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Biodegradable Cationic and Ionizable Cationic Lipids: A Roadmap for Safer Pharmaceutical Excipients

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Cationic and ionizable cationic lipids are broadly applied as auxiliary agents, but their use is associated with adverse effects. If these excipients are rapidly degraded to endogenously occurring metabolites such as amino acids and fatty acids, their toxic potential can be minimized. So far, synthesized and evaluated biodegradable cationic and ionizable cationic lipids already showed promising results in terms of functionality and safety. Within this review, an overview about the different types of such biodegradable lipids, the available building blocks, their synthesis and cleavage by endogenous enzymes is provided. Moreover, the relationship between the structure of the lipids and their toxicity is described. Their application in drug delivery systems is critically discussed and placed in context with the lead compounds used in mRNA vaccines. Moreover, their use as preservatives is reviewed, guidance for their design is provided, and an outlook on future developments is given.

1. Introduction

Cationic and ionizable cationic lipids are small amphiphilic molecules that are valuable auxiliary agents for a wide range of pharmaceutical applications. As they form lipophilic complexes with anionic therapeutic agents like nucleic acids, anionic small molecules, peptides, proteins, and heparins, they are utilized to improve cellular membrane permeability of these drugs. Current prominent examples are the ionizable cationic lipids ALC-0315 [[(4-hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate))] and Lipid H (SM-102) (9-heptadecanyl 8-{(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino}octanoate) that are used for coronavirus disease 2019 (COVID-19) mRNA vaccines BNT 162b and mRNA-1273, respectively.[1] Furthermore, cationic lipids like benzalkonium chloride and cetrimide are broadly employed as preservatives and antiseptics because of their antimicrobial properties.[2]

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The use of cationic and ionizable cationic lipids in pharmaceutical products, however, is a double-edged sword, as these excipients are of considerable safety concerns. Because of their permanent or pH-dependent cationic nature, they perturbate cellular and nuclear membranes, trigger the release of degrading enzymes from lysosomes, cause mitochondrial permeabilization and dysfunction, generate reactive oxygen species (ROS), alter cytoplasmatic enzyme functions, and damage DNA.[3] To address this substantial shortcoming of cationic and ionizable cationic lipids, biodegradable alternatives have been introduced that are rapidly degraded in vivo to preferably endogenous metabolites. The design of such lipids is inspired by natural cationic or ionizable cationic compounds like arginine, lysine or betaine that are generally regarded as safe. Their conjugation to endogenous lipids like fatty acids or cholesterol results in amphiphilic lipids. As ester and amide bonds are cleaved in vivo by numerous enzymes such as lipases, esterases, and proteases, they are the preferred linkages between these natural building blocks.

Since the FDA approved ethyl Nα-lauroyl-L-arginate as biodegradable food preservative being effective against a broad range of Gram-positive and Gram-negative bacteria, yeasts, and molds in 2005,[4] the potential use of biodegradable cationic and ionizable cationic lipids as pharmaceutical excipients has been evaluated by numerous research groups. As these excipients exhibit the same properties as their non-biodegradable counterparts but causing relatively low adverse effects, they will likely substitute currently used non-biodegradable lipids in the future. Within this review, we provide an overview on the different types of biodegradable cationic and ionizable cationic lipids, their synthesis and cleavage by endogenous enzymes. Applications in drug delivery systems and as antimicrobial agents are discussed. A guideline on their design and application is provided and an outlook on future developments is given.

2. Building Blocks and Formation of Cationic and Ionizable Cationic Lipids

Generally, biodegradable cationic and ionizable cationic lipids are composed of biocompatible building blocks that are conjugated via a linkage such as an ester or amide bond.[5] Representative building blocks and linkages are depicted in **Figure 1**. Endogenous enzymes can break these linkages and degrade

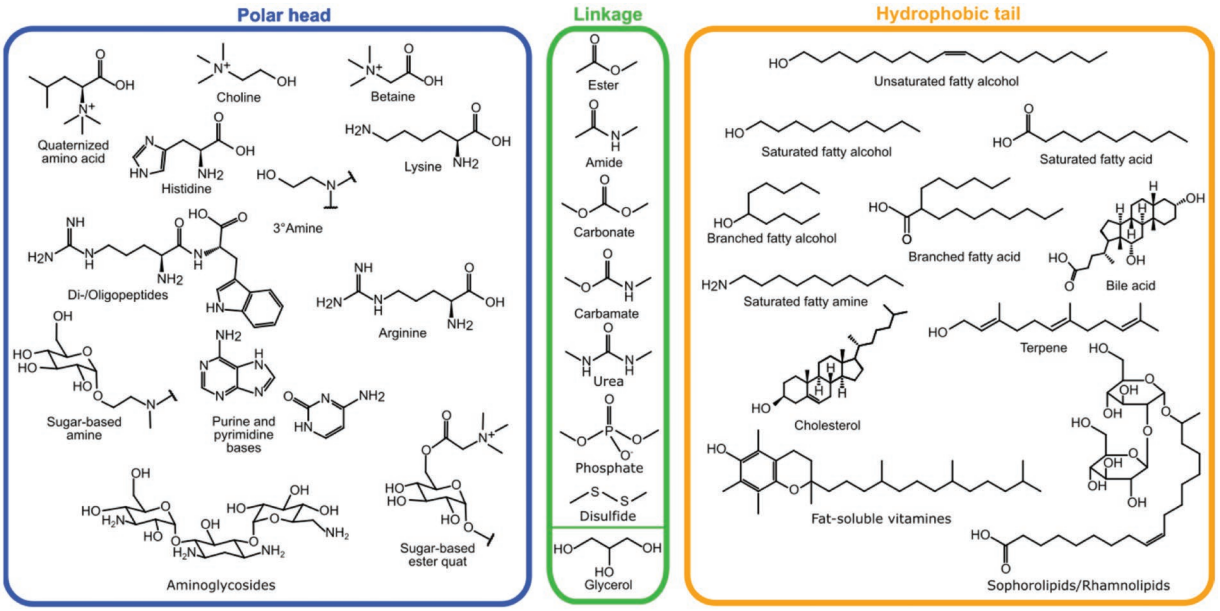


Figure 1. Representative building blocks of biodegradable cationic and ionizable cationic lipids.

biodegradable lipids into nontoxic products that are in most cases the synthetic precursors.[6] For a holistic design in terms of biocompatibility and sustainability, compounds from natural sources or industrial waste products, eco-friendly reactants and reusable biological catalysts are favorable.[5c,7] For more detailed insights into conventional syntheses and green chemistry approaches for the preparation of biodegradable cationic and ionizable cationic lipids, the reader is referred to recent review articles.[8]

2.1. Polar Head

The polar head of biodegradable cationic and ionizable cationic lipids carries either a permanently charged quaternary ammonium group or an ionizable moiety with an acid dissociation constant (pKa) that provides protonation at physiological pH. The terms "cationic," "ionizable cationic," and "ionizable" are often used synonymously. There are, however, significant differences between a permanently charged cationic head and a pH-sensitive ionizable cationic head regarding function, toxicity, and applications. In this review, permanently charged heads are, therefore, referred to as "cationic," whereas pH-sensitive polar heads are referred to as "ionizable cationic."

The basic amino acids arginine, lysine, and histidine are the most common building blocks as they are endogenous, nontoxic, and cheap. The guanidine, primary amine, and imidazolium groups, respectively, allow to choose from cationizable moieties with various characteristics. Esterification or amidation reactions are typically conducted to conjugate a hydrophobic tail to amino acids. This requires a protection of the carboxylate group in case of amidation or a decreased reactivity of the amine, for instance, by pH adjustment, in case of esterification.[9] However, such reaction protocols imply harmful chemicals and drastic conditions. Moreover, the presence of hazardous by-products, fairly low yields, and high production costs make such synthesis routes unfavorable.[5c,6a,8b,c,10] To overcome these limitations, biocatalytic production has gained increasing interest. Such green synthesis approaches mediated by immobilized lipases and proteases allow recovery of the catalyst and facilitate the purification of the product.[5c,7b]

Although microbial production is a well-established method for several surfactants like rhamnolipids and sophorolipids, no cationic lipids from microbial sources are described yet.[11] Semisynthetic approaches to modify compounds of microbial source are a useful extension to biocatalytic approaches. Aminoglycosides, which are typically derived from fermentation, have been used as polar head for lipopeptides to strengthen their antimicrobial profile but also to repurpose them as polar heads for gene delivery.[12] The presence of several hydroxy and amine groups allows the application of various synthesis routes like esterification, amidation, epoxide ring opening, and Michael addition but may raise concerns in terms of specificity.[13] Moreover, possible degradation into the aminoglycoside precursor may promote the emergence of resistant bacteria.

Di- and oligopeptides of L- and D-amino acids mimicking antimicrobial peptides (AMPs) can be acylated via the same pathways as monomeric amino acids. Polar heads, which consist of multiple amino acids that are not present in nature, are predominantly derived from solid phase synthesis using Fmoc chemistry.[14] Nonbasic amino acids are commonly quaternized in addition to esterification or amidation. Alkyl halides that predominantly originate from petrochemical sources are highly reactive building blocks for this purpose.[15] However, the resulting products cannot be considered as biocompatible anymore and yet, it has not been precluded whether these compounds bioaccumulate in a similar way as quats. On the contrary, betaine, choline, and carnitine surfactants carry a quaternary ammonium group but allow cleavage into natural or endogenous compounds.[7a,16] Consequently, these surfactants

can combine the advantages of a permanent cationic charge and biocompatibility.

Cationic and ionizable cationic sugar-based surfactants exhibit structural similarities to aminoglycoside-based ones and therefore, similar concerns regarding specificity during synthesis may arise.[17] To obtain ionizable cationic sugar-based surfactants, an amination between the sugar and a fatty amine is most frequently carried out, yielding an ionizable amine or, after alkylation, a cationic quaternary ammonium. Moreover, one of the hydroxy groups can be modified to an ester bond. In this case, the acid component is typically introducing the cationic or ionizable cationic group in proximity to the ester yielding an ester quat. Despite the abundant availability from renewable sources and high biocompatibility of sugars, the dependence on toxic solvents and low yields are limiting the use of such sugar-based surfactants.[18]

Tertiary amines are a vital part of the ionizable cationic lipids used for mRNA vaccines.[19] However, they are neither synthesized from endogenous or natural structures nor are they degraded into typical biocompatible building blocks. The cleavage of fatty acids from currently used ionizable cationic lipids renders these auxiliary agents per definition "biodegradable." Since synthetic ionizable cationic lipid building blocks remain after this cleavage, as discussed in more detail in Section 3.2., this type of biodegradation does not improve their safety. The synthesis of these auxiliary agents follows distinctly different pathways than for other biodegradable lipids. Synthesis protocols were established under the premise that large libraries can easily be built up.[20] Common approaches are Michael addition,[21] epoxide ring opening,[22] alkylation of amines,[23] as well as thiol–ene[24] and copper mediated click reactions.[25] The syntheses themselves do not introduce a cleavable linkage, so that the building blocks must contain one to provide biodegradability. Despite the large libraries established in academia, manufacturing companies experienced difficulties when moving to mass production as the large-scale synthesis is tedious and requires specialized expertise.[26] Consequently, the ionizable cationic lipid is by far the most expensive excipient used in mRNA vaccines.[27]

2.2. Linkage

In general, the type of linkage is governed by the precursors and the synthesis protocol but is commonly rendered with respect to biodegradability.[28] To date, the role of linkages in terms of functionality is investigated to a limited extent. However, the impact of linkage type and orientation on application-relevant properties like biodistribution has already been addressed in recent research and may become of higher interest in the future design of biodegradable ionizable cationic lipids.[28,29] Esters and amides are the predominant linkages as numerous well-established synthesis protocols are available and the degradation by endogenous enzymes is mostly provided. Further, carbamates are present in the lipids 3β[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and N4-cholesteryl-spermine (GL67) that are frequently used for in vitro transfection.[30] Other linkages are used to a rather low extent. However, also for these linkage groups biodegradability as well as synthesis from endogenous and safe compounds are provided.[31] Moreover, glycerol can be utilized as linker because it can serve as backbone to connect for example two hydrophobic tails to a polar head group via esterification.[32]

2.3. Hydrophobic Tail

The hydrophobic tail allows to tune hydrophobicity, geometry, and self-assembly of a surfactant. Saturated straight-chain alkyl tails are the predominant hydrophobic tail structure. A fatty amine, alcohol or acid can be used as educt, depending on the respective structure of the head and the desired linkage. Unlike many fatty acids that are derived from renewable fats, the major share of fatty alcohols is derived from petrochemical sources. However, current developments point toward an increase of naturally derived fatty alcohols.[33] While vegetable fats are mostly composed of straight-chain alkyl residues, terpenes provide a high variety of branched tails from biomass feedstocks.[33a,34] Branching of the alkyl tail significantly alters the surfactant geometry and plays a key role in the design of the functional lipids used in nucleic acid therapeutics.

In addition, glycolipids like sophorolipids and rhamnolipids offer a valuable addition to the available tail structures. Such biosurfactants can be produced in large quantities, using bacterial strains or yeasts. Introduction of a cationic or an ionizable cationic group allows to strengthen intrinsic microbial properties or give rise to new applications like gene delivery. The conjugation of basic amino acids has already been applied by conjugating the carboxylate moiety via an ester or amide bond in the hydrophobic tail of the biosurfactant but yet only via conventional synthesis routes.[35] As the carbohydrate structure remains, the resulting surfactant carries two polar domains.

Moreover, fat-soluble vitamins, bile acids, and cholesterol provide endogenous structures with bulky or branched alkyl residues.[6a,36] Steroid structures are mostly conjugated at the C3-positioned hydroxyl group because of its lower steric hindrance in comparison with other available modification sites. Despite their complexity and rigidity, steroid structures can be selectively acylated, using enzymes like lipases.[37] Rigidity and hydrophilic regions within the hydrophobic structure distinguish these tails from alkyl and alkenyl structures.[38] Moreover, their physiological role implies intrinsic functional properties like an active uptake in the intestine that can be of substantial advantage with regards to their application.

3. Biodegradation

3.1. In Vitro Degradation

In vitro degradation studies of cationic and ionizable cationic lipids by using various isolated enzymes such as lipases and esterases with a broad substrate scope are well described in the literature. The latter hydrolyze ester, amide, and thioester-bearing compounds in aqueous media. Preferentially, they cleave ester bonds of short chain fatty acids, giving rise to alcohols and acids.[39]

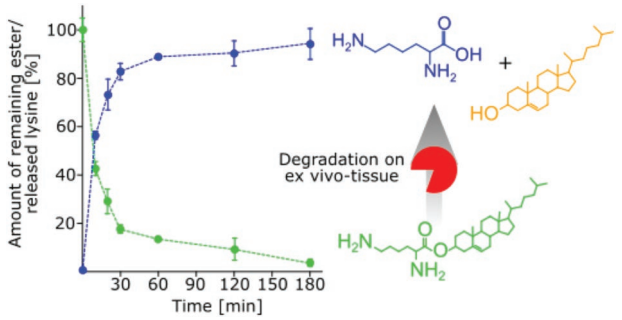


Figure 2. Cleavage of cholesteryl lysinate into nontoxic building blocks during incubation with porcine intestinal mucosa by brush boarder membrane bound enzymes. Results adapted with permission.[6a] Copyright 2020, Elsevier.

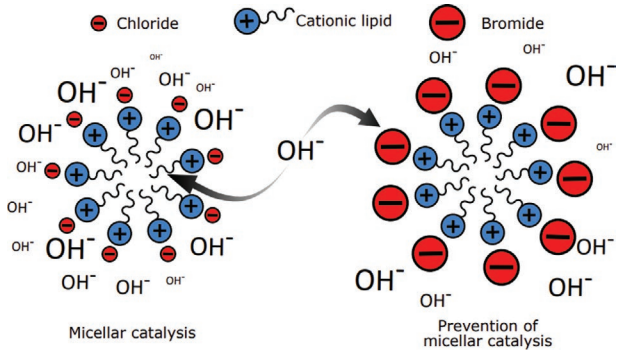


Figure 3. Schematic illustration of counterion-dependent micellar catalysis.

The water-insoluble lipases are the most common lipid hydrolyzing enzymes in the human body. They are predominantly present in pancreatic secretions, breaking down long-chain triglycerides into free fatty acids and glycerol. Apart from these enzymes, more specific serine proteases such as trypsin cleaving proteins at the carboxyl side of the amino acids lysine or arginine, as well as the butyrylcholinesterase (BChE) are involved in the degradation of cationic and ionizable cationic lipids.[6a,40] The degradation by trypsin has recently been exploited for ionizable cationic lipids based on the amino acids lysine and arginine.[5d,6a,40c,41] Furthermore, cell membrane bound enzymes seem to contribute to the degradation of biodegradable lipids.[5d,6a] Lysine esters were also cleaved by enzymes being present on excised porcine intestinal mucosa into their nontoxic building blocks (**Figure 2**).[6a]

The same principle is applied to choline-based cationic lipids that are cleaved by BChE present in human serum and mucosal membranes.[40b,42] The enzymatic degradation process of such amino acid-based cationic and ionizable cationic lipids, however, is strongly dependent on the structure of the hydrophobic tail. Longer hydrocarbon tails decelerate the degradation process.[6a,40b,c] Chain elongation and bulkiness of the lipidic tails cause steric hindrances at the cleavage site. Furthermore, chain elongation leads to higher lipophilicity of the molecule and, thus, to decreased critical micellar concentrations (CMCs). Above CMC, the predominantly occurring micellar structure provides additional protection of lipids from enzymatic cleavage. The resulting kinetics of the hydrolysis can deviate from Michaelis–Menten kinetics.[40b] This deviation might be caused by a decrease in the "effective" substrate concentration as a result of micelle formation. Cleavability of biodegradable lipids can therefore also be adjusted by varying the hydrophobic tail to control the CMC. Furthermore, cationic and ionizable cationic lipids can interact with enzymes resulting in conformational changes lowering enzymatic activity.[40b]

Another mechanism being involved in the degradation of cationic and ionizable cationic lipids is a simple pH dependent hydrolysis. For instance, betaine-based esters are resistant to acid hydrolysis unless the pH is extremely low.[7a,43] Under alkaline conditions, by contrast, these esters are unstable due to hydrolysis that occurs even at neutral pH.[7a,44] Also in this case, the degradation process depends on the chain length of the lipid as a longer alkyl residue accelerates the hydrolysis due to micellar catalysis. The higher the fraction of the surfactant in a micellar state, the higher is the hydroxyl ion concentration around the micelle and consequently the local pH.[45]

The micellar catalysis is, on the one hand, strongly dependent on the anions in solution and, on the other hand, on the surfactant counterion (**Figure 3**). The degradation process is accelerated by chloride or acetate as counterions, whereas bromide prevents degradation. The large, polarizable bromide strongly interacts with the surface of the micelle and, thus, prevents access by the hydroxyl ion and vice versa for a small nonpolarizable ion such as acetate.[43] This observation demonstrates the tremendous impact of counterions on the stability of cationic and ionizable cationic lipids.

Furthermore, amino acid-based carbonates and carbamates are promising alternatives to commercially available non-biodegradable alkyltrimethylammonium surfactants. The base-catalyzed hydrolysis of such compounds generates favorable alcohols and cholines instead of carboxylic acids formed by esters or amides.[31a,46] Carbonates and carbamates are stable at neutral pH but liable to acid-catalyzed hydrolysis. Consequently, such compounds remain stable in the systemic circulation but decompose into non-toxic building blocks after entering endosomes, where the pH is 1–2 levels lower.[31c,47]

Figure 4 illustrates the hydrolysis characteristics of different types of hydrolysable surfactants with respect to their linkage

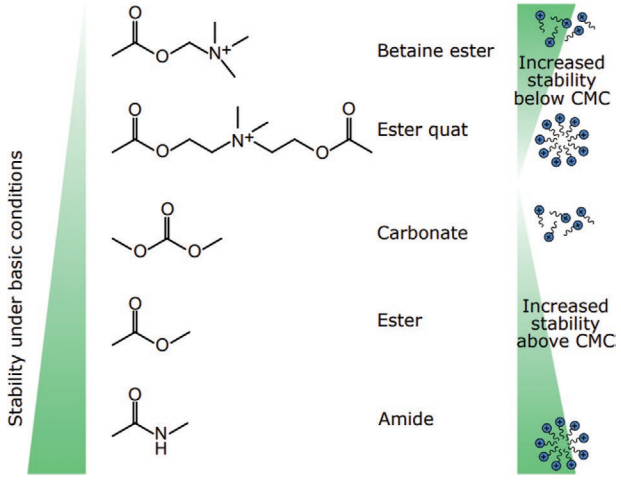


Figure 4. Stability of linkages under alkaline conditions and influence of CMC on stability.

bond and micellar catalysis. Lipid esters are highly prone to alkaline hydrolysis. Above their CMC the instability increases due to micellar catalysis. The same applies to ester quats. Conventional esters, carbonates, and amides, by contrast, exhibit higher stability under basic conditions that increases in a micellar state. Overall, enzymatic and pH-dependent degradation of amino acid-based esters seem to be a reliable process for the design of biodegradable lipids.[44]

Recently, esterase-cleavable ionizable cationic lipid-esters bearing a tertiary amino function were introduced and are, since the COVID pandemic, highly recognized. These amino ester-derived lipid-like compounds also offer the opportunity to tune their degradation by varying the hydrophobic tails of the lipids.[48]

3.2. In Vivo Degradation

The introduction of biodegradable structures to cationic and ionizable cationic lipids preferably leads to rapid elimination from plasma and tissues after these auxiliary agents have fulfilled their task without any side effects.

Among amino acid-based cationic and ionizable cationic lipids, only a few studies investigated their fate in vivo. At least one component of the lipid must be modified to distinguish between endogenously occurring structures and the applied ones. For instance, long chain alkanoylcholines were studied using radio labeled choline as head structure demonstrating a rapid degradation to choline and fatty acids.[49] The latter were complexed with physiological lipids present in intestinal tissue and subjected to further metabolism, whereas radio labeled choline was found in the bloodstream. This study confirmed that choline-based surfactants are substrates for BChE by showing similar results as an in vitro study. In accordance with these in vitro results, the hydrolysis rates decreased with increasing chain length suggesting that structural changes can effectively alter the degradation process according to the requirements given by the application.

In case of ionizable tertiary amine-based ester lipids, the elimination is well studied in mice, rats and monkeys. These studies showed a rapid clearance from blood, confirming the in vivo cleavage of the ester linkage (**Figure 5**),[48,50] whereas ionizable cationic lipids without ester bonds remained stable.[51] The formed metabolites were eliminated without accumulating in plasma or tissues.[50a] Lipid nanoparticles (LNPs) based on such biodegradable ionizable cationic lipids are currently used for the delivery of COVID-19 mRNA vaccines.[52]

After ester cleavage of ALC-0315, the doubly de-esterified metabolite still exhibits a lipophilic ionizable cationic character that is further metabolized by glucuronidation, followed by urinary excretion.[53] Nonetheless, considerable amounts of ALC-0315 were found in the liver two weeks after administration, whereas SM-102 and its degradation products were more rapidly eliminated via the renal and biliary route.[53,54] This might

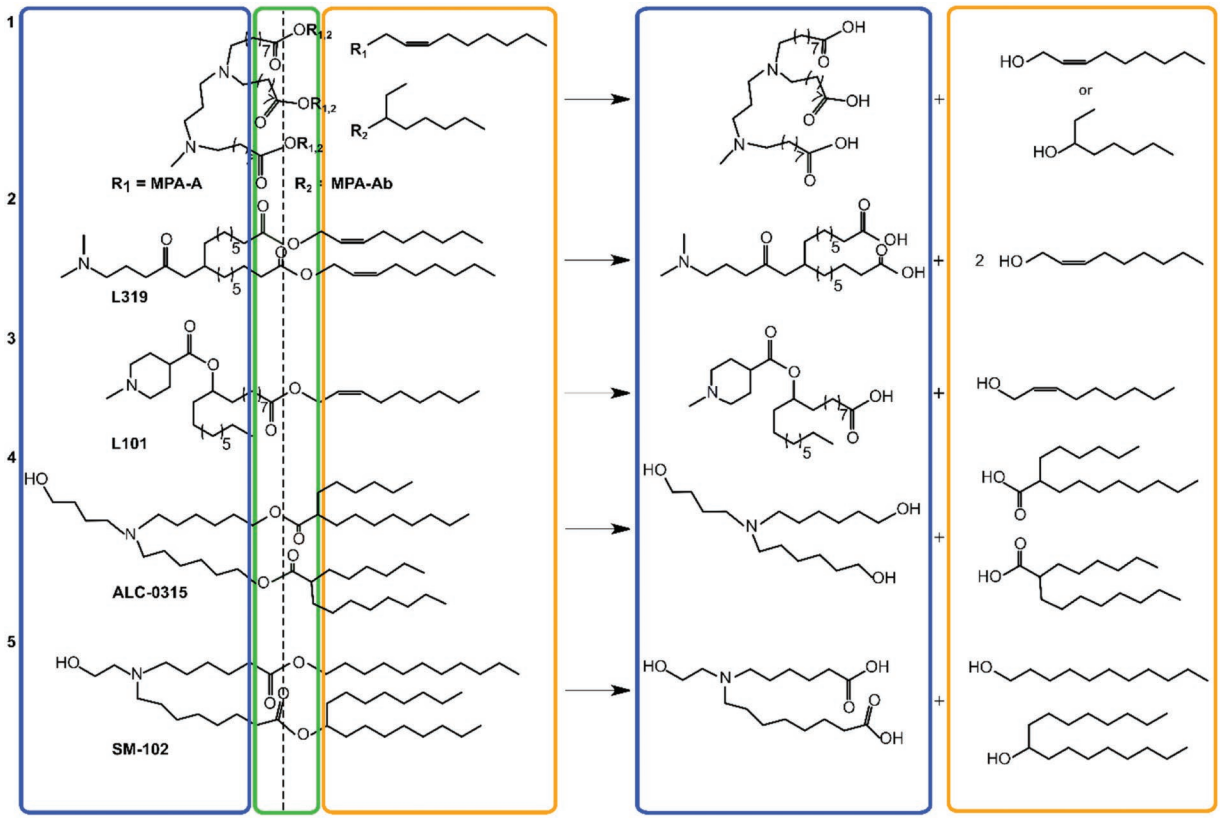


Figure 5. Cleavage of different ionizable cationic lipids into their polar head (blue frame) and hydrophobic tail (yellow frame) at the dashed line of the linkage (green frame).

Table 1. Overview of cationic and ionizable cationic lipids and their biodegradation studies.

Lipid class	Lipid	Linkage type	Biodegradation		Cationic metabolites	Refs.
			In vitro	In vivo		
Cationic and ionizable cationic lipids with a natural head group	Arginine-based	Ester	Lipases Trypsin		Arginine	[40c,41]
	Betaine-based	Ester	pH		Betaine	[6b,7a,43]
	Choline-based	Ester	Esterase pH	Rats	Choline	[16a,31a,40b,46,49]
		Carbonate	pH			[31a]
	Lysine-based	Ester	Lipases Trypsin Caco2-cells Brush border membrane-bound enzymes		Lysine	[5d,6a]
Cationic and ionizable cationic lipids with a synthetic head group	ALC-0315	Ester	Esterase	Rats	Nonendogenous compound	[53]
	L319	Ester	Esterase	Mice Rats Monkeys	Nonendogenous compound	[50a]
	L101	Ester	Esterase	Mice	Nonendogenous compound	[50b]
	MPA-A MPA-Ab	Ester	Esterase	Mice	Nonendogenous compound	[48]
	SM-102	Ester	Esterase	Rats	Nonendogenous compound	[54]

be explained by the linear fatty alcoholic tail of SM-102 that contributes to a higher accessibility for enzymatic cleavage of the first ester bond and subsequently also the second as a result of a reduced steric hindrance.

An overview of in vitro and in vivo biodegradable cationic and ionizable cationic lipids is given in **Table 1**.

4. Structural Function and Toxicity

Toxic effects of cationic and ionizable cationic lipids are mainly caused by their tendency to disrupt integral membranes due to adsorption and ionic interactions at the cell-aqueous interface (**Figure 6**A,B).[8c,55] Several studies indicated that an interaction with cell membrane phospholipids alters physiological properties and functions (Figure 6C) resulting in concentration-, time-, and pH-dependent cell lysis.[55a,56] In this regard, CMC appears to be an important parameter, since interactions with biointerfaces and cellular components largely differ depending on whether micelles or molecular disperse solutions are present.[55b] Micelles are able to extract lipids from the membrane to form mixed micelles disrupting the membrane and ultimately resulting in cell lysis (Figure 6D). This toxic effect increases with the length of the hydrophobic chain of lipids because chain elongation decreases CMC in most cases.[55b,57] As an approximation, the CMC is halved by the addition of one methylene group to the alkyl chain of a straight-chain single-tailed ionic surfactant.[43] Furthermore, the CMC decreases with increasing hydrophobicity of the polar head structure, but intra- and intermolecular interactions may alter this trend.[58] The presence of aromatic or bulky substituents as well as of hydrogen bond-donor and -acceptor groups can strongly influence molecular packing at interfaces as well as micellar stabilization.[55b]

Furthermore, toxic effects caused by cationic and ionizable cationic lipids can occur at concentrations below their CMC suggesting that their action does not necessarily involve cell membrane disruption.[59] Studies revealed high levels of toxicity with reduced chain lengths.[5b,41,55a] Lipids with short hydrocarbon chains can insert and subsequently translocate faster across lipid bilayer membranes, reaching sites of metabolic activity such as polynucleotides and mitochondrial membranes more rapidly than their longer analogues. Consequently, the toxicity level increases (Figure 6E).

In summary, concentrations near the CMC primarily cause acute toxicity whereas lower concentrations can lead to a persistent postexposure toxicity.[59]

4.1. Polar Head

The toxicity of cationic and ionizable cationic lipids is strongly related to their polar head.[60] The polar head can be categorized into quaternary ammonium (A), amine (B), guanidinium (C), and heterocyclic head groups (**Figure 7**).[61]

Although the cytotoxic effect of cationic and ionizable cationic lipids is directly correlated to the polar head, the relationship is rarely discussed hindering the advancement of such lipids toward clinical trials.[60,61] Among polar head groups, quaternary ammonium domains (A) are known to be more toxic than their tertiary amine counterparts because these structures interact with critical enzymes such as protein kinase C (PKC) to a tenfold higher extent.[47c,62] Cationic lipids with quaternary ammonium groups as polar domain induce apoptosis to

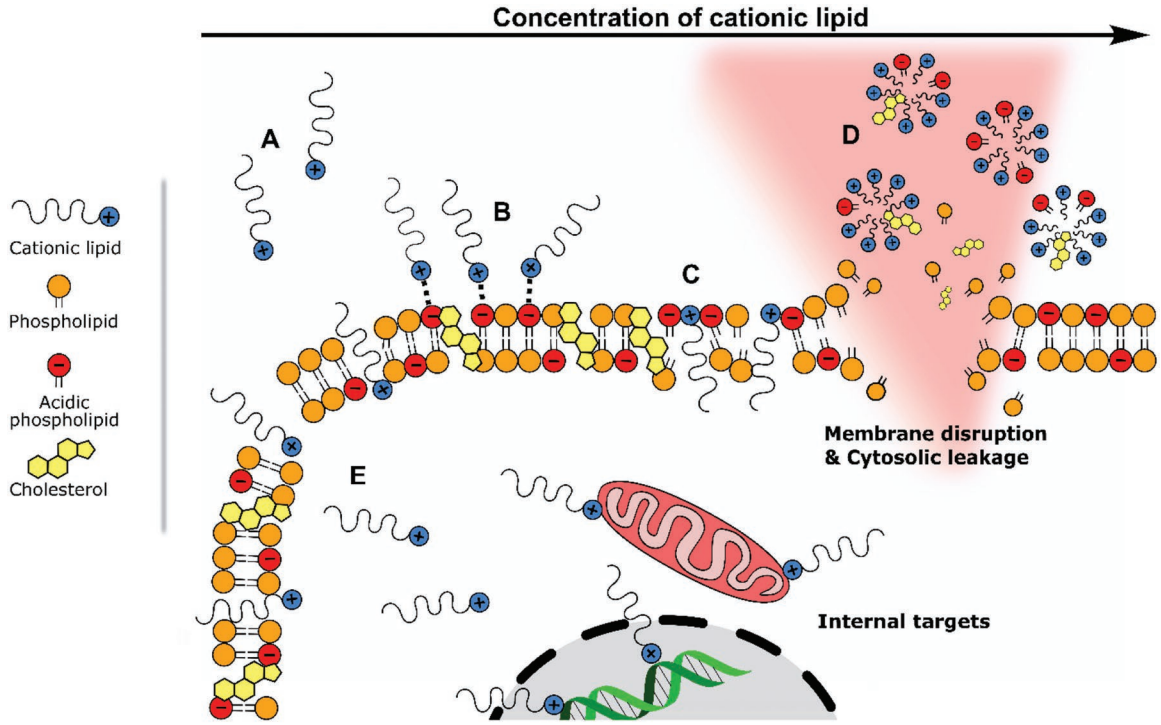


Figure 6. Mechanism of toxicity. A) Cationic lipid molecules approach a bilayer, B) ionic interaction with negatively charged membrane lipids, C) membrane intercalation alters the membrane fluidity, D) mixed micelle formation results in membrane disruption, cytosolic leakage, and ultimately in cell death, and E) translocation through bilayer to internal targets.

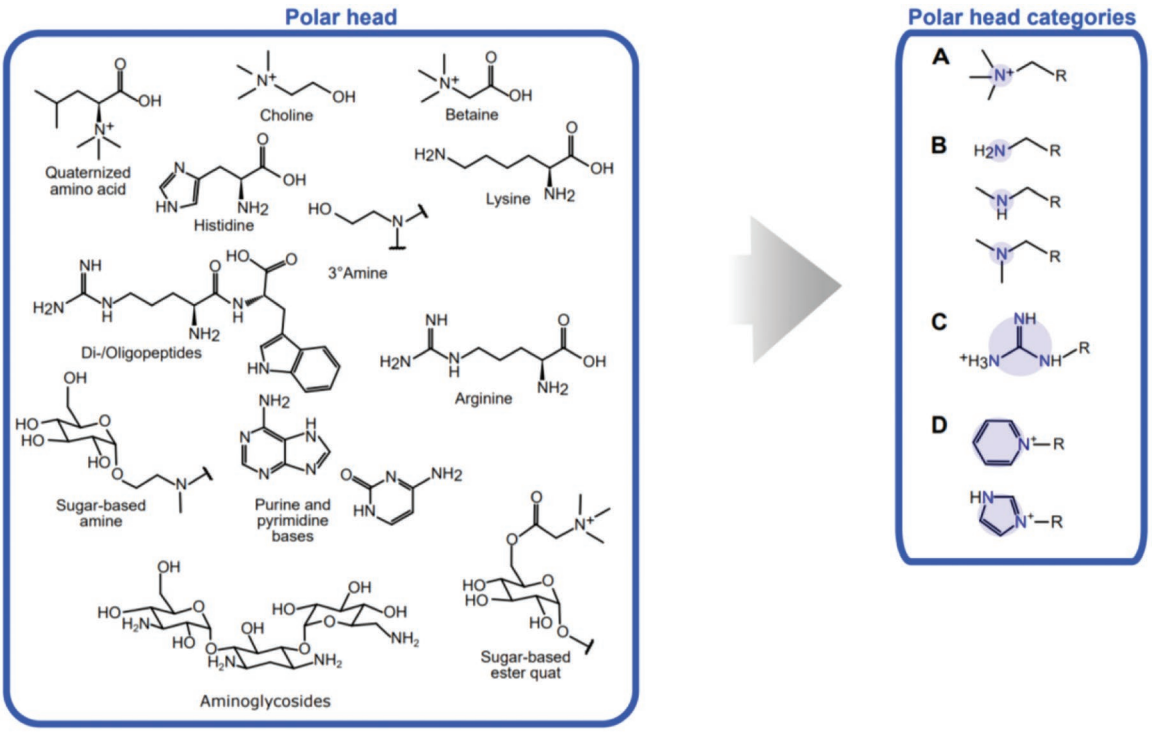


Figure 7. Structures of polar domains categorized in quaternary ammonium (A), amine (B), guanidinium (C), and heterocyclic heads (D). Light blue areas illustrate charge localization of cationic or ionizable cationic heads.

a higher extent compared with their counterparts with amino acids. Their cytotoxic mechanism is mainly related to a caspase activation-dependent signaling pathway and mitochondrial dysfunction. Enhanced ROS generation and cell cycle arresting effects at the S phase further contribute to the toxicity.[60] While it remains questionable whether the cytotoxicity caused by the cationic species can be fully avoided,[56] structures derived from common components of human metabolism are certainly less toxic.[63] Consequently, even biodegradable cationic lipids bearing quaternary ammonium structures are promising candidates, which have stronger toxicity reducing effects than non-biodegradable compounds.[50a]

Possible alternatives include enzymatically hydrolysable cationic lipids with quaternary ammonium functions derived from betaine, carnitine, and choline (A). These compounds combine high efficiency with lower toxicity.[16a,56] However, amine head groups (B) bearing ionizable cationic lipids such as peptide- or amino acid-based ones were shown to be superior to quaternary ammonium moieties in terms of efficiency and toxicity.[63b,64] Generally, primary, secondary, and tertiary amines can be considered as less toxic.[47c] For instance, N-acyl amino acid-based lipids from aspartic and glutamic acid are mild surfactants widely used in cosmetics and personal care formulations due to their low toxicity and mildness to skin and eyes.[55b] Recently, it was shown that only surfactants with a positive charge on the α-amino group of lysine exhibit pH sensitive hemolytic activity and improved kinetics within the endosomal pH range, indicating that the positive charge position is critical for toxic effects.[55a]

The major mechanism for in vivo toxicity of tertiary amines-based lipids is considered to be caused by nonspecific adhesion to proteins.[50b,65] These head structures form N-oxides and consequently aldehydes that react with RNA (**Figure 8**).[1]

Based on their lipophilicity, these head structures are more likely to accumulate in the human body than other more hydrophilic head group-bearing ionizable cationic lipids. Moreover, tertiary amine head groups are often derived from synthetic materials that are cleaved to nonendogenously related metabolites with unknown fate and effects. However, studies attest such compounds to be well-tolerated since no significant changes in clinical parameters were observed in mice and rats.[48,50] Solely one study showed minor to mild organ damage in form of single-cell hepatocellular necrosis and vacuolation without correlation to clinical chemistry parameters.[50a] It is important to note that the studies were conducted at low concentrations ranging from 1 to 10 mg kg^{-1} but lack standardized toxicological studies according to OECD guidelines that would be beneficial for comparison.[67] Recent repeat-dose Good Laboratory Practice toxicity studies with the COVID-19 vaccine Comirnaty in rats indicated that portal vacuolation is caused primarily by the accumulation of ALC-0315 in the liver.[68] Although it seems arguable to attribute all side effects of mRNA vaccines to the head structure of the ionizable cationic lipids, it is certain that charge delocalization in polar head structures is a promising strategy to decrease toxicity. The guanidinium (C) function in arginine-based ionizable cationic lipids is therefore a promising candidate[47c] as these lipids are remarkably less cytotoxic (> 50 times) in comparison with conventional cationic and ionizable cationic surfactants.[40c] Arginine-derived ionizable cationic lipids showed lower eye and skin irritation than the synthetic surfactant sodium dodecyl sulfate.[65] The same principle applies to surfactants with a heterocyclic head (D) as the positive charge is spread by delocalization.[47c] Heterocyclic heads, which comprise an imidazole moiety like histidine or pyridine, demonstrated higher transfection efficiency than classical transfection systems and reduced cytotoxicity.[47c,69]

4.2. Linkage

The linkage bond can be divided into ester, amide, carbonate, carbamate, and ether structures. The bond is an important determinant of the chemical stability of the lipid. The introduction of biodegradable linkages, including pH-, redox-, and enzyme-sensitive linkages appears beneficial in terms of efficiency and lipid-associated cytotoxicity.[70]

In general, biodegradable linkages are associated with lower toxicity than non-biodegradable structures such as ether bonds. Therefore, the most widely used linkage for biodegradable cationic and ionizable cationic lipids are of the ester or amide type as these structures are cleaved in vivo.[47c] Furthermore, their synthesis procedures are rather simple. However, these linkages carry the risk to decompose too rapidly in the systemic circulation. Structural modifications like an additional spacer between the polar head and the acyl chains were investigated to alter the characteristics of the lipids such as improved gene delivery efficiency and lower toxicity.[63b,71] Furthermore, Maier et al.[50a] noted that the placement of the linkage, an ester group in this case, affects the pKa of the ionizable amino group and, moreover, the toxicity of the compound. Centrally located degradable functions within the hydrocarbon chain yielded hydrolysis products that are more hydrophilic than the parent lipid.[50a] **Table 2** provides an overview of the different linkage types along with their biodegradability and associated toxicity.

4.3. Hydrophobic Tail

Generally, the hydrocarbon chain of cationic and ionizable cationic lipids can be categorized into saturated and unsaturated single- or double-chain lipids. Moreover, the lipid chain can be linear, branched or steroid-based (**Figure 9**).

The impact of the hydrophobic tail on toxicity and the mechanism of toxicity are controversially discussed in literature. For instance, Häckl et al.[56] reported that the cytotoxicity of carnitine-based cationic lipids appears to be primarily driven by the increased interaction between micelles and cell membranes. A lower CMC rendered by chain length elongation therefore results in increased toxicity.[56] Similar results were obtained by Kurpiers et al.[6a] regarding lysine ester-surfactants. The hemolytic activity increased with increasing chain length or larger volume of the attached alcohol moiety. The toxicity increased in the following order: decyl lysinate < oleyl lysinate < cholesteryl lysinate < hexadecyl lysinate. The authors assumed that the double bond of the oleyl residue might reduce the membrane disrupting effect. Another study confirmed CMC-dependent toxicity because toxic effects increased in concentrations at or above the CMC of the arginine ester-surfactants.

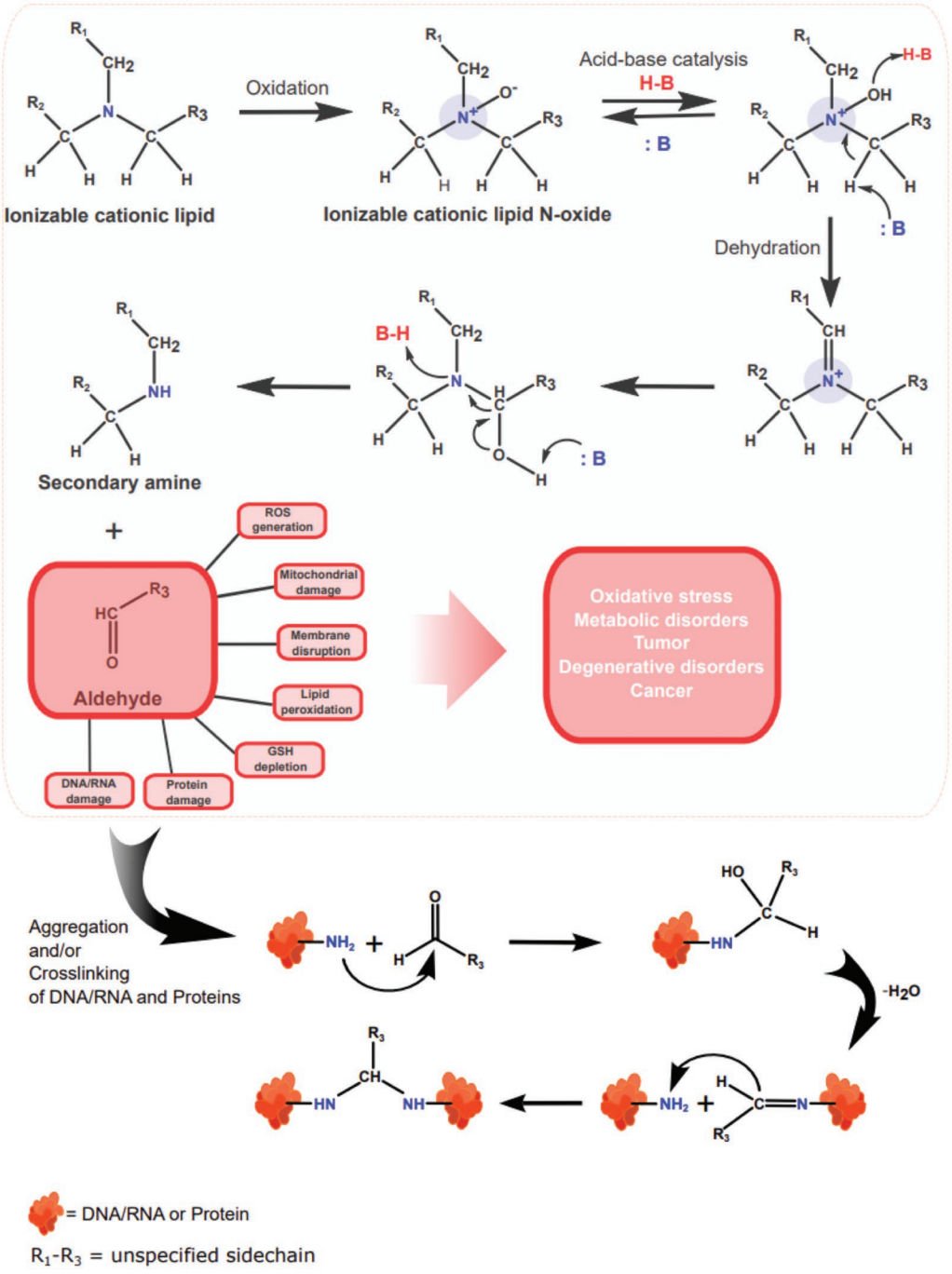


Figure 8. N-oxide formation through tertiary amine oxidation and acid/base-catalyzed dehydration at the amine to generate aldehydes and secondary amines (top).[66] Aldehyde-related toxicity (mid) and mechanism of DNA/RNA and protein aggregation and/or crosslinking (bottom).

In contrary to the chain length-dependent toxicity, however, the arginine hexadecanoyl ester showed lower toxicity than the arginine nonyl-ester toward Caco-2 cells (34% vs > 80% cell viability).[40c] Similar results were reported by Pérez et al.,[5b] as the hemolytic activity of the lysine-based surfactants decreased with increasing length of the hydrophobic tail. This observation was explained by surfactant intercalation into the membrane and, thus, an alteration of the membrane molecular organization, causing increased membrane permeability and cell lysis.[5b,77] Inácio et al.[59] reported a nonlinear dependence of toxicity of cationic amphiphiles of the non-biodegradable C_nTAB surfactants (n = 10 to 16). Toxic effects were observed even at concentrations well below their CMC suggesting that their action does not involve cell membrane disruption. The authors expect short hydrocarbon chain surfactants to insert and subsequently translocate faster across lipid bilayer membranes, reaching sites of metabolic activity such as polynucleotides and mitochondrial membranes more rapidly than their

Table 2. Overview biodegradable linkage and their associated toxicity.

Type of linkage	Biodegradation	Toxicity	Leaving groups	Refs.
Ester	Hydrolysis Enzymatic Cells In vivo	+[a)]	Acid Alcohol	[5d,40c,41,48,72]
Amide	Hydrolysis Enzymatic Cells	++[a)]	Acid Amine	[72–73]
Carbonate	Hydrolysis Enzymatic	Unknown	Alcohols Carbon dioxide	[31a,b,72,74]
Carbamate	Stable in neutral pH Acid-catalyzed hydrolysis	Unknown	Alcohol Amine Carbon dioxide	[28b,31d,47b,75]
Ether	Non/Limited	+++[a)]	None	[47c,76]

[a)]Level of toxicity scaled from low (+) to high (+++).

longer analogues. Furthermore, surfactant concentrations close to the CMC cause acute toxicity while lower concentrations can lead to a persistent postexposure toxicity.[59]

It has been proposed that high concentrations of cationic and ionizable cationic surfactants cause necrosis whereas low concentrations induce primarily apoptosis by causing structural changes of plasma membrane, such as phosphatidylserine translocation.[78]

Recently, a single-tailed lipid was reported to be less toxic than its branched analogue which might be attributed to the unsaturated state of the single-tailed lipid chain.[48] Kurpiers et al.[6a] observed the same effect for unsaturated chains.

Double-tailed lipids, on contrary, appeared to generate generally fewer toxic effects than their single-tailed counterparts.[47c,79] The steroid hydrophobic tails in cationic and ionizable cationic lipids, such as derivatives of cholesterol, are PKC inhibitors. This characteristic might be linked to their toxicity that is reported to be higher than that of linear-chain analogues.[62]

In summary, the effect of the hydrophobic domain on toxicity has not been adequately addressed up to date. In any case, the influence of hydrophobic chain length on toxicity may depend on the physicochemical features provided by the head and the linkage domains.[47c,71]

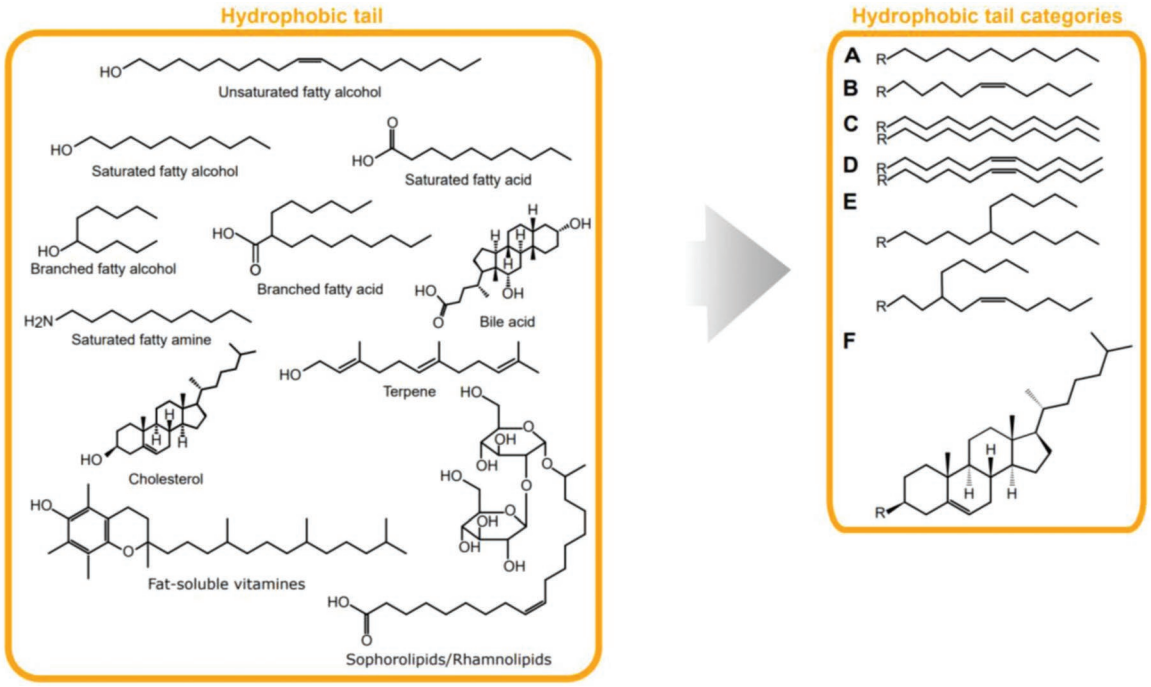


Figure 9. Structures of hydrophobic tails categorized into single-chain (A), unsaturated single-chain (B), double-chain (C), unsaturated double-chain (D), branched chains (E), and steroid derivatives (F).

5. Use as Complexing Agents for Drug Delivery

Many hydrophilic active pharmaceutical ingredients (APIs) do not reach their target as they are degraded in biological fluids or not able to surpass lipophilic biological barriers.[80] Amongst others, lipid-based formulations are a widely employed strategy to protect the API and to shuttle it across lipophilic biological barriers.[81] To bridge the gap between the hydrophilic nature of the API and lipophilic matrices, an amphiphilic surfactant can be bound to the API via electrostatic interactions, resulting in a hydrophobic complex.[82] In the case of nucleic acids, the negatively charged phosphate backbone is complexed with a cationic or ionizable cationic lipid and buried in an LNP, often in a one step-process. The same strategy can be applied to small molecules, peptides, proteins, and other hydrophilic APIs. However, the highly ordered and exclusively negatively charged phosphate backbone of nucleic acids as well as their ability to transform between a condensed and decondensed state, distinguish nucleic acids from other APIs. Hydrophobic complexes of these APIs are most frequently preformed and subsequently incorporated into a lipid-based formulation (**Figure 10**).

5.1. Complexation of Nucleic Acids

LNPs that carry nucleic acids, commonly contain a helper lipid, cholesterol, a PEGylated lipid, and an ionizable cationic lipid. After complexation and LNP formation, the lipidic matrix protects the nucleic acid from degradation by nucleases present in the bloodstream. Moreover, the ionizable cationic lipid is mostly uncharged in the bloodstream, contributing to an inert surface. After cellular uptake, LNPs enter endosomes where the ionizable cationic lipid becomes protonated as a result of decreasing pH. The acquired positive charge allows interaction with the inner leaflet of the endosomal membrane and endosomal escape.[83] Subsequently, the intact nucleic acid is released into the cytoplasm. Because of this pH-dependent bifunctionality, research focused on ionizable cationic lipids resulting in a plethora of structures for this excipient.[84] A pKa value between 6.2 and 7 and a kink in the hydrophobic tail, first induced by an alkenyl structure, later by a biodegradable ester bond reducing toxicity, were identified as crucial parameters for successful nucleic acid delivery.[50a,84b,85] These advancements led to the "first-in-class" siRNA therapeutic Onpattro and subsequently, to the mRNA vaccines launched during the COVID pandemic.[19,52,84b]

The optimum pKa-value of the polar head provides an uncharged, bioinert surface at neutral pH on the one hand, and allows protonation and subsequent interaction with the inner leaflet of the endosomal membrane on the other hand, when the pH is decreasing.[83b] However, as often exclusively amines, predominantly tertiary ones, were screened, this optimum may only apply to ionizable cationic lipids, which achieve endosomal escape via interaction with acidic lipids on the inner leaflet of endosomes and subsequent disruption of the endosomal membrane.[83b,86] Alternatively, compounds with high buffering capacity can provide endosomal release. Although the mechanism is not entirely understood, disruption

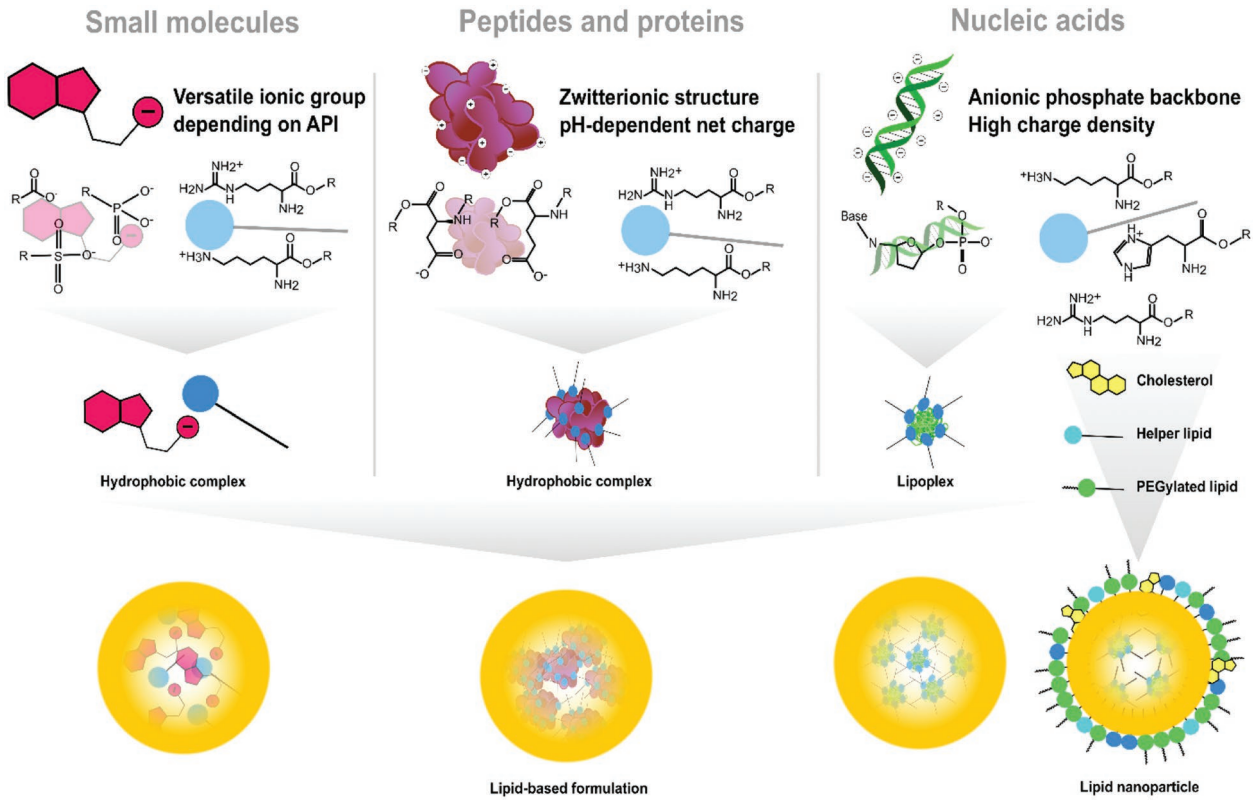


Figure 10. Schematic overview of the complexation of small molecules, peptides, proteins, and nucleic acids and the subsequent processing.

of endosomal membranes by osmotic swelling is the most popular theory.[86a,87] Accordingly, histidine has promising characteristics from a theoretical point of view because the imidazolium ring provides buffering capacity[88] as well as a pKa-value that can be shifted to fit the optimal range.[89] Consequently, histidine-based transfection agents may be shaped to allow endosomal escape via both membrane disruption and osmotic lysis. By rendering one of the mechanisms predominant or by providing a synergism, endosomal escape may be shifted to earlier endosomal stages to prevent that the nucleic acid is subjected to low pH and catalytic enzymes.[90]

Moreover, Ripoll et al.[91] showed high storage stability at 4 °C for formulations comprising an ionizable cationic lipid with imidazole head. A stabilization of the secondary structure of the nucleic acid via π-stacking and subsequent conformational stabilization is claimed to be responsible for increased stability in comparison with currently used ionizable cationic lipids. A mechanism preventing this degradation pathway appears reasonable because nucleic acid hydrolysis is dictating the storage time in the non-frozen state.[92] Nonetheless, other mechanisms like a lower degree of water entrapment within the LNP may increase stability as well and were not investigated within this study.[93] Further, tertiary amines contribute to the degradation of nucleic acids by an oxidative mechanism that can be prevented by applying imidazole as ionizable cationic moiety.[66] Although ester bonds are present in the current lead compounds to decrease their toxicity, earlier studies showed rapid degradation of nucleic acids when being formulated with ionizable cationic lipids carrying this rather labile linkage.[94] The degradation occurred after cellular uptake by endosomal enzymes, demonstrating that a compromise between a sufficient stability and a complete degradation and elimination must be provided. The rate of degradation can also be controlled by introducing a steric hindrance in proximity to the linkage, for example with a branched tail structure. Moreover, the influence of the linkage on efficacy and biodistribution is investigated in ongoing research.[28a,93]

Preformation of the complex and subsequent incorporation into a lipid-based formulation, allows the complexing agent to fulfill only its eponymous task and may prevent hydrolysis as the entrapment of water in the vehicle can be avoided.[5d] Endosomal escape, but also interactions with physiological environment after application, can be rendered separately by surface modification of the nanoparticle. Since their surface is rendered independent from the nucleic acid complexation, lessons learned from other cargos can be applied to nucleic acid delivery. In this case, lysine- and arginine-based complexing agents seem to be advantageous because they provide higher complex stability and therefore, might result in a stronger retention of the cargo inside of the oil droplet.[95] Surface modifications and cargo retention are of particular interest when the oral route is desired for nucleic acid delivery.[96] However, also for other mucosal administration sites like the pulmonary one, the vehicle must pass a mucus layer requiring bioinert properties. Nevertheless, biointeraction subsequently is required to provide efficient cell uptake.[96b] As suggested in a recent study, a biointeractive structure underneath a bioinert one may increase both mucus penetration and cell uptake.[97]

Regardless of whether LNPs or preformed complexes are considered, in vivo evidence for the potency of biodegradable cationic and ionizable cationic lipids that can be metabolized into natural or even endogenous metabolites, is limited. The few available in vivo studies (**Table 3**) lack data on the elimination of the lipid and its metabolites, as well as a comparison with the lead compounds ALC-0315 and SM-102. Considering the ongoing research on nucleic acid therapeutics, an increasing demand for ionizable cationic lipids can be anticipated. The rapid and complete elimination of the parent compound and its metabolites will enable applications that require a higher dose and dosing frequency than vaccines. Studies, which investigate a larger number of such biodegradable lipids in vivo and compare them to the current lead compounds, are required to fulfill this demand without sacrificing the efficacy.

5.2. Complexation of Small Molecules, Peptides, Proteins, and Other Hydrophilic Macromolecules

Although hydrophobic ion pairing has become a well-established strategy, cationic and ionizable counterions and in particular biodegradable ones are seldomly applied.[82,105] Biodegradable ionizable cationic counterions with safe building blocks that have been used for hydrophobic ion pairing as a process step for drug delivery are listed in **Table 4**. Moreover, few studies probed interactions between such counterions and APIs by spectrophotometric and fluorometric methods.[106]

In most studies using biodegradable ionizable cationic surfactants, hydrophobic ion pairing was conducted in just water or in aqueous media at almost neutral pH.[40c,107a–c,e,h,109] Even though this provides ionization of guanidine and primary amine moieties present on the complexing agent as well as of anionic carboxylates of aspartic and glutamic acid, lysine, and arginine residues attributed to the macromolecular API are protonated as well (**Figure 11**). These positive charges might repel the likewise charged counterion and result in insufficient saturation of ionic sites. Consequently, permanently charged quaternary ammonium ions as present in betaine or carnitine esters are likely the better choice as they allow to work at a sufficiently high pH to provide the maximum net charge of zwitterionic macromolecules.[110]

The predominant rationale to conduct hydrophobic ion pairing is to increase the lipophilicity of hydrophilic APIs.[111] Unsaturated, branched, and bulky structures as well as the presence of two or more hydrophobic tails increase the lipophilicity of the complex to a higher extent, presumably as due to an effective shielding of polar residues.[81a,112] Moreover, a high logP of the counterion correlates with a high logP of the resulting complex.[112b,113] Applied biodegradable counterions could already provide an up to 10^8-fold increase in logP.[6a,40c] Still, these studies could not reach logP-values that were achieved in studies using conventional counterions.[112b,114] The use of branched or bulky alkyl tails and the conjugation of a second alkyl tail offer possibilities to further increase the potential of this counterion class, which could provide a rationale to advance beyond in vitro investigations.

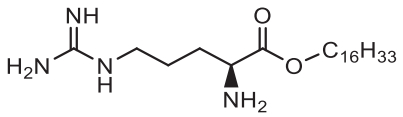
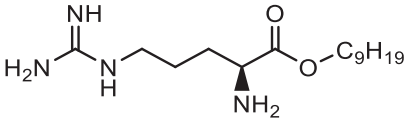
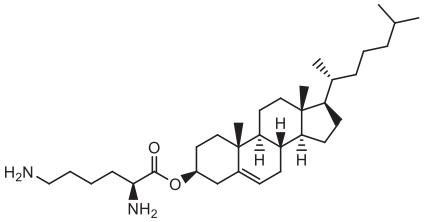
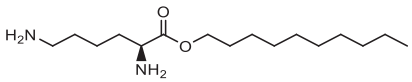
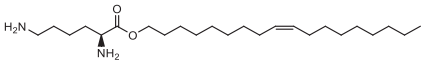
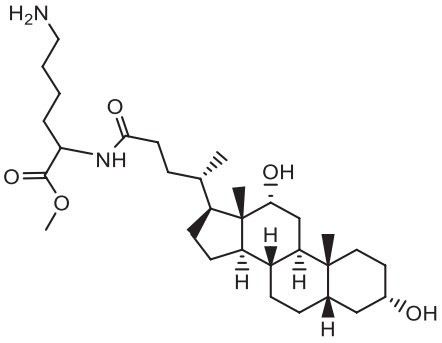
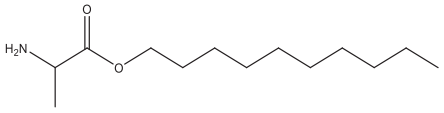
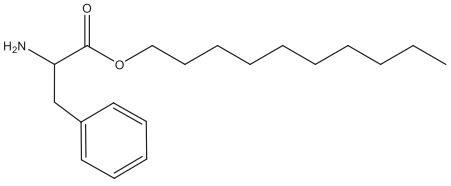
Besides an increase in lipophilicity, an active uptake mediated by the counterion is a well-explored strategy for substrates

Table 3. In vivo studies with ionizable cationic lipids present in marketed nucleic acid therapeutics and of ionizable cationic lipids that are composed of natural or endogenous building blocks.

Ionizable cationic lipid	pKa	Degradability	Further LNP constituents	Species	Efficacy	Elimination	Notes
Dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA)	6.30[98] 6.35[85b] 6.44[99]	Non-biodegradable	Phospholipid Cholesterol PEGylated lipid	Mouse[85b,99,100] Monkey[99]	ED_{50}: 0.005 mg kg^{-1}[99] ED_{50} < 0.03 mg kg^{-1}[99]	Slow elimination from administration site and accumulation in liver and spleen found in mice;[85b] 61% of dose remaining 48 h postinjection in rats[98]	Excipient in Onpattro; formulation shelf life of 27 months (4–8 °C), presumably as siRNA results in lower amount of entrapped water[93]
ALC-0315	6.09[100]	Biodegradable into nonendogenous metabolites	Phospholipid Cholesterol PEGylated lipid	Mouse[100]	6- and 14-fold at doses of 0.3 and 1.0 mg kg^{-1} in comparison with DLin-MC3-DMA[100]	Fast elimination from plasma but slow elimination from the liver[53]	Excipient in Comirnaty
Lipid H (SM-102)	6.68[85b,98]	Biodegradable into nonendogenous metabolites	Phospholipid Cholesterol PEGylated lipid	Monkey[85b]	Threefold higher response expressed as antibody expression than DLin-MC3-DMA[85b]	Rapid but incomplete elimination from muscle, liver and spleen in mice;[85b] 1.3% of dose remaining 48 h postinjection in rats[98]	Excipient in Spikevax; improved tolerability in rats compared with DLin-MC3-DMA[85b]
(S)-N-(4-guanidino-1-(hexadecylamino)-1-oxobutan-2-yl) oleamide (C18:1/C16-dialkylated norarginine)	12.2[101]	Biodegradable into natural metabolites	Cholesterol Cholesteryl hemisuccinate PEGylated lipid	Mouse[101]	ED_{50}: 0.10, 0.16, and 0.25 mg kg^{-1} determined as knockdown of the factor VII, transthyretin, and apolipoprotein B100 genes[101]	Not determined	High tolerability determined as hepatotoxicity[101]
Kanamycin A-lipid derivatives[12b,102]	6.19, 7.42, 8.16, 9.03 for kanamycin A[103]	Biodegradable into natural metabolites[12b,102]	Dioleylphosphatdiyl ethanolamine[12b,102]	Mouse[12b,102]	Up to 0.90 and 8.34 ng chloramphenicol acetyltransferase expression per 100 mg protein in trachea and lungs, respectively[102]	Not determined[12b,102]	Pulmonary administration[12b,102]
N-Octadecyl histidine[104]	6.58, 8.02[104]	Biodegradable into endogenous metabolites[104]	Phospholipid Dioleylphosphatdiyl ethanolamine Cholesterol PEGylated lipid[104]	Mouse[104]	Investigated via the pharmacological effect[104]	Not determined[104]	No acute toxicity with pDNA doses of 500 µg kg^{-1}[104]

that are used as counterions without modification,[115] as well as for counterions that carry a substrate as building block.[107b,c,109c] Predominantly, anionic bile acids and their ionizable cationic derivatives have been applied to increase uptake via the apical sodium-dependent bile acid-transporter (ASBT).[107a,d,e,h,116] Mostly, cholic or deoxycholic acid were utilized, likely because of their higher lipophilicity in comparison with other bile acids, except of lithocholic acid. Nevertheless, the rather weak point charge and high pKa-value of the carboxylate are unfavorable. Taurocholate, on the contrary, bears a strong sulfonate group but is fairly hydrophilic and, thus, the resulting complexes most likely also. Their ionizable cationic derivatives provide a promising alternative to overcome these inherent disadvantages of native bile acids. The potential of

Table 4. Biodegradable ionizable cationic surfactants used as counterions for hydrophobic ion pairing of small molecules, peptides, proteins, and other hydrophilic APIs.

Drug(s)	Counterion	Structure	Refs.
Daptomycin Heparin	Hexadecyl argininate		[40c]
Daptomycin Heparin	Nonyl argininate		[40c]
Insulin	Cholesteryl lysinate		[6a]
Insulin	Decyl lysinate		[6a]
Insulin	Oleyl lysinate		[6a]
Ibandronate Insulin Oxaliplatin Pemetrexed Teriparatide[a)] Zoledronate	Methyl deoxycholyl lysinate		[107]
Tolfenamic acid	Decyl alaninate		[108]
Tolfenamic acid	Decyl phenylalaninate		[108]

[a)]Formation of ternary complex between API, ionizable cationic lipid, and anionic deoxycholic acid.

such derivatives to increase oral bioavailability was already shown but with the aid of a polar head, which would be cleaved into toxic ethylenediamine[109b,c] or a lysine-derivative with unknown toxicity.[109a] Nevertheless, by simply using an endogenous or biocompatible polar head this approach could provide active uptake mediated by the bile acid, a favorable polar head assuring stability of the complex and cleavage into safe degradation products.

Based on the strategy of targeting intestinal transporters, the application of counterions, which exert an effect on the

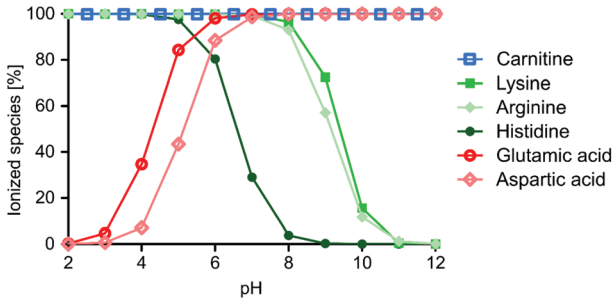


Figure 11. Ionization state of amino acids at given pH-values. In the case of the basic amino acids, only positively charged species are depicted whereas only anionic species are depicted in the case of acidic amino acids, both irrespective of the net charge. Values are calculated values from chemicalize.com (accessed: 29.03.2022, 10:10).

epithelium of the respective application site seems promising. Compounds such as long chain acyl carnitines that are well recognized as permeation enhancers and, in addition, bear a permanent cationic charge, may be an attractive choice.[117] As a hydrophobic complex, the permeation enhancer could exert its effect while being colocalized at the membrane with the API. Consequently, hydrophobic ion pairing could significantly increase the efficiency of such formulations in comparison with a physical mixture.[118]

To date, anionic counterions are predominantly used because of their rather low toxicity.[81a,112b,119] Cationic and ionizable cationic counterions are required if exclusively anionic sites are present on the active agent, e.g., heparin. Nonetheless, peptides and proteins commonly carry both anionic and cationic charges and, thus, the use of cationic or ionizable cationic surfactants is not compelling. It seems advantageous to ionically complex the charge that is predominantly available on the API because this presumably allows to bind a higher amount of counterions. For peptides and proteins with low isoelectric points that predominantly carry anionic charges, enlarging the available toolbox with biodegradable cationic and ionizable counterions can contribute to a safe and efficient delivery of such compounds.

6. Use as Preservatives

Cationic surfactants like benzalkonium chloride that are used as disinfectants and preservatives, exhibit considerable toxicity toward mammalian cells. Moreover, the lack of biodegradability results in environmental accumulation, which finally leads to the emergence of resistant strains.[120] Intensive efforts to design biodegradable cationic and ionizable cationic surfactants resulted in the approval of ethyl lauroyl arginate as a safe food preservative by the FDA in 2005 and the EFSA in 2007.[121] In addition, several amino acid-based lipids exhibiting activity against ESKAPE strains were identified to be promising candidates as disinfectants for the use in hospitals.[5c,122]

The predominant focus in the design of antimicrobial cationic and ionizable cationic lipids is on the polar head. The abundance of acidic, anionic phospholipids in prokaryotic membranes results in a negative charge of the outer leaflet.[123] Consequently, a positive charge of the lipid at physiological pH is necessary to allow electrostatic adsorption to bacterial membranes. Accordingly, the pKa-value and the charge density of the polar head are key parameters for this initial adsorption.[124] Lysine and arginine are often used as biocompatible building blocks in antimicrobial ionizable cationic lipids. The guanidine group of arginine provides a bidentate structure and up to 5 hydrogen bond donors, depending on the site of modification. As a result, arginine attracts more phosphate groups and water in the membrane and achieves more stable clusters with phosphates. Consequently, interfacial binding and membrane perturbation are stronger than for amino groups as present in lysine.[125] The membrane perturbation subsequently results in leakage of cytoplasmic constituents and leads to cell death.[120c] Consequently, replacement of lysine with arginine is presumed to enhance the interaction with phospholipid bilayers and, thus, the antimicrobial activity.[126] Furthermore, increasing the number of cationic charges results in an increased charge density and stronger antimicrobial activity (**Figure 12**A).[127] However, the effect of multiple cationic and ionizable cationic groups seems limited after introducing a certain number of them.[128]

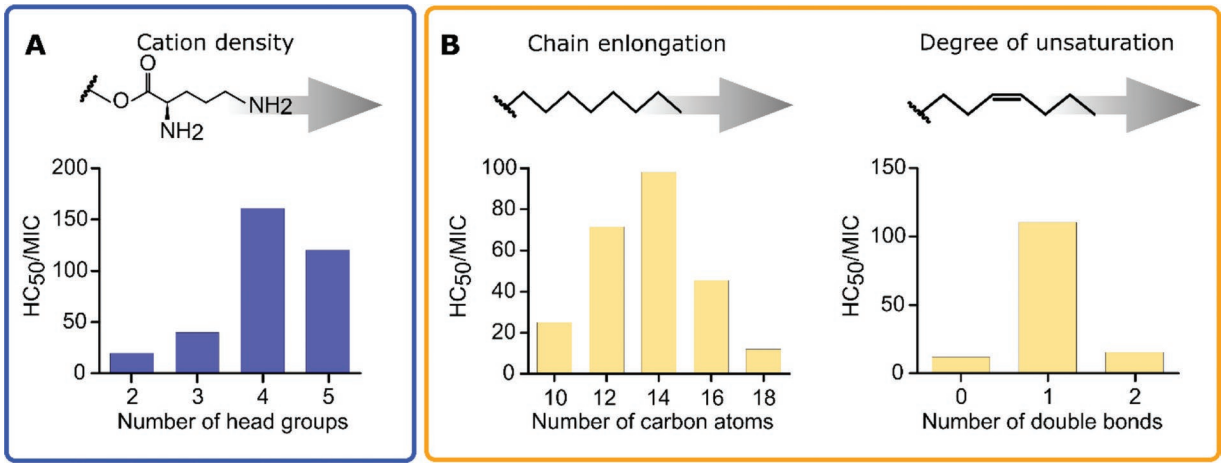


Figure 12. Alteration of the therapeutic index expressed as the ratio of hemolytic activity (HC_{50}) and minimum inhibitory concentration (MIC) after common modifications of A) the head[128a] and B) tail of antimicrobial lipids.[129]

Although increasing the charge density of the polar head is a straightforward strategy to increase the antimicrobial activity, it has limitations in governing the selectivity. Instead, the introduction of nonionic amino acids, often using AMPs as a model, has been extensively studied.[130] Reduction of the amino acid number to decrease production costs and acylation to increase protease stability have led to the class of "short" and "ultrashort lipopeptides." In addition to the approved lipopeptide daptomycin, compounds of this class progressed to phase III studies as oral antibiotics and to preclinical stages as dermal antibiotics.[130d,131] Despite their investigation in clinical trials, none of the tested lipopeptides entered the market since the approval of daptomycin. Most trials were discontinued because the new compounds merely showed noninferiority to established antibiotics.[131]

The AMP-based design is commonly selectivity-driven and, unlike most approaches discussed in this review, attempts to increase stability because labile AMPs are considered as precursors.[130a,132] Nevertheless, they consist of biocompatible building blocks and endow linkages that are theoretically biodegradable. The antibacterial mechanisms of lipopeptides as well as of the preceding AMPs are not entirely understood and unlikely to be consistent across different lipopeptides.[133] Anyhow, this might also preclude cross-resistance.

Hydrophobic aromatic amino acids such as tryptophan are an inevitable part of AMPs to render the cationic-hydrophobic balance. Moreover, they enable unique interactions with cationic moieties.[134] Tryptophan itself and cationic-π interactions, in particular with arginine, allow distinct interactions with lipid bilayers resulting in a prolonged association.[134c,135] Although the acylation reduces the need for hydrophobic amino acids, tryptophan is therefore still often used in the design of lipopeptides.[132a,136]

Altering the stereochemistry of amino acids in the sequence of AMPs resulted in higher antimicrobial activity, lower hemolytic activity, and increased stability.[14a,137] Indeed, the same advantageous features were found for lipopeptides after substitution of L- with D-amino acids.[14b,c,132b,138] However, increasing the stability of antibacterial agents is a double-edged sword as a possible bioaccumulation promotes resistant strains. Despite their lower susceptibility, resistances against quaternary ammonium substances and lipopeptides have already emerged.[120b,139] Therefore, a suitable compromise must be found between increased stability and yet reliable degradability into nontoxic building blocks. Merging the AMP-based and biocompatibility-driven designs and experimental setups might help to identify highly selective compounds that have a sufficient shelf-life to provide antimicrobial activity but also allow biodegradation to decrease toxicity and prevent bioaccumulation. Moreover, the potential of lipopeptides to exert bacterial activity via multiple modes of action makes them promising candidates to combat existing bacterial resistances.[136,140]

The concerns on multidrug resistant-bacteria should urge authorities to recognize resistance development as primary outcome parameter in clinical trials.[141] In that sense, parameters like bioaccumulation also deserve more attention. A positive evaluation on that measures could allow lipopeptides and biodegradable cationic and ionizable cationic lipids to enter the market if they ensure at least noninferiority in comparison with established treatment options. However, a deeper understanding of the mechanisms of action is needed to rationally design lipopeptides that exert their activity via multiple pathways to prevent resistance development.[142]

The hydrophobic tail of cationic and ionizable cationic lipids drives the membrane insertion after electrostatic adsorption. Further, the tail influences the geometry and self-assembly of the surfactants and therefore, aids to render these lipids selective.[124a,129,143] An optimum length between 10 and 16 carbon atoms, depending on whether Gram-positive or Gram-negative strains are investigated, has been identified.[5c,122a,144] However, increasing the length of the hydrophobic tail beyond a certain number of carbon atoms decreases or even ceases antimicrobial activity.[31a,122a,144,145] Introduction of more than one alkyl tail or the formation of gemini surfactants increase the antimicrobial activity. Nevertheless, efforts in this direction did not result in a significantly higher selectivity than their single-tailed counterparts as cytotoxicity is increasing as well.[32,73c,127a,145a,146] The presence of unsaturated bonds in the hydrophobic tail decreased the hemolytic activity of cationic and ionizable cationic surfactants while maintaining antimicrobial activity.[129,147] Introduction of multiple unsaturated bonds, however, often results in a severe loss of antimicrobial activity (Figure 12B).[129] The diversity of conjugated alkyl tails is yet mostly restricted to saturated and to a lower extent unsaturated linear ones. Branched or bulky alkyl tails likely alter the self-assembly properties as well and, moreover, could influence how cationic and ionizable cationic lipids insert into membranes and influence their curvature. In that sense, terpenes might provide promising branched structures and, in addition, exert themselves selective activity against pathogenic microbes.[148]

Beyond tuning the head and tail group, a switchable activity as achieved for supramolecular assemblies[149] as well as the use of linkages that are exclusively cleaved by eukaryotic cells to deactivate or vice versa an activation by enzymes exclusively present on prokaryotic cells or drug-resistant bacteria[150] offer further opportunities to obtain highly selective and biodegradable antimicrobial lipids. However, these strategies are pursued to a fairly low extent and, thus, have not yet found their way into clinical trials.

7. Guidelines for the Design of Biodegradable Cationic and Ionizable Cationic Lipids

7.1. Production and Scalability

To meet the need for ionizable cationic lipids, multiple contract manufacturing organizations have required the expertise to produce these excipients in a large scale.[26c] Considering the ongoing extensive research on nucleic acid-based therapeutics, however, a constantly increasing quantity of such lipids will be needed in the future. Moreover, outstanding challenges such as extrahepatic delivery are likely to require new functional properties and equally complex synthesis procedures that are yet not established as large scale process.[151] The higher the demand for such lipids, the more important is the availability of the precursors and scalability of the synthesis. Amino acids, the most common endogenous polar heads, are produced in large quantities from renewable sources like corn starch[152] or

from waste products.[153] To introduce quaternary ammonium groups, betaine and choline are preferred over synthetically quaternized amino acids to avoid halogenated alkyl compounds during manufacturing. Likewise, a great variety of hydrophobic tails can be derived from renewable feedstocks.[17,33a] Apart from providing long lasting solutions, such natural precursors mostly have common moieties for conjugation like carboxylates, hydroxyl, and amine groups. This structural similarity facilitates to translate synthesis protocols between different building blocks. Consequently, these building blocks should allow to build combinatorial libraries for the identification of new structure–function relationships and lead compounds on a small scale. However, libraries of biodegradable lipids based on endogenous building blocks are still lacking. Considering the key role of combinatorial screening approaches to identify functional lipids for nucleic acid delivery, a focus on similar approaches but using endogenous building blocks is on demand to boost the development of safe cationic and ionizable cationic lipids.

The increasing awareness for green chemistry in the industrial sector will entail large scale production of a growing number of chemicals via sustainable approaches.[33a,154] Scalability from small to large scale, as well as availability of raw materials should be considered early in the design of these excipients. Otherwise, the production of these excipients could hamper the accessibility of new therapeutics.[155]

7.2. Degradability and Safety

The currently marketed ionizable cationic lipids DLin-MC3-DMA, ALC-0315, and SM-102 demonstrate the differences in the term "biodegradability." Despite an ester linkage, DLin-MC3-DMA is not biodegradable. Consequently, it is not sufficient to merely introduce a cleavable linker but evidence for the cleavage must be provided as well. The biodegradability of the ionizable cationic lipids in the mRNA vaccines are considered a major advancement over DLin-MC3-DMA.[52] Still, in vivo-studies revealed slow clearance of ALC-0315 from the liver whereas SM-102 is rapidly cleared.[53] The lower velocity of degradation is likely attributed to two instead of one branched tail structure. Ultimately, only in vivo-studies provide certainty on the fate and toxicity of such excipients. However, the establishment of an in vitro setup, which allows to reliably assess the degradability of cationic and ionizable cationic lipids, is certainly helpful for a rational preselection of these excipients.

After degradation, the accumulation of toxic metabolites must be prevented. From a chemical point of view, the metabolites of ALC-0315 and SM-102 carrying the tertiary amine are still relatively lipophilic and require further metabolization to be excreted.[54] To prevent the rise of possibly toxic or accumulating metabolites a priori, cleavage into endogenous compounds is likely the safest way.

7.3. Functional Properties

Adjusting the pKa of the tertiary amine and the geometry of the tail were key factors to achieve highly effective functional lipids for nucleic acid delivery.[50a,83b,99,156] Fixed building blocks limit the possibilities to fine-tune the properties of the functional groups. In particular, when the requirements are well-established, deviating characteristics of the building blocks could hamper the development of new safe excipients. Nonetheless, for instance histidine exhibits properties matching the required ones and accordingly yielded encouraging in vitro- and in vivo-results.[104,157] Once the requirements for a particular application have been established, it is therefore key to find nontoxic building blocks, meeting these requirements for the development of auxiliary agents that offer improved safety without sacrificing efficacy. To ensure this, a complete characterization of new excipients must include a comparison with the current lead compounds in terms of both efficacy and safety. A determination of unified parameters like an ED_{50} and half-life in plasma and various tissues facilitates comparison between new and established auxiliary agents.

Furthermore, unique structures and interactions found in nature hold tremendous promise for the design of biodegradable cationic and ionizable cationic lipids and could boost the identification of structure–function relationships. For example, the structural features of AMP have inspired the design of new antibiotics that advanced to clinical trials.[130d,e,158] Certainly, also the drug delivery field benefits from such interactions as demonstrated by active uptake achieved with bile acids or targeting achieved with peptides and proteins.[109c,151c,159] In addition, deviations from established functions may reveal new advantages such as an increased shelf-life of mRNA.[91] Such intrinsic properties allow a rational selection of the building blocks and may improve functional properties of these lipids. However, maintained function of the respective building block must be guaranteed after synthesis. An overview of benefits and opportunities associated with natural and endogenous building blocks is given in **Figure 13**.

8. Future Trends

Having learned from established biodegradable cationic and ionizable cationic lipids, their mode of biodegradation will have to be addressed in more detail. Ideally biodegradable lipids should be stable as long as they serve for a certain purpose such as the formation of lipophilic complexes with hydrophilic macromolecular drugs or as preservatives. As soon as they have fulfilled their task, they should be degraded as rapidly as possible. This can be easily addressed in the case of complex formation with anionic macromolecular drugs because a hydrolytic cleavage of these conjugates is restricted by the lipophilic nature of these systems. A rapid degradation of the cationic or ionizable cationic lipid in lysosomes or cytoplasm is taking place after cellular uptake of these complexes. When such lipids are used as preservatives, however, we are confronted with a completely different situation. The time point of initiation and the velocity of biodegradation are keys to success for preservatives. Betaine esters, for instance, were shown to exhibit on the one hand a pronounced antimicrobial activity in alkaline media and on the other hand to be rapidly hydrolytically cleaved at pH > 5.[160] Consequently, they cannot serve as preservatives. Even not all biodegradable cationic and ionizable cationic lipids that

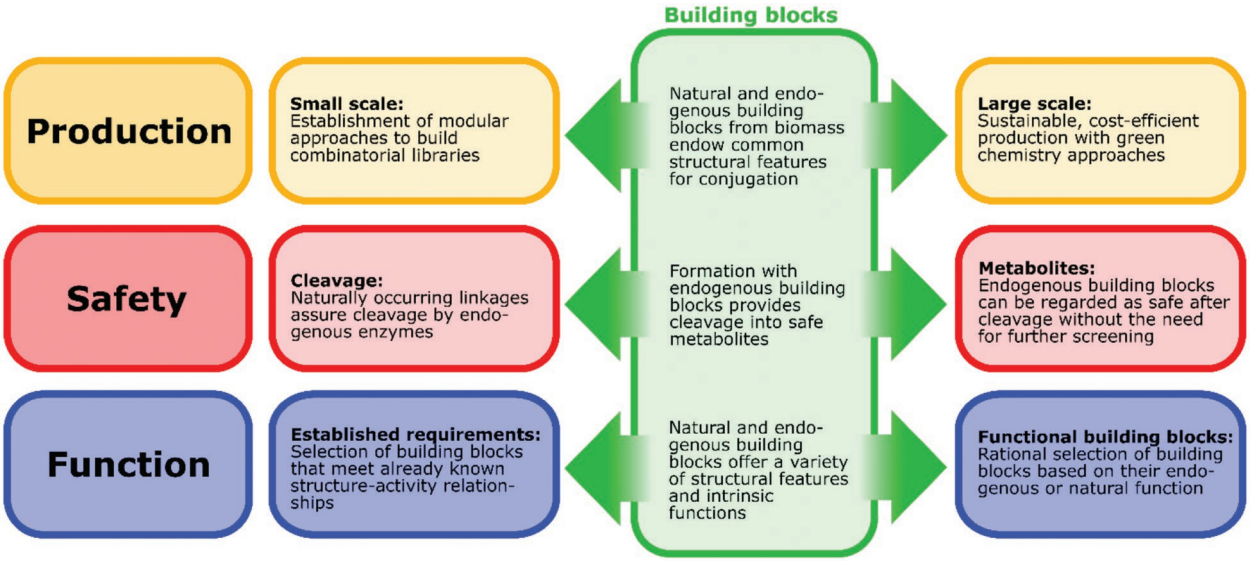


Figure 13. Benefits and opportunities offered by natural and endogenous building blocks for the design of biodegradable cationic and ionizable cationic lipids.

are stable over a broad pH range in aqueous media, can be utilized as preservatives. Only those are of relevance, which are sufficiently resistant against enzymatic degradation by microorganisms. Taking the high similarity of most microbial and human enzymes into account, this is certainly not the case for all of them. As the number of microorganisms in pharmaceutical products is low, the microbial degradation of cationic and ionizable cationic lipids during storage might have a marginal influence on their concentration in the formulation. When these lipids are more rapidly metabolized by microorganisms than they can eradicate them, however, they will not be effective at all. Bacteria expressing lactamase, for example, metabolize beta-lactam antibiotics very rapidly and consequently remain unaffected by these potent drugs.[161] Predictions for the spread of resistance suggest that we are currently moving into a post-antibiotic era[162] and that preservatives will not be spared from this development. In order to overcome such resistances, the combination of biodegradable cationic and ionizable cationic lipids that are cleaved by different enzymes might therefore be advantageous. On contrary, lipids that are degraded too slowly remaining even days after administration in significant concentrations in the human body are also not beneficial. Ethyl Nα-lauroyl-L-arginate was shown to be entirely metabolized within 12 h in humans,[163] which seems to be a good compromise between a too rapid degradation limiting antimicrobial efficacy and a too slow degradation causing adverse effects. In the long term, even the design of biodegradable cationic and ionizable cationic lipids might be possible so that this compromise is not necessary anymore. Such compounds would need to bear a cleavage site that is exclusively recognized by human enzymes and not at all by microorganisms that should be eradicated. As the registration of new pharmaceutical excipients is as complex and costly as that for new APIs, however, only very few of these novel biodegradable cationic and ionizable cationic lipids will reach the global market. Recently launched initiatives such as the Novel Excipient Review Pilot Program of the FDA (https://www.fda.gov/drugs/development-approval-process-drugs/novel-excipient-review-pilot-program) that shall foster the development of excipients for scenarios in which excipient manufacturers and drug developers have cited difficulty in using existing excipients, perhaps accelerate this process.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biodegradable lipids, cationic lipids, complex formation, ionizable cationic lipids, lipid nanoparticles, preservatives

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