complex of Chol-R9 and siRNA against VEGF inhibited tumor growth after intratumoral injections in a CT-26 mouse tumor model. In 2011, Cline et al. conjugated cholic acid, spermidine, and lysine to prepare diwalled and tetrawalled umbrella compounds (Figure 96).[823] Then, these umbrella compounds were reacted with the octaarginine peptide to obtain a variety of lipopeptides. Lipopeptide **408**/siRNA complex showed similar silencing activity to that of Lipofectamine 2000.[823] In 2017, Sharma et al. modified CGKRK, a tumor targeting peptide, with saturated and unsaturated fatty acids to synthesize the lipopeptides including **409**.[824] The oleic acid-CGKRK lipopeptide is a lead material for siRNA delivery found in cell studies. In 2020, Zhao et al. formulated cholesterol peptide (CP, **410**, Figure 96), cabazitaxel, IKBKE siRNA, and cholesterol-hyaluronic acid (CHA) to assemble siRNA nanocomplex (CHA/CP/siRNA/cabazitaxel).[817] This formulation could target CD44 overexpressed on triple-negative breast cancer (TNBC) cells. Moreover, this nanocomplex could codeliver IKBKE siRNA and cabazitaxel to TNBC cells, which significantly inhibited tumor growth in an orthotopic MDA-MB-231 TNBC mouse mode.[817]

4.3. Lipoproteins

Lipoproteins are endogenous nanocomplexes in the human body, which can be divided into chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) based on their density.[825,826] Lipoproteins serve as natural transporters for proteins, vitamins, and hormones. Lipoprotein particles are composed of surface components (apolipoproteins, phospholipids, and cholesterol) and core components (cholesterol esters and triglycerides).[827−829] Researchers have applied lipoproteins to deliver small molecular drugs and nucleic acids for therapeutic applications.[711,830−832] For example, Yang et al. prepared an HDL-mimicking peptide−phospholipid scaffold (HPPS) by integrating amphipathic α-helical peptides, cholesteryl oleate, and phospholipids; HPPS loaded with cholesterol-modified bcl-2 siRNA greatly reduced the expression of bcl-2 in KB cells.[833] Based on this study, Cruz et al. constructed HPPS encapsulating siRNA against spalt-like transcription factor 4 (SALL4).[834] The HPPS-siRNA nanoparticles showed significant inhibition of tumor growth in a hepatocellular carcinoma mouse model. In another study, McMahon et al. formulated templated lipoprotein particles (TLP) using gold nanoparticle, ApoA-I, two phospholipids, and cholesterol, which were then complexed with the DOTAP−RNA mixture to produce siRNA-TLP.[835] These siRNA-TLP nanoparticles targeting the androgen receptor (AR) significantly inhibited tumor growth in a LNCaP xenograft mouse tumor model. In 2017, Huang et al. applied calcium phosphate, phospholipids, apolipoprotein E3, and siRNA to formulate siRNA-CaP-rHDL nanoparticles.[836] These siRNA-CaP-rHDL promote the penetration of the blood−brain barrier and deliver siRNA to glioblastoma cells. In a C6 glioblastoma mouse model, the nanoparticles loaded with siRNA against activating transcription factor-5 (ATF5) reduced the ATF5 protein level and improved the mouse survival time. Recently, Jiang et al. developed SLNP, a lipoprotein-like nanoparticle using multiple components including calcium phosphate, phospholipids, stromal cell-derived factor 1 (SDF1) mimic peptides, and apolipoprotein E (apoE).[829] These SLNP nanoparticles effectively delivered miR34a to glioma initiating cells (GICs) and extended overall survival in a patient-derived GICs glioma mouse model.

5. CONCLUSIONS AND OUTLOOK

Synthetic chemists and material scientists have spent over three decades on developing novel structures and formulations of lipids, lipid derivatives, and lipid-derived macromolecules for RNA delivery. With a better understanding of structural and biophysical properties for effective RNA delivery as well as the underlying mechanisms involved in the cellular uptake and endosomal release of the nanoparticles, more and more lipid-based RNA delivery systems are in different stages of clinical trials. In particular, MC3, ALC-0315, and SM-102-based lipid nanoparticles have been approved by the FDA and EMA for clinical use. These significant advances open up numerous opportunities for lipids and lipid derivatives and their related RNA-based therapeutics. (i) New chemical structures of lipids and lipid derivatives can be conceived and synthesized based on the current knowledge of lipid components such as cationic or ionizable head groups, linker groups, and hydrophobic tails. Many types of helper lipids and lipopolymers can be incorporated into nanoparticle formulations. (ii) A large number of chemical reactions such as Michael addition, epoxide-ring-opening, reductive amination, click chemistry, or one-pot multicomponent reactions permit the rapid synthesis of libraries of lipids with diversified chemical structures, paving a way to evaluate the structure−activity relationship. Incorporation of some biocompatible or targeting ligands, such as mannose, cell penetrating peptides, aptamers, or vitamins into the head group domains of cationic/ionizable lipids can improve RNA delivery efficiency with higher biocompatibility and lower immunogenicity. (iii) Detailed studies on LNPs properties such as physicochemical properties, enzymatic stability, morphology, and shelf life are needed. These studies will expand our understanding of the features of LNPs and provide useful design criteria for future LNPs development. (iv) Pharmaceutical properties of LNPs such as pharmacokinetics, pharmacodynamics, immunogenicity, and safety should also be carefully characterized. Knowledge obtained from these studies will not only elucidate the LNPs characteristics and their interactions with the host but also benefit the development of a wide variety of LNPs. (v) LNP-formulated RNA therapeutics can be broadly applicable to diverse diseases. Current clinical trials are investigating a series of LNPs-RNA candidates in cancer immunotherapy, protein replacement therapy, and gene editing. Importantly, LNPs-RNA formulations can potentially overcome "non-druggable" targets to treat diseases that cannot be addressed with current medicines, such as neurodegenerative disorders and genetic diseases. In summary, with the increasing chemical diversity and formulation number of lipids and lipid derivatives, more and more RNA-based therapeutics are translating from bench to bedside and improving the quality of life.

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Notes

The authors declare the following competing financial interest(s): Y.D. is a scientific advisory board member of Oncorus Inc and serves as a consultant of Rubius Therapeutics.

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Katarina E. Jankovic is a second-year undergraduate honors student in the program of neuroscience at The Ohio State University. She is an undergraduate researcher in the Dong group. Her research interests include cell specific drug delivery systems with a focus on mRNA therapeutics.

Yizhou Dong is an Associate Professor in the Division of Pharmaceutics and Pharmacology, College of Pharmacy at The Ohio State University. He received his B.S. in pharmaceutical sciences from Peking University, Health Science Center, and his M.S. in organic chemistry from Shanghai Institute of Organic Chemistry. In 2009, he received his Ph.D. degree in pharmaceutical sciences from the University of North Carolina at Chapel Hill (UNC-CH) under the supervision of Professor K.-H. Lee. From 2010 to 2014, he was a postdoctoral fellow in the laboratory of Professors Robert Langer and Daniel Anderson at Harvard Medical School and Massachusetts Institute of Technology. His research focuses on the design and development of nanomaterial and biotechnology platforms for the treatment of genetic disorders, infectious diseases, and cancers. He has authored over 70 papers and patents. Several of his inventions have been licensed and are currently under development as drug candidates for clinical trials. He serves as a board member of the Gene Delivery and Editing (GDE) Focus Group of the Controlled Release Society and an editorial board member for *Bioactive Materials*. He is a scientific advisory board member of Oncorus Inc. He is the recipient of Young Innovator in Cellular and Molecular Bioengineering from the Biomedical Engineering Society, Maximizing Investigators' Research Award from the National Institute of General Medical Sciences (NIGMS), Ohio State Early Career Innovator of the Year, and American Association of Pharmaceutical Scientists (AAPS) Emerging Leader Award.

ABBREVIATIONS

AML	acute myeloid leukemia
APC	antigen-presenting cell
ApoB	apolipoprotein B
ApoE	apolipoprotein E
ASGPR	asialoglycoprotein receptor
ASO	antisense oligonucleotides
BBB	blood−brain barrier
Boc	*tert*-butyloxycarbonyl
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
CAR-T cell	chimeric antigen receptor T cell
Cas9	CRISPR associated protein 9
Cbz	benzyloxycarbonyl
CD	circular dichroism
CDI	*N,N'*-carbonyldiimidazole
cirRNA	circular RNA
CME	clathrin-mediated endocytosis
CMC	critical micelle concentration
CNE	cationic nanoemulsion
COVID-19	coronavirus disease 2019

CRISPR	clusters of regularly interspaced short palindromic repeats
CPP	cell penetrating peptide
CTL	cytotoxic T lymphocyte
CvME	caveolae-mediated endocytosis
DC	dendritic cell
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	*N,N'*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DOPE	1,2-dioleoyl-*sn*-glycerol-3-phosphatidylethanol-amine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
dsRNA	double-stranded RNA
EC	endothelial cell
EVs	extracellular vesicles
ECM	extracellular matrix
ED$_{50}$	median effective dose
EDCI	1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide
EGFP	enhanced green fluorescent protein
ePC	ethylphosphatidylcholine
EUA	emergency use authorization
FIND	Fast Identification of Nanoparticle Delivery
FVII	factor VII
FXN	frataxin
GalNAc	*N*-acetylgalactosamine
GHS	glutathione
HA	hemagglutinin
HAI	hemagglutination inhibition
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
hCSF	human cerebrospinal fluid
HDL	high density lipoprotein
HMPT	hexamethylphosphorus triamide
IDL	intermediate density lipoproteins
IFN	interferon
LDL	low density lipoprotein
LNP	lipid nanoparticles
LPHNP	lipid−polymer hybrid nanoparticle
Luc	luciferase
MHC I	major histocompatibility complex I
MHC II	major histocompatibility complex II
miRNA	micro RNA
MOG	monooleoyl glycerol
mRNA	mRNA
MTO	mitoxantrone
ncRNA	noncoding RNA
NHP	nonhuman primate
NLE	neutral lipid emulsion
OVA	ovalbumin
HIV	human immunodeficiency virus
Pal	palmitoleyl
PBA	phenylboronic acid
pBAE	poly(*β*-amino ester)
pDNA	plasmid DNA
PEG	polyethylene glycol
PEI	poly(ethylenimine)
Ka	acidity constant
PLGA	poly(lactic-*co*-glycolic acid)
PLL	poly-L-lysine
PPTS	*p*-toluenesulfonate

PMP	platelet-derived microparticle
RBC	red blood cell
RBD	receptor binding domain
RISC	RNA-induced silencing complex
RNase	ribonucleases
RNAi	RNA interference
RVG	rabies virus glycoprotein
SA	sialic acid
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
scFv	single-chain variable fragment
siRNA	small interfering RNA
sgRNA	single-guide RNA
SORT	selective organ targeting
SR-SAXS	small-angle X-ray scattering
STING	stimulator of interferon genes
TLP	templated lipoprotein particle
TAA	tumor associated antigen
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TTR	transthyretin
VLDL	very low density lipoprotein

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on July 19, 2021, with an error in Table 3. The corrected version was reposted July 22, 2021.