Highlights:

- Progress in the rational design of cationic lipids
- Outlook on the influence of cationic lipid domains on nucleic acids complexation and delivery
- Outlook on the influence of cationic lipid composition on its ultimate geometry and supramolecular structures of lipoplexes

Review Article

Title:

Cationic lipids for gene delivery: many players, one goal

Authors' List

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Abstract

Lipid-based carriers represent the most widely used alternative to viral vectors for gene expression and gene silencing purposes. This class of non-viral vectors is particularly attractive for their ease of synthesis and chemical modifications to endow them with desirable properties. Despite combinatorial approaches have led to the generation of a large number of cationic lipids displaying different supramolecular structures and improved behavior, additional efforts are needed towards the development of more and more effective cationic lipids for transfection purposes.

With this review, we seek to highlight the great progress made in the design of each and every constituent domain of cationic lipids, that is, the chemical structure of the headgroup, linker and hydrophobic moieties, and on the specific effect on the assembly with nucleic acids. Since the complexity of such systems is known to affect their performances, the role of formulation, stability and phase behavior on the transfection efficiency of such assemblies will be thoroughly discussed. Our objective is to provide a conceptual framework for the development of ever more performing lipid gene delivery vectors.

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Abbreviations:

NAs, nucleic acids; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; ASO, anti-sense oligonucleotide; CMC, critical micellar concentration; SAR, structure-activity relationship; DOTMA, 2-di-O-octadecenyl-3-trimethylammonium propane; DOTAP, 1,2-dioleoyloxy-3-[trimethylammonium]-propane; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; Chol, cholesterol; GSH glutathione; DSC, differential scanning calorimetry; SAXS, small angle X-ray scattering; CR, charge ratio; CME, clathrin-mediated endocytosis; CvME, caveolae-mediated endocytosis

Keywords: non-viral gene delivery; cationic lipids; lipoplexes; transfection; structure-activity relationship; complexation

1. Introduction

Over the last few decades, the delivery of nucleic acids (NAs) as molecular therapeutics has gathered much attention. The concept of introducing exogenous NAs into host cells (i.e., transfection) has been extensively exploited to tune the expression of specific proteins in target cells for manifold purposes (Buck et al., 2019). For instance, gene delivery means are nowadays essential for the exploitation of some therapeutics, such as those relying on the use of the CRISPR-Cas9 gene editing technology (Ahmad and Amiji, 2018) or gene vaccines (Jackson et al., 2020; Walsh et al., 2020).

As a rule of thumb, gene delivery techniques and technologies allow the delivery of NAs, including plasmid DNA (pDNA) and messenger RNA (mRNA), as well as short regulatory RNAs, such as small interfering RNA (siRNA), micro RNA (miRNA) and short hairpin RNA (shRNA), and anti-sense oligonucleotides (ASOs) (for additional information please refer to (Duvall et al., 2013; Giacca, 2010; Ginn et al., 2018)). Despite the delivery of naked NAs represents the safest way to transfect cells, such procedure is rather ineffective because they are inherently anionic at physiological pH such that they cannot passively enter cells. In addition, in this form they are very prone to nucleases-mediated degradation (Al-Dosari and Gao, 2009). Hence, the main challenge facing us is to develop more and more effective and little toxic delivery means that protect and facilitate the transfer of NAs into target cells. The delivery technologies developed so far belong to one of these classes, namely i) physical methods (Mellott et al., 2013) and ii) vectors (Bono et al., 2020; Lukashev and Zamyatnin, 2016).

Physical methods rely on the application of exogenous physical stimuli that allow NAs to cross the cell membrane and reach the cytosol (for instance inducing a transient disruption of the plasma membrane) and/or the nucleus (e.g., by means of microneedles) without the use of any carrier (Wells, 2004). Although such methods have been found somewhat effective (Mehier-Humbert and Guy, 2005), major drawbacks relying on their inherent toxicity and the *in vivo* translatability have limited their widespread application as well.

Conversely, vectors are vehicles able to shield the NAs into particle-like assemblies and ferry them into cells (Patil et al., 2019; Pezzoli et al., 2012; Pezzoli and Candiani, 2013). Vectors are broadly categorized as viral and non-viral. Viral vectors, that is, engineered viruses in which a gene cassette encoding desirable traits is in place of the viral genome, are at present the most effective NAs vehicles, because they take advantage of the inherent ability of wild-type viruses to productively infect cells (for additional information on viral vectors please refer to (Finer and Glorioso, 2017; Lukashev and Zamyatnin, 2016; Mancheño-Corvo and Martín-Duque, 2006)). However, some drawbacks related to viral tropism (i.e., the specificity of a virus for infecting a particular cell type), inflammatory potential, rather limited packing capacity and poor safety profile, have prompted the search for the other class of gene carriers (Jin et al., 2014). Cationic lipids and polymers have gained increasing attention and have thus become the most studied and used vectors (Bono et al., 2020). These carriers spontaneously self-assemble with anionic NAs through electrostatic interactions to form nano- or microparticles, namely lipoplexes and polyplexes, respectively, which provide NAs protection against nuclease degradation and drive the genetic cargo into cells. The main reasons why non-viral vectors are really on the rise rely on the greater packing capacity as compared to viral counterparts and, even more exciting, on the ease of tailoring most of their specific features (e.g., size, charge, molecular structure) in order to tune and improve their gene transfer behavior (Hill et al., 2016).

With this in mind, this review takes stock of cationic lipids for non-viral gene delivery. They are positively charged amphiphiles with a molecular architecture somewhat similar to that of natural lipids, with the major difference being the cationic headgroup (Martin et al., 2005; Niculescu-duvaz et al., 2003; Rao, 2010). When exposed to an aqueous environment and above a certain critical micellar concentration (CMC), they spontaneously arrange in intriguing three-dimensional (3D) assemblies, namely lamellar, micellar, or inverted hexagonal phases, depending on the composition and structure of the lipid itself (Wasungu and Hoekstra, 2006). Since the seminal work of Felgner and colleagues in late 80s about the use of cationic lipids for lipofection (i.e., the transfection of cells using lipid-based transfectants) (Felgner et al., 1987), much has been done in the way of developing a number of lipids and lipid formulations for the delivery of a wide range of NAs of different molecular weight (M_w), including large pDNAs (M_w < 10 kbase pairs) (Buck et al., 2019; Hirko et al., 2005) and messenger RNAs (mRNAs) (M_w < 10 kbases) (Guan and Rosenecker, 2017; Hajj and Whitehead, 2017), and short sequences such as siRNAs ($M_w \approx$ 15-30 mers) (Rietwyk and Peer, 2017; Shim et al., 2013; Zhang et al., 2007). Broadly speaking, cationic lipids consist of three different domains, that is, a *cationic headgroup*, covalently bound through a *linker* to a *hydrophobic tail* (Martin et al., 2005) (**Figure 1**). Interestingly, each of them plays a pivotal role in the delivery process of NAs, so that each and every of these moieties can be suitably tailored to fine-tune the behavior of the resulting complexes. It is worthy of note that, even if the effect of each domain on the overall lipoplex behavior has been extensively but separately studied (Zhi et al., 2018, 2013, 2010), the joint effects of the combination of the three have seldom been faithfully depicted, so that the reader may find it difficult to see the forest and the trees. Nonetheless, due to the inherent complexity of the cells-to-lipoplexes interplay and *vice versa*, a comprehensive big picture depicting reciprocal interactions between such living and non-living matter is far from being drawn up (Ewert et al., 2010). In this context, the thorough understanding and knowledge of the uptake mechanisms of transfectant/NAs particles and their intracellular trafficking would lead to the rational design of more effective non-viral lipid-based vectors.

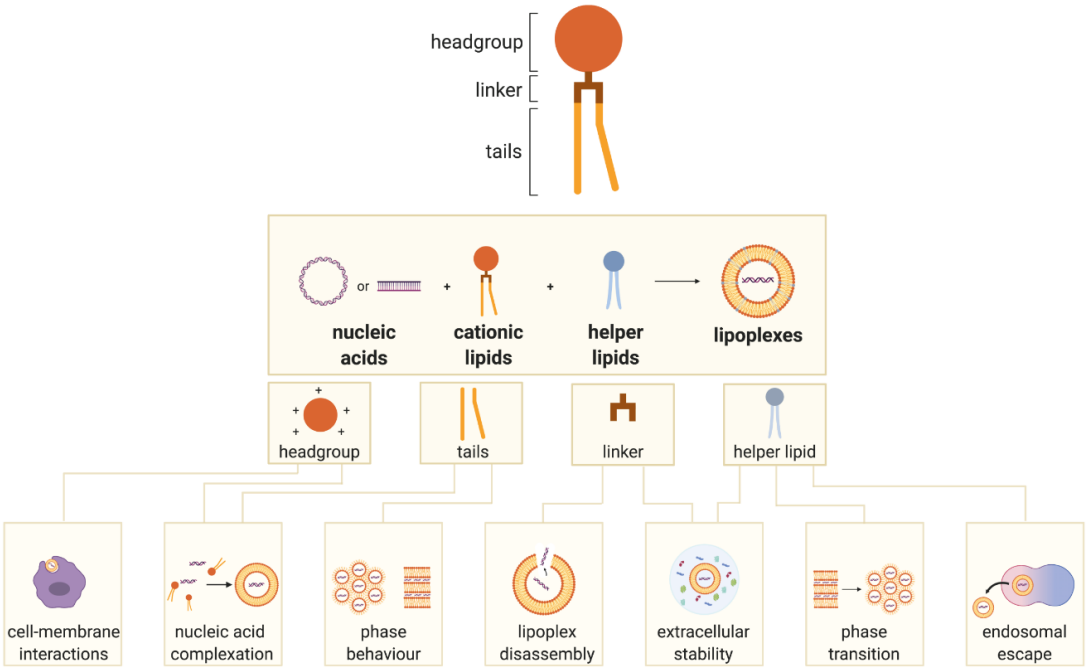

Figure 1 - Schematic representation of the three basic domains of a cationic lipid and their role in the complexation and delivery of nucleic acids.

2. Molecular structure of cationic lipids

2.1. *Headgroups*

Polar headgroups play a prominent role in binding anionic NAs by means of electrostatic interactions and give rise to complexes, the so-called lipoplexes, made of cationic lipids and NAs. The headgroup features, such as dimension and charge density, are responsible for the lipoplex stability, interaction with the cell membrane and endosomal escape mechanisms, along with NAs compaction, that is, they have a huge impact on the overall performance of lipoplexes (Zhi et al., 2013).

Depending on the chemical composition, the most prominent classes of polar heads are quaternary ammonium salts, amines (primary, secondary, and tertiary), guanidine, heterocyclic compounds, and a combination thereof (**Figure 2**). More recently, the rational design of novel cationic lipids bearing biomacromolecular headgroups has led to the rise of novel multifunctional carriers with unique delivery properties (Ortiz Mellet et al., 2010).

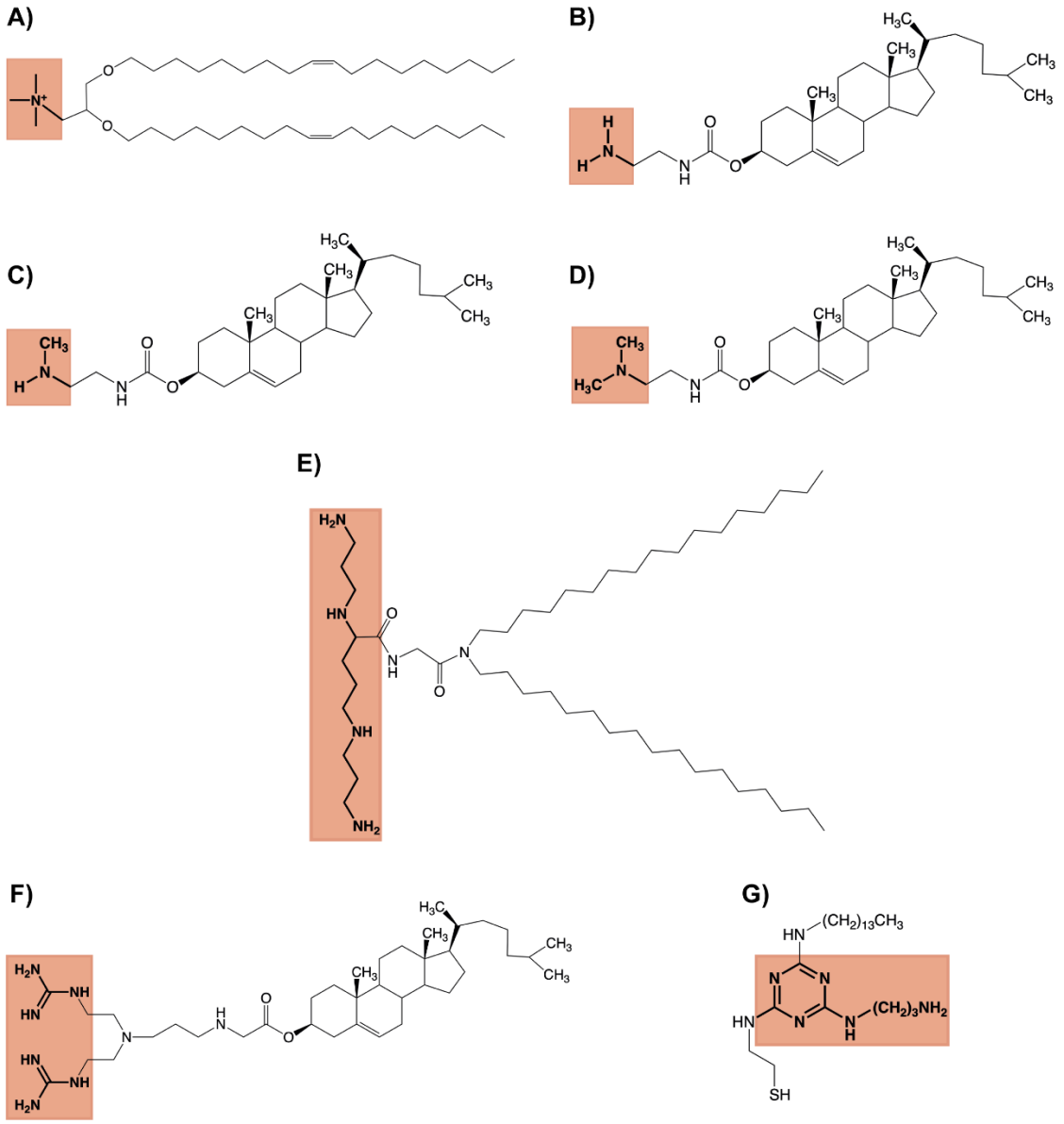

Figure 2 - Chemical structure of cationic lipids with different headgroup domains, namely: A) DOTMA; B) AC-Chol; C) MC-Chol; D) DC-Chol; E) DOGS; F) BGTC; G) triazine ring-based cationic lipid. Specifically, the headgroup domain of each lipid consists of a (A) quaternary ammonium salt, (B) primary amine, (C) secondary amine, (D) tertiary amine, (E) polyamine, (F) guanidinium group, and a (G) melamine group (i.e., a heterocycle). Colored areas highlight polar headgroups.

Cationic lipids bearing multivalent headgroups have been proposed as effective transfectants because able to bind NAs tightly, pack and seclude them from the intracellular environment, so that they are considered more effective in transfection than their monovalent counterparts (Koynova and Tenchov, 2011; Rosenzweig et al., 2001).

When in solution, the NAs binding properties of the polar head are strongly dependent on the pH of the solution, as this influences the protonation of the headgroup itself. The best way to get insights into the acid-base behavior of a cationic headgroup relies on the acidic dissociation constant (pK_a) of the conjugate acid. Specifically, the higher the pK_a of the conjugate acid, the stronger the base. In practice, when cationic lipids are dissolved at pH < pK_a, their headgroups are protonated, otherwise called cationic, such that the electrostatic interaction with the NA counterions occurs.

Furthermore, the presence of protonatable groups within the headgroup chemical structure may confer to the amphiphile some buffering activity that can be conveniently exploited to favor NAs release once in the endosome. For instance, when in the acidic environment of the endosome (pH= 5.5-6), weakly basic sponges display a H^+ buffering activity, thus resulting in a Cl^- accumulation within the endosomal compartment, ultimately leading to the swelling of the vesicle (Budker et al., 1996; Freeman et al., 2013). Since the poor release of the genetic material from endosomes is believed to be one of the most critical issues hindering the gene transfer efficiency of lipoplexes, cationic lipids able to exploit such osmotic-driven process, generally known as proton sponge effect, are widely preferred to strong bases indeed.

In this light, the headgroup chemistry is a key aspect in lipid design.

2.1.1. Quaternary ammonium

Quaternary ammonium (NR_4^+) is an organic cation carrying a permanently positively charged nitrogen atom (N) covalently bound to four organic substituents (R) (**Figure 2**). Due to their lasting positive net charge at physiological pH, which allows for strong NAs binding and high solubility in aqueous environments (Dizman et al., 2004), quaternary ammonium headgroups are so far the most frequently used polar heads to build cationic lipids (Sakurai et al., 2000). The wide range of gene delivery vectors bearing a quaternary ammonium headgroup includes 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) (Felgner et al., 1994) depicted in **Figure 2A**, 1,2-dioleoyloxy-3-[trimethylammonium]-propane (DOTAP) (Stamatatos et al., 1988), dimethyl-dioctadecyl ammonium bromide (DDAB) (You et al., 1997) and cetyl-trimethyl ammonium bromide (CTAB) (Pinnaduwage et al., 1989). The former molecule (**Figure 2A**) was the first quaternary ammonium-bearing lipid to be synthesized and it is perhaps the most renowned among them all.

In order to strengthen the NAs binding behavior of cationic lipids and foster cell membrane interactions of lipoplexes, some authors devised the hydroxylation of the quaternary ammonium head of lipid vectors (Berchel et al., 2015; Felgner et al., 1994). Along with those first evidences, recent works have confirmed the improved transfection abilities arising from such molecular modification with respect to the quaternary ammonium-bearing precursors. For instance, Maiti and colleagues synthesized novel *gemini* surfactants with hydroxyl-modified quaternary ammonium headgroup, and speculated that the poor hydration of the head region, that is, its low cross-sectional area, was responsible for the higher transfection efficiency the lipids displayed when compared to the commercially-sourced Lipofectamine 2000 (Maiti et al., 2018). In turn, the decrease in the headgroup hydration resulting from the hydrogen bonding between juxtaposed hydroxyl groups was thought to drive the formation of lipoplexes which were more prone to phase transition, such that they eventually fuse with the endosome and release the NAs (Jones et al., 2013; Zhang et al., 2014). Conversely, along

with this destabilizing effect, quaternary ammonium headgroups bearing hydroxyls have proved to condense more effectively NAs due to the formation of hydrogen bonding with NAs, such that the stability of complexes was improved (Narang et al., 2005).

2.1.2. Primary amines, secondary amines, tertiary amines and polyamines

Amines are derivatives of ammonia classified as primary, secondary, or tertiary whether one, two or three hydrogen atoms have been replaced by an organic group (**Figure 2B-D**). Although their acid-base properties are strongly dependent on the number and type of substituents, amines are generally considered as weak bases. Broadly speaking, secondary amines (**Figure 2C**) are slightly more basic than primary ones (**Figure 2B**), while tertiary amines (**Figure 2D**) are less basic than their secondary analogues due to the steric hindrance of their substituents. The amines basicity mirrors their pK_a values, that is, 10.6, 10.8 and 9.8 for primary, secondary and tertiary amines, respectively, when the substituent is the simple methyl group (Hall, 1957). Since amine headgroups invariably exhibit a neutral or low cationic charge at physiological pH, cationic lipids bearing such kinds of cationic heads have long half-life in the body circulation, but also a relatively poor NAs binding ability (Buck et al., 2019).

In an attempt to shed light on the transfection effectiveness of the differently substituted amine-bearing lipids, Kearns *et al.* synthesized a whole array of cationic cholesterol (Chol)-based derivatives and found that primary and secondary amines were the most effective (Kearns et al., 2008). More recently, these results were eventually proven wrong by Lin and colleagues who showed that tertiary amines featured as the most effective transfectants (Lin et al., 2019; Liu and Huang, 2010; Semple et al., 2010). Interestingly, because tertiary amines are weaker bases, it has been hypothesized that they undergo protonation in an acidic environment, thus conferring lipids with some buffering capacity that is beneficial for endo/lysosome escape and NAs release within the cell.

Polyamines-bearing lipids have emerged as promising transfectants as well (Vijayanathan et al., 2014). As an example, Cooper and co-workers reported that the newly synthetized penta-amine N^{15}-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (CTAP) showed a 100-fold increase in transfection efficiency with respect to conventional DC-Chol/1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) formulation (Cooper et al., 1998). Because CTAP displays a high charge density, which is dependent on the number of total amine groups that are fully protonated at physiological pH, the authors speculated that this would lead to a more effective neutralization, condensation and encapsulation of NAs (Stewart et al., 2001). Apart from the charge density, Martin *et al.* pointed out the importance of the headgroup shape on the transfection behavior of polyamine-containing amphiphiles (Martin et al., 2005). Although branched polyamines are less prone to folding problems, that is, they display the most stable conformation (Fujiwara et al., 2000; Zhi et al., 2013), they are generally less efficient than linear compounds. Byk *et al.* compared cationic lipids having linear, T-shaped, branched and globular headgroups, and reported that the former showed the highest NAs condensation ability and transfection efficiency *in vitro* (Byk et al., 1998). Since the seminal work by Behr's team about the effective linear polyamine-bearing lipid dioctadecylamidoglycylspermine (DOGS) (**Figure 2E**) (Behr et al., 1989), spermine headgroup has thus been extensively exploited to design gene delivery vectors (Markov et al., 2012; Maslov et al., 2012; Niyomtham et al., 2015).

2.1.3. Guanidinium headgroup

Guanidine is a strong organic base typically present in arginyl residues involved in DNA-binding histones (Goebel and Klapo, 2007). It is worthy of note that guanidine ensures strong

NAs binding properties (Cotton et al., 1973; Wender et al., 2008) because it exists almost exclusively in the form of guanidinium cation over a wide range of pH (Xu et al., 2017), with the charge delocalized over three N that donate lone pair electrons at once (**Figure 2F**). Guanidinium-bearing carriers are also able to form bidentate hydrogen bonding with the anionic phospholipids of the cell membrane, which favors the internalization of lipoplexes into cells (Rothbard et al., 2005). Nonetheless, it has also been reported that guanidinium cation may negatively impact the effectiveness of gene delivery systems because of the (too) tight binding with NAs (Bono et al., 2019).

From an historical perspective, bis-guanidinium-spermidine-Chol (BGSC) and bis-guanidinium-tren-Chol (BGTC) (**Figure 2F**) were the first two guanidinium-bearing lipid transfectants developed (Vigneron et al., 1996). Multivalent forms arising from the conjugation of the guanidinium group with other cationic moieties, such as pyridinium and amines, have proven to be more efficient in transfection and less cytotoxic than their monovalent analogues (Banerjee et al., 2001). As an example, Huang's group synthesized the cationic lipid N,N-distearyl-N-methyl-N-2[N′-(N^2-guanidino-l-lysinyl)] aminoethyl ammonium chloride (DSGLA) with a dual head displaying both guanidinium and lysine residues, and showed that it gave rise to a more effective siRNA binding as compared to the conventional DOTAP (Chen et al., 2010).

One of the most relevant chemical variations of the guanidinium headgroup relies on the use of amidine substituents, which are organic compounds having the general formula $RC(NR')NR''_2$. They are obtained through the replacement of an amine of the guanidine with a generic R group. Depending on the substituent, the pK_a may vary from 5 to 12, while protonation invariably occurs at the imino nitrogen (=NR') (Quek et al., 2013). In this regard, the patented *gemini* cationic surfactant known as TRX, which bears an amidine in the head region, did induce a higher percentage of GFP-expressing cells and showed reduced cytotoxicity than the gold standard Lipofectamine (Koiwai et al., 2005).

2.1.4. Heterocyclic headgroups

Pyridine, imidazole and their derivatives have been widely used as cationic heterocyclic headgroups. Due to their chemical nature, they can act as acids as well as bases, with imidazole being 100 times more basic than pyridine (Ouellette and Rawn, 2018). Once in acidic solutions, pyridine and imidazole become protonated and give rise to pyridinium and imidazolium ions, respectively. Of note, the delocalization of the positive charge throughout the heterocycle results in a slightly hydrophobic head, which improves the NAs binding-release behavior (Gaitor et al., 2017) by acting as pH-sensitive moiety. In this regard, relying on such peculiar feature, Liu *et al.* synthesized a series of cyclen-based cationic lipids bearing an imidazole headgroup (Liu et al., 2013). Of note, being the pK_a of this moiety very close to the endosomal pH, these lipids were able to give rise to the proton sponge effect and to efficiently transfect cells. Likewise, Berchel and co-workers synthesized α-amino-phosphonate lipids displaying two pH-sensitive moieties that were in the protonatable aza-heterocycle (imidazole or pyridine) (Berchel et al., 2017). Acting as weak bases, such compounds are mostly neutral at physiological pH, while they become cationic when the pH drops to 6 and even lower. Again, this resulted in the escape of lipoplexes from the endosomes. It has been recently proposed that the coupling of different polar heads, such as 1,2,3-triazolium and the conventional quaternary ammonium headgroup, could give rise to multi-cationic lipids very effective for gene delivery applications (Gosangi et al., 2017).

Besides, among heterocyclic groups, melamine is an organic nitrogenous compound used in the production of plastics, dyes, fertilizers, fabrics, and it is part of the core structure for a

number of drugs. Due to delocalized cationic charge of the s-triazine ring and the easy N-derivatization of melamine scaffold with different substituents, melamine has also been profitably used to synthesize cationic lipids for gene delivery (**Figure 2G**) (Candiani et al., 2007). Likewise, triazacyclononane (TACN)-based lipids have emerged as suitable transfectants as well. Bearing three N with different pK_a values, this class of lipids exhibited delivery abilities comparable to that of Lipofectamine 2000. The reason for that relies on the presence of a N that, at physiological pH, confers some basicity (pK_a = 11) to the lipid and promotes interactions with the DNA, while the other N-based groups have $pK_a \approx 6$ and provide some buffering ability to lipoplexes once they are in the endosomes (B. Wang et al., 2014; Zhang et al., 2011).

2.1.5. Multifunctional headgroups

Despite the vast majority of cationic lipids have been designed with amine-derived headgroups, it is generally thought that amino acid-based transfectants can achieve similar or even higher transfection efficiency and lower cytotoxicity than commercially sourced lipid transfectants, such as DOTAP and Lipofectamine (Brito et al., 2009; Zhang et al., 2014; Zhao et al., 2017). Lysine, arginine, histidine and ornithine have been extensively used as polar headgroups for the synthesis of lipidic transfectants (Zillner et al., 2012). In this direction, Silva *et al.* synthesized a series of serine-derived *gemini* surfactants, which have been used alone or in combination with the helper lipid DOPE to condense pDNA and transfect cells with some success (Silva et al., 2014, 2013). In an attempt to select the most effective amino acid to be used as the polar head, Obata and co-workers synthesized a series of cationic lipids bearing lysine, histidine and arginine (Obata et al., 2008). Notably, they showed that histidine-bearing lipids were not suited to form lipoplexes. This was probably because of the low basicity of the tertiary amino group of histidine that would account for weaker interactions with NAs than the primary amino group of lysine or arginine. Other authors reported similar results and provided some evidence about the higher transfection efficiency of lysine- and arginine-bearing cationic lipids with respect to histidine analogues (Jiang et al., 2016; Sheng et al., 2014). It is worthy of note that the amino acid lysine is sensitive to pH changes due the presence of two protonatable amines displaying different pK_a values. In this light, Walsh and co-workers reported the synthesis of a series of ionizable lysine-based lipids that exhibited a pH-dependent protonation behavior (Walsh et al., 2013). At physiological pH such lipids did proficiently interact with siRNA, but when endocytosed, the lipids became increasingly cationic and were able to disrupt the endosomes because of the lowering of the pH.

Amphiphiles with di- or tripeptide headgroups have also been largely investigated. For instance, Zhao and co-workers developed a tri-ornithine peptide-bearing cationic lipid (Zhao et al., 2017), that was found to be far more effective than the quaternary ammonium-bearing counterpart in binding and delivering NAs to cells. Of note, such behavior has been attributed to the presence of the tri-ornithine peptide that does undergo protonation at different pH and facilitate the endosomal escape of lipoplexes.

Aminoglycosides-bearing lipids have been found very effective as well. Because aminoglycosides are a heterogeneous class of polycations with strong NAs binding ability (Arya et al., 2001; Bono et al., 2019; Ghilardi et al., 2013) and renowned antibacterial properties (Bera et al., 2010; Fosso et al., 2014; Houghton et al., 2010), they have been extensively used to give rise to multivalent gene delivery vectors. Just to give some examples, Lehn's group synthesized and patented very effective lipid derivatives using paromomycin-, neomycin B, and kanamycin A as headgroups (Mével et al., 2012; Sainlos et al., 2003). More recently, our group synthetized an array of aminoglycosides-modified calixarene lipids that

showed transfection efficiencies similar or even higher than that of the gold standard branched polyethylenimine (*b*PEI), low cytotoxicity and excellent antimicrobial properties against Gram-negative bacteria (Bono et al., 2018).

Altogether, these findings pointed to ionizable headgroups as the most efficient moieties in the design of lipids for gene delivery applications. It has been shown that they endow lipoplexes with endosomal membrane-destabilizing properties that, in turn, are responsible for the ultimate NAs release in the cytoplasm. As a matter of fact, cationic lipids bearing protonatable headgroups with pK_a < 7 (e.g., primary and tertiary amines, imidazole and pyridine) are by far the most effective in transfection.

2.2. Linkers

Being the *trait d'union* between the hydrophilic polar headgroup and the non-polar tail(s), the linker moiety plays a pivotal role in the behavior of the cationic lipid as a whole, such as the stability, biodegradability, cytotoxicity, and transfection efficiency (Pezzoli et al., 2012; Srinivas et al., 2009). In practice, some features of the linker, including the overall charge, length and steric hindrance, are responsible for the conformational flexibility of the amphiphile. In other words, the relative orientation of the hydrophobic and cationic moieties affects the interaction of the lipid with NAs, and in turn, the ultimate gene transfer efficiency (Buck et al., 2019; Draghici and Ilies, 2015; Fujiwara et al., 2000).

Depending on the structure, linkers are grouped into many types, such as ethers, esters, carbamates, and amides (**Figure 3**). Of note, some of them have been engineered to be sensitive to specific stimuli, including pH and redox variations and the action of enzymes, underpinning behavioral responsiveness to environmental cues (Candiani et al., 2007; Guo et al., 2014; Nagasaki et al., 2003; Terada et al., 2006).

The most significant advances in the linkers design are highlighted and thoroughly discussed here below.

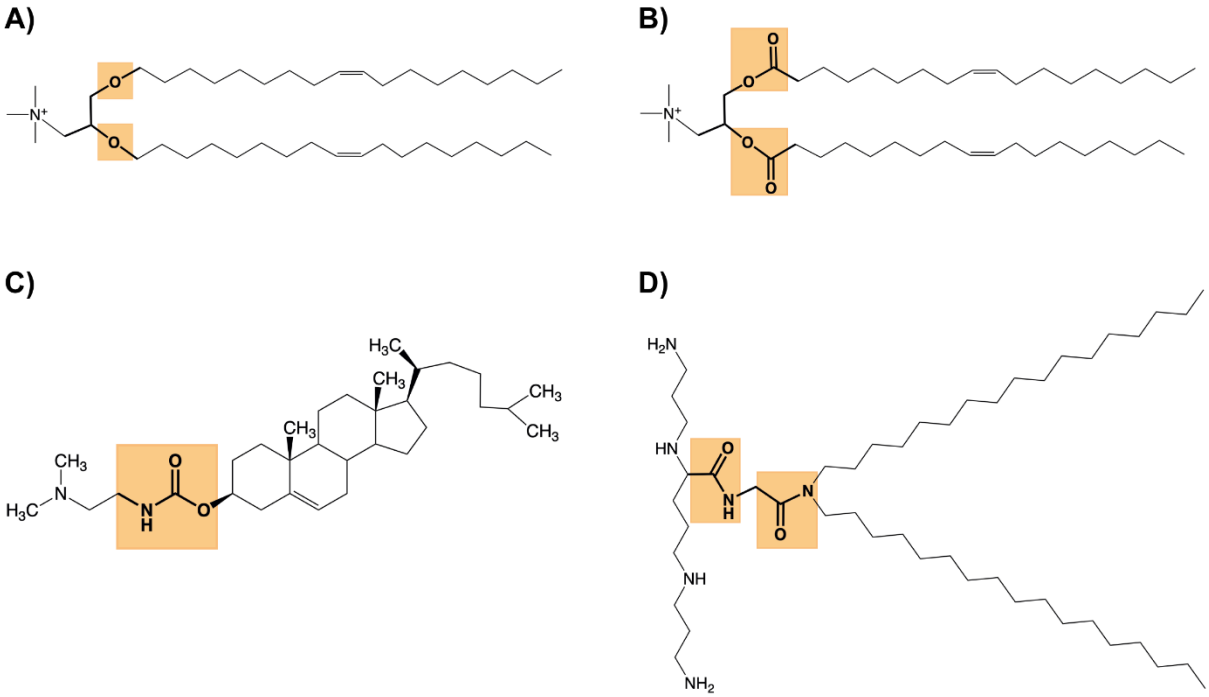

Figure 3. Chemical structure of cationic lipids displaying different linkers, namely: A) DOTMA; B) DOTAP; C) DC-Chol; D) DOGS. Specifically, the headgroup region is connected to the hydrophobic portion(s) by means of a (A) ether, (B) ester (C) carbamate, and (D) amide. Colored areas highlight linkers.

2.2.1. Ethers

Ether bonds are characterized by the presence of an oxygen (O) atom linked to two alkyl groups (**Figure 3A**).

It has been shown that the transfection efficiency of the diether-linked cationic lipid, DOTMA (**Figure 3A**), was 10-fold more effective than that of the diester-containing lipid analogue, DOTAP, when lipoplexes were formed without the addition of helper lipids (Song et al., 1997). By the same token, Ghosh and colleagues reported that ether-containing lipids were much more effective in transfection than the easily-degradable ester- and carbamate-bearing lipids (Ghosh et al., 2000). Despite the superior transfection efficiency of cationic lipids bearing ether linkages, such transfectants do not undergo substantial hydrolysis *in vitro* and *in vivo* (White et al., 1996), which results in some cytotoxicity (Zylberberg et al., 2017). Indeed, because of the high chemical stability of ethers, the cleavage of the C-O bond is uncommon in the absence of specialized reagents or under extreme conditions.

So far, a number of strategies have been envisioned in order to enhance the biodegradability of common ether linkages. Among stimuli-sensitive linkers, acid-labile vinyl-ethers have been widely exploited. The hydrolysis rate of this kind of ethers displays a pseudo first-order dependence on the pH, as the reaction rate accelerates approximately to an order of magnitude for each unit of pH reduction (Gerasimov et al., 1997). In the acidic milieu of lysosomes (pH ≈ 4.5), the hydrolysis of the linker occurs through the protonation of the β-carbon of the vinyl-ether (Meyer and Wagner, 2006), which favors some lipid structural changes leading to the release of NAs (Shin et al., 2012, 2003). One practical and most successful example of cationic lipid bearing vinyl-ether groups is represented by O-(2R-1,2-di-O-(1Z,9Z-octadecadienyl)-glycerol)-3-N-(bis-2-aminoethyl)carbamate) (BTCA), which has been found to mediate endosomal escape of lipoplexes (Sullivan et al., 2002). Recently, in order to improve the overall biocompatibility of α-tocopherol-based cationic amphiphiles, Patri's group used ether-β-hydroxy-triazole linker that underwent total hydrolysis at the endo-lysosomal pH. This resulted in the endosomal escape of NAs that, in turn, gave rise to transfection efficiencies similar to that of Lipofectamine 3000 and very low cytotoxicity (Muripiti et al., 2018).

2.2.2. Esters

Esters are carboxylic acid derivatives in which the hydroxyl group is replaced by an O-alkyl (alkoxy) moiety (**Figure 3B**). Acting as hydrogen-bond acceptors, esters are very soluble in water. Of note, ester linkages are prone to both acidic intracellular hydrolysis and endogenous esterase- or lipase-mediated cleavage (Speight, 2017). This kind of linker is therefore easily (bio)degraded. A typical example of ester-bearing cationic lipid is DOTAP in **Figure 3B**. Given the very promising results in transfections (Fletcher et al., 2006; Leventis and Silvius, 1990), different DOTAP-based lipoplexes entered preclinical and clinical trials (Firouzmand et al., 2013; Lu et al., 2012). On the other hand, the poor chemical stability of the vectors displaying this kind of linkage may even undermine their overall delivery efficacy (Sun et al., 2013). In this regard, a significant reduction in transfection efficiency was reported when alkoxy linker in the 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA) lipid was replaced with an ester linker (Semple et al., 2010).

In an effort to develop pH-sensitive lipoplexes for efficient gene delivery, some scientists have developed novel cationic lipids incorporating ortho-ester linkages. Being one of the most acid-labile linkers, ortho-esters show strong pH-responsiveness, along with great biocompatibility. As an example, Chen *et al.* showed that, at low pH, transfectants bearing ortho-ester linkages were prone to acidic hydrolysis and split apart, such that the interactions

between cationic lipids and the DNA became so weak that the genetic cargo was promptly released into the cytosol (Chen et al., 2013, 2007).

2.2.3. Carbamates (or urethanes)

Lipoplexes based on cationic lipids incorporating carbamate linkers, also referred to as urethanes, exhibit improved stability with respect to simple ester counterparts. This results in excellent transfection properties and poor cytotoxicity (Jin et al., 2014). A typical example of carbamate-bearing cationic lipid is DC-Chol in **Figure 3C**. Similar to the other linkers, also carbamates undergo hydrolysis in the endosomal compartment because sensitive to pH shifts. In addition, Gao and Huang suggested that carbamate linkers are promptly cleaved by intracellular esterases (Gao and Huang, 1991), so that such kind of linkers are biodegraded once having entered the cells. All these features have spurred more interest in the design of cationic lipids with these linkers.

Medvedeva and colleagues first reported about the superior transfection behavior of carbamate-containing lipids as compared to ether, ester analogues and even the gold standard Lipofectamine. Interestingly, such lipids were found to be less cytotoxic than the other counterparts (Medvedeva et al., 2009). Similar results were also reported by other groups dealing with carbamate-containing *gemini* quaternary ammonium headed lipids (Shi et al., 2016; Zhao et al., 2014). On the basis of their high transfection efficiency and stability in extracellular fluids, lipids with a carbamate linker entered gene therapy clinical trials (McLachlan et al., 2011; Zabner et al., 1997).

2.2.4. Amides

An amide linkage is a covalent bond occurring in peptides and proteins, with the general formula sketched in **Figure 3D**. A representative cationic lipid with an amide linkage is DOGS**,** in which the linker is used to tether the saturated alkyl chain and the spermine headgroup (Behr et al., 1989). DOGS-based lipoplexes have been reported to be much more effective in transfecting cells when compared to DOTAP-based analogues (Paliwal et al., 2015), and this may be due to their pH-buffering behavior.

As a rule of thumb, amide hydrolysis occurring in the acidic environment of the endosome was found to be very similar to that of ester linkers, that is, the mechanism consisting in the protonation of the O atom of the amide followed by the attack of water on the carbonyl carbon, giving rise to a carboxyl acid and an ammonium salt (O'Connor, 1970). Similarly to carbamates, lipoplexes prepared with pH-sensitive amide-bearing cationic lipids possess superior stability and reduced cytotoxicity with respect to those containing ester and ether linkages (Ghosh and Brindisi, 2015). In this light, Vacus and co-workers suggested that the ability of amide linkers to form intermolecular hydrogen bonding is responsible for the high melting temperature of the lipids and for the lipoplex stability (Boukhnikachvili et al., 1997). Such speculations were later confirmed by Gopal *et al.*, who demonstrated that amide-bearing cationic lipids were much more stable and effective in transfection than ester-tethered transfectants (Gopal et al., 2011).

Besides, it has been shown that the relative orientation of the amide linker with respect to the cationic headgroup has a striking effect on the transfection behavior of lipoplexes (Srujan et al., 2011; Vijay Darshan et al., 2014). Specifically, the presence of reverse isomeric amide groups in the lipid structure showed reduced transfection efficiency with respect to normal-oriented amide ones (i.e., the amine group of the amide is in close proximity to the polar head), which, in turn, proved to be as effective as Lipofectamine 2000 in transfecting a wide variety

of cells. This was conceivably due the higher rigidity of inverse amide-containing lipids due to the Coulombic repulsion of the two moieties (Srujan et al., 2011).

2.2.5. Miscellaneous

Although less common, pH-sensitive, acid-labile linkers with hydrolysis rates similar to those of carbamates and vinyl-ethers are acetals/ketals (Semple et al., 2010; Zhu et al., 2002) and hydrazones (Aissaoui et al., 2004).

Of note, since ketal bonds display a higher hydrolysis rate at endosomal pH as compared to neutrality, cationic lipids incorporating this linkage were found to be stable in blood (Zhu et al., 2002). Likewise, Aissaoui *et al.*, studied the sensitivity of the acylhydrazone function to the acidic environment of lysosomes and showed that the hydrolysis rate increased under such conditions (Aissaoui et al., 2004).

In addition to acid-labile linkers, redox-sensitive disulfide (-S-S-) linkers are amongst the most appealing options in order to achieve a spatially and temporally controlled intracellular cleavage of cationic lipids, lipoplex disassembly, and NAs release. Some evidence suggests that reducible disulfide bonds in lipids undergo intracellular reduction owing to the presence of reducing agents (Zhang et al., 2012). Therefore, from the inception of the first cationic lipid containing a S-S linkage, namely 1,2-dioleoyl-sn-glycero-3-uccinyl-2-hydroxyethyl disulfide ornithine (DOGSDSO) (Tang and Hughes, 1998), an increasing number of disulfide-bearing lipids have been synthesized (Shirazi et al., 2011; Wetzer et al., 2001). To this aim, our group designed a SS14 bioreducible *gemini* surfactant that has been used to shed light on the mechanism of action of redox-sensitive transfectants (Candiani et al., 2010, 2008). Of note, we found that the effectiveness of SS14 was strictly dependent on intracellular glutathione (GSH) levels.

Lipids bearing disulfide linkers have also been profitably used to deliver siRNAs and silence genes (M. Wang et al., 2014). In this regard, Gujrati *et al.* demonstrated that the use of the sulfur-containing amino acid cysteine in the linker domain did not only control the release of siRNA in the cytosol, but also contributed to the overall stability of the complex itself (Gujrati et al., 2014). Additionally, cysteine residues could be profitably used as the anchoring group to tether a given targeting moiety to the carrier (Wang et al., 2009).

On the other side, lipids with enzyme-cleavable linkers have been extensively exploited to achieve a sustained delivery of NAs into specific targets. The main advantage of such approach is that the amount of NAs released from lipoplexes depends on the enzyme concentration and localization (Fouladi et al., 2017). As an example, it has been shown that the lipid PEG-peptide-DOPE (PEG-PD) underwent disassembly because of substantial cleavage of the peptide moiety by means of the matrix metalloproteinase-2 (MMP-2), an enzyme specifically expressed at high levels at target sites (Terada et al., 2006). Comparable outcomes were found in other studies using similar vectors (Bruun et al., 2015; Hatakeyama et al., 2009; Koutroumanis et al., 2013).

The use of the cationic lipid backbones featuring photosensitive linkers represents a valuable alternative to the other environment-sensitive amphiphiles described herein above. In this context, Nagasaki and co-workers developed an array of cationic amphiphiles to ascertain the role of photocleavable (UV-sensitive) linkages on the lipoplex activity. Interestingly, they found that the UV-induced linker cleavage allowed NAs to escape from endocytic vesicles, such that their transfection efficiency was up to 20 fold-higher than that of Lipofectin (Nagasaki et al., 2003).

Together, these findings entail that each and every linker moiety has an impact on the stability of the lipoplexes, which, in turn, plays a role in tuning the cytotoxicity and the transfection efficiency of lipoplexes. Even though different kinds of linkers show specific pros and cons, biodegradable, environment-responsive moieties, such as carbamate and disulphide linkers, are the most promising candidates because giving rise to low cytotoxicity and high transfection efficiency.

2.3. Tails

The tail moiety is the hydrophobic domain of cationic lipids. Structure-activity relationship (SAR) studies have shown that non-polar tails play a role in the phase transition, and thus in the fluidity, overall stability and cytotoxicity of the resulting lipoplexes (Jones et al., 2013). Depending on the structure, hydrophobic tails are classified as aliphatic chains or cyclic (steroid-based) domains.

2.3.1. Aliphatic chains

The aliphatic tails of cationic lipids are typically saturated (e.g., stearyl) or unsaturated (e.g., oleyl). Despite the number of chains displayed by the lipid, their length and the degree of unsaturation have been found to influence the transfection performances of the resulting lipoplexes (Jones et al., 2013), there is no general consensus as to which an ideal tail design should be (Felgner et al., 1994; T. Ren et al., 2000; Song et al., 1997).

As a general rule, the shorter the saturated chain of the cationic lipid, the higher the effectiveness. On this matter, the optimal chain length of aliphatic tails was unfortunately found to vary dramatically from study to study. Some authors concluded that short hydrocarbon chains, such as those composed of 10 to 14 carbon atoms, are the most effective in transfection (Gopal et al., 2006; Venkata Srilakshmi et al., 2002). On our side, we checked the effectiveness of an array of cationic lipids with hydrocarbon tails of various length. Of note, we found a bell-type transfection trend with an optimum performance corresponding to C_{14}, while shorter and longer aliphatic tails were less effective (i.e., $C_{14} > C_{12} > C_{10}$ and $C_{14} > C_{16} > C_{18}$) (Candiani et al., 2007).

Yet, the identification of the optimum number of aliphatic chains the cationic lipid should display to achieve the optimized transfection is still matter of debate. In fact, it is generally accepted that lipids with two hydrophobic chains, such as DOTMA, DOTAP, DOPSA, DORIE, DOGS and others, are more effective in transfection than single-tailed lipid counterparts. Nevertheless, their performances are largely dependent on factors other than the simple number of hydrophobic chains, such as geometrical and chemical features (Ewert et al., 2010). A possible explanation as to why the transfection efficiency of two-tailed cationic lipids was high may rely on their potential to form stable aggregates in aqueous solutions (Li et al., 2013; H. Wang et al., 2014). By contrast, single-chained cationic lipids are more prone to form unstable lipoplexes characterized by higher cytotoxicity and reduced transfection efficiency (Lv et al., 2006; Pinnaduwage et al., 1989). Of note, the combination of single- and double-tailed lipids to give mixed liposomes had a positive synergistic effect on the transfection efficiency (Li et al., 2013; Wu et al., 2016).

Although seldom used, multi-tailed lipids have shown some intriguing results in transfection (Byk et al., 1998; Gaucheron et al., 2002). In this regard, it has been recently shown that the cationic lipid bearing three saturated alkyl chains N-[6-amino-1-oxo-1-(N-tetradecylamino)hexan-(2S)-2-yl]-N'-{2-[N,N-bis(2-aminoethyl)-amino]ethyl}-2,2-ditetradecylpropandiamide (DiTT4), used in combination with DOPE, gave rise to very effective lipoplexes (Wölk et al., 2015b, 2015a).

Moreover, it has been shown that the symmetry of the two alkyl chains bound to the headgroup plays some role in the behavior of two-tailed cationic lipids (Chandrashekhar et al., 2011; Hiwale et al., 2017; Le Corre et al., 2014; Wang and Macdonald, 2004). SAR studies revealed that the degree of asymmetry between the two tails strongly impacts the phase behavior of the resulting lipid as a consequence of the differences in the overall tail free volume (Zhang et al., 2011). Indeed, in two-tailed lipids with chains of different lengths, the packing density decreases, and voids created by the presence of shorter chains severely reduce the rigidity of the assembly overall, thus affording a greater intermembrane mixing (Le Corre et al., 2014). This is the reason why several authors have suggested using asymmetric lipids to attain high transfection efficiency (Dileep et al., 2001; Le Corre et al., 2014; Wang et al., 2006).

Unsaturated alkyl chains are also believed to increase the transfection performances of lipids because they are known to increase the membrane fluidity and display a good fusogenic behavior (Arpicco et al., 2004; Heyes et al., 2005). The effectiveness of lipoplexes containing unsaturated lipid tails depends on three variables, that is, the number of double bonds, their position along the chain and their configuration (Zhi et al., 2010). Even though few reports have showed a direct correlation between the degree of unsaturation and transfection efficiency (Inoh et al., 2010), it is generally accepted that lipoplexes made of lipids with monounsaturated chains are the most efficient in transfection (Arpicco et al., 2004; Delepine et al., 2000; Zhi et al., 2010; Zuhorn et al., 2002). For the sake of comparison, Loizeau and colleagues examined different cationic lipids with invariable heads but different hydrophobic tails, such as saturated, mono- and poly-unsaturated alkyl chains of different lengths, and found that C_{18}-long, monounsaturated single-tailed amphiphiles were the most effective in terms of DNA condensation and transfection efficiency (Loizeau et al., 2013). Besides, the solid geometry of the unsaturated aliphatic chains is another important factor to be taken into account (Giacometti et al., 2017; Zhu et al., 2008). In this regard, many authors have shown that the cis configuration is the most effective in transfection (Obika et al., 2001; Zhu et al., 2008; Zhu and Mahato, 2010).

2.3.2. Cyclic (steroid-based) domains

Since the development of the popular DC-Chol (Gao and Huang, 1991), many other steroid-based transfectants have been designed (Kearns et al., 2008; Kim et al., 2014; Vigneron et al., 1996). Steroids in general, and cholesteryl specifically, are often used in place of aliphatic chains because inherently rigid, biodegradable (Martin et al., 2005; Zidovska et al., 2009), biocompatible (Choi et al., 2001; Lee et al., 2004), and fusogenic (Ohvo-rekila et al., 2002; Silvius, 2003).

Han *et al.* developed two novel Chol-based lipids, i.e., cholesteryloxypropan-1-amine (COPA) and cholesteryl-2-aminoethylcarbamate (CAEC), to efficiently deliver siRNA molecules into cells (Han et al., 2008), while Bhattacharya's group synthesized a series of *gemini* Chol-based cationic surfactants that displayed transfection efficiencies similar to those of the single-tailed analogues and to the gold standard Lipofectamine (Bajaj et al., 2007; Biswas et al., 2011).

Besides, despite Chol has been the most employed non-aliphatic domain so far, other steroids, such as vitamins (Tan Ren et al., 2000), bile, cholestane and lithocholic acid (Fujiwara et al., 2000; Walker et al., 1996), have been used to synthesize cationic amphiphiles for gene delivery applications. For instance, vitamin D_2 and D_3 have been used to synthesize an array of lipid transfectants as effective as DC-Chol (Tan Ren et al., 2000). More recently, other studies pointed to the use of α-tocopherol, a derivative of vitamin E, to synthesize cationic lipids with superior transfection properties (Kedika and Patri, 2012, 2011; Zheng et

al., 2016). Because similar to Chol, bile acids have also been used with some success (Walker et al., 1996).

Taken together, these findings disclose both aliphatic and cholesterol-based tails as suitable hydrophobic domains to design cationic lipid transfectants. In this regard, there is a consistent body of evidence that the single-tailed cholesterol-based motif and two-tailed monounsaturated aliphatic chains are the most suited lipophilic moieties to design cationic lipids for gene delivery.

3. Helper lipids

Although sole cationic lipids have been shown to successfully deliver NAs, they are often combined with helper lipids, also called co-lipids, which are typically zwitterionic lipids such as DOPE or 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine (DOPC), among others, to increase their effectiveness. Indeed, helper lipids assist the formation of different supramolecular assemblies that strongly affect the colloidal stability of lipoplexes by promoting their interaction with cell membranes (Balazs and Godbey, 2011; Buck et al., 2019; Du et al., 2014; Mochizuki et al., 2013).

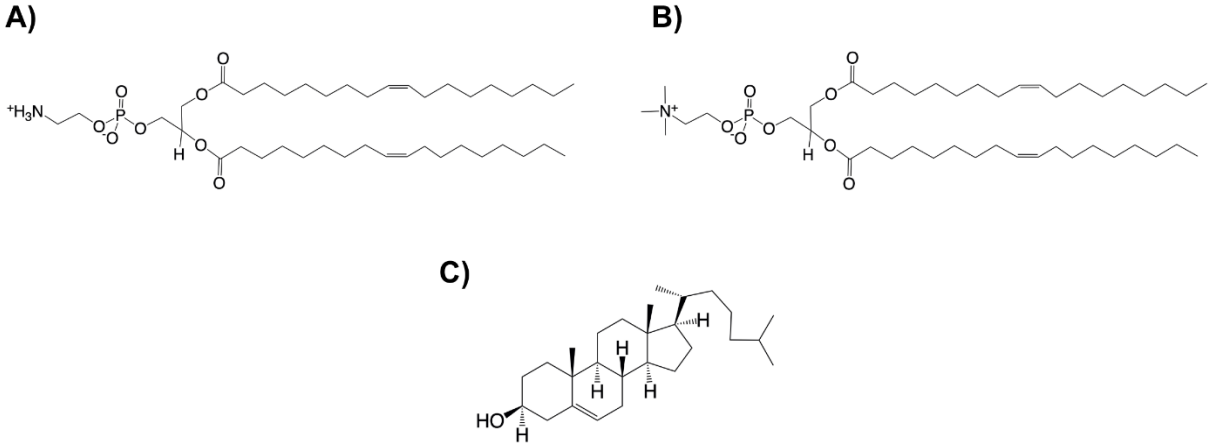

Figure 4. Chemical structure of different helper lipids, namely: A) DOPE; B) DOPC; C) Chol.

DOPE consists of a relatively small phosphoethanolamine headgroup bound to two bulky and unsaturated oleyl chains by means of ester linkages (**Figure 4A**), and acts as a fusogenic lipid. Quite for this reason, it has been frequently incorporated in early designs of lipoplexes in order to achieve an unstable geometry at acidic pH and enable the endo-lysosome destabilization, and the consequent NAs release (Hoekstra et al., 2007). Mochizuki and colleagues revealed that DOPE-containing lipoplexes underwent conformational changes when the pH was lowered from 7 to 4, as it occurs in the late endosomes environment (Mochizuki et al., 2013). Due to the above mentioned behavior, DOPE has been added to several lipid formulations which are on the market, such as Lipofectin (a lipid formulation consisting of 1:1 (w/w) mixture of DOTMA and DOPE) and Lipofectamine (a 3:1 (w/w) mixture of DOSPA and DOPE) (Dalby et al., 2004; Wang et al., 2018).

DOPC is a lipid with a zwitterionic behavior given by the occurrence of both an anionic phosphate and a cationic choline moiety (**Figure 4B**) (Bhattacharya and Bajaj, 2009). It is inherently prone to give rise to stable structures, which are considered to be less effective in transfection than those containing DOPE (May et al., 2000). To shed more light on the effects of helper lipids, some authors investigated the transfection behavior of DOTAP in combination with DOPE or DOPC and, as hypothesized, they found out the superior effectiveness of the

former formulation (Hattori et al., 2005). In this regard, it was found that DOPE-containing lipoplexes exhibited quick endosomal trafficking and DNA accumulation within the nucleus, while lipoplexes containing DOPC settled into the late endo-lysosomes (Du et al., 2014; Zhang and Anchordoquy, 2004; Zylberberg et al., 2017).

Chol (**Figure 4C**) has also been used as the helper lipid in many formulations. Chol is a natural, waxy steroid found in all animal cell membranes. In nature, the hydroxyl group of each Chol molecule interacts with the water surrounding the membrane, while the bulky steroid and the hydrocarbon chains are embedded within the membrane, alongside the non-polar fatty-acid chains of the other lipids. Through the interaction with the fatty-acid chains of phospholipids, Chol increases membrane packing and allows retaining membrane integrity (Koynova and Tenchov, 2009). Besides, being Chol an uncharged amphiphile, it does not interact directly with NAs, but rather it supports cationic lipids to interact with them (Xu and Anchordoquy, 2008). In addition, it has been reported that Chol may favor the interactions with plasma and endosomal membranes (Pozzi et al., 2012). All these findings support the idea that the formulation of cationic lipids with Chol may improve the overall transfection performances of lipoplexes (Betker et al., 2013; Dabkowska et al., 2012; Faneca et al., 2002).

Although it is commonly accepted that some cationic lipids work exclusively with specific helper lipids and not with others, Mukherjee *et al*. have investigated the possible effect on transfection of the co-presence of common co-lipids (Mukherjee et al., 2005). Of note, it turned out that, when acting in synergy, the helper lipids DOPE, DOPC, and Chol improved the gene transfer properties of some newly synthesized cationic lipids with different aliphatic chain lengths but failed to do so to the same extent when taken individually. Similar results were confirmed by other authors who used mixtures of the cationic lipid DDAB and the helper lipids DOPE, DOPC, Chol and two kinds of phosphatidylcholines (Safari and Hosseinkhani, 2013).

In light of such promising results about the use of co-lipids to improve the transfection performances of cationic lipids, recent studies have focused on the design of novel helper lipids, for instance bearing triggerable elements (Le Gall et al., 2014; Réthoré et al., 2007; Zheng et al., 2015). Just as an example, Zheng and colleagues synthesized the zwitterionic amphiphile trans-2-aminoacyclohexanol (TACH) and used it in combination with DOTAP in an attempt to improve its gene delivery efficiency. Due to the pH-responsiveness displayed by the former molecule, the formulation of DOTAP with TACH proved to be more effective than the conventional DOTAP:DOPE and DOTAP:Chol mixtures (Zheng et al., 2015).

Overall, even though different kinds of helper lipids have been found to enhance to some extent the performance of cationic lipids, DOPE and Chol have emerged as the most widely used because they favor the interaction of the lipoplexes with the cellular membranes.

4. Structure and properties of lipoplexes: from complexation to transfection efficiency

The performances of a given vector are definitely affected by the chemical features of the amphiphile, that is, the chemical structure of each domain and the interplay among them.

Relying on the composition of the lipid, different supramolecular structures can be obtained. When these are mixed with NAs, things get even trickier. In the next sections, the interplay between lipid chemistry, some rearrangements that give rise to supramolecular assemblies, and how these together affect the final transfection outcomes are discussed in some depth. Specifically, by focusing on the steps that lead to complexation and on the factors that influence the structural and physico-chemical features of the resulting lipoplexes (i.e., charge ratio (CR), temperature and complexation buffer), we seek to highlight how each and

every feature affects the ability of lipoplexes to overcome cellular barriers and achieve successful gene transfer.

4.1. Cationic lipids: from structure to aggregation phase

When dispersed in water at a certain concentration above their critical micellar concentration (CMC), amphiphiles naturally self-assemble into thermodynamically stable vesicles, which are the result of some structural rearrangements that minimize the exposure of the hydrophobic moieties to the protic solvent. Depending on the geometrical packing constraints imposed by hydrophilic and hydrophobic moieties that are specific to each lipid, different supramolecular assemblies have been proposed. An easy way to predict the conformation of a given amphiphile is by means of the so-called packing parameter (P), which is defined according to the Eq. 1 (Hsu et al., 2005):

$$P = \frac{v}{a_0 \times l_c} \qquad \text{(Eq. 1)}$$

where:
v is the molecular volume of the hydrocarbon tails;
a_o is the surface area occupied by the polar headgroup;
l_c is the length of the hydrophobic chain(s).

In short, this equation emphasizes the relevance of the ratio between the volume occupied by the hydrophobic region and that of the hydrophilic domains. Lipids with P < 0.5 are cone-shaped (e.g. lysophosphocholine, LPC) (Kang et al., 2016) because they have bulky headgroups with a single short hydrocarbon chain (**Figure 5A**), and do associate in micelles with a positive membrane curvature. Conversely, lipids bearing headgroups which exhibit about the same cross-sectional area than hydrophobic tails (0.5 < P < 1) are referred to as cylindrical-shaped lipids (e.g., DOTAP and DOPC) (**Figure 5B**) and are prone to assemble into a lamellar structure, that is, a bilayer with nearly zero curvature (Majzoub et al., 2016). Conversely, inverted cone-shaped lipids have larger hydrophobic moieties with respect to polar heads such that P > 1 (e.g., DOPE) (**Figure 5C**). They associate with each other to give inverted hexagonal phases (Cullis and Hope, 1986; Pezzoli et al., 2012).

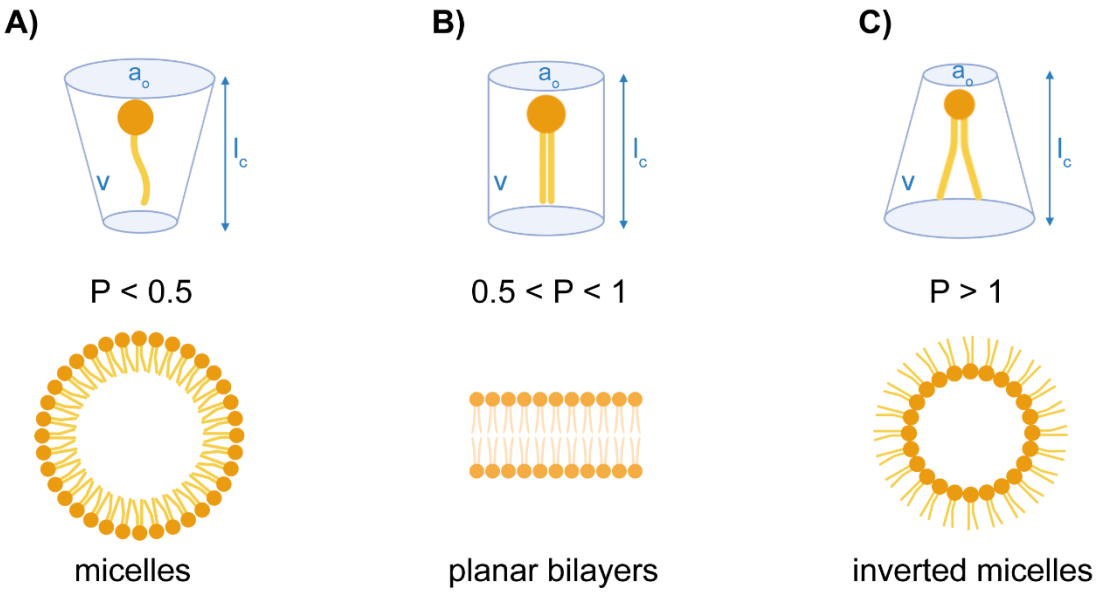

Figure 5. Schematic representation of the molecular shape that cationic lipids may have, and the supramolecular structure they may form, depending on the packing parameter (P). Specifically, a_o is referred to the area of the polar headgroup, l_c represents the length of the hydrophobic domain, while v refers to the hydrocarbon volume. Depending on the P value, cationic amphiphiles are depicted by A) cone-like geometry, B) cylindrical shape, and C) inverted cone geometry. They may aggregate to give rise to the formation of micelles, planar bilayers, and inverted micelles, respectively.

Despite the prediction of the 3D structure of lipids which is relatively simple, the conformational changes occurring just after mixing NAs and lipids are not as trivial to predict (Koynova and Tenchov, 2009). Lipoplexes are usually obtained by adding NAs to the cationic lipids in order to enable the natural assembly of the two components. So far, a number of studies have shown the ability of different cationic lipids to bind and condense NAs of various size, including DNA molecules in the form of pDNA and oligonucleotides (Caracciolo and Amenitsch, 2012; Koynova and Tenchov, 2010; Meidan et al., 2000; Tros de Ilarduya et al., 2010; Wang et al., 2015; Weisman et al., 2004), and RNA molecules, such as siRNA and mRNA (Guevara et al., 2020; Midoux and Pichon, 2014; Semple et al., 2010; Zhang et al., 2007). Therefore, when mixing NAs with cationic lipids, the size and the 3D arrangement of the former may affect the physico-chemical features and the supramolecular structure of the resulting complexes (Ewert et al., 2010; Rao, 2010; Scholz and Wagner, 2012).

Although conceptually simple, lipoplex formation is a multi-step and multi-length scale process that relies upon the temperature, volume and ionic strength of the medium, and relative concentrations and types of NAs and cationic lipids (Fuj and Sakura, 2012; Ilies et al., 2002; Tros de Ilarduya et al., 2010). Complexation primarily consists of two major phases: i) a long-lasting but abrupt (i.e., in the order of milliseconds) interaction between the anionic phosphates of NAs and the cationic headgroups of cationic lipids. This step is spontaneous and driven by the entropic gain associated to counterions release in solution (Gao et al., 2010; Sennato et al., 2005); ii) a slower, endothermic process of irreversible rearrangement and stabilization of the lipoplex itself (Dan, 2015; Kang et al., 2016; Koynova and Tenchov, 2009). Biophysical studies with small-angle X-ray scattering (SAXS) and differential scanning calorimetry (DSC) revealed that the binding of cationic lipids to NAs results in lipid rearrangement and mixing (Mrevlishvili et al., 1998; Wasungu and Hoekstra, 2006). During this step, the hydrophobic portions of cationic lipids may be provisionally exposed to water

and rearrange into unstable conformations. In turn, these undergo rearrangement by means of hydrophobic interactions (Matulis et al., 2002) to give different thermodynamically favored assemblies (Caracciolo and Amenitsch, 2012; Fuj and Sakura, 2012), such as spaghetti-and-meatballs, lamellae and rod-like structures (Elouahabi and Ruysschaert, 2005). Of note, the organization of lipids and NAs in specific 3D architectures (**Figure 6**) impacts the ability of lipoplexes to overcome some delivery barriers, and thus their transfection efficiency (Ma et al., 2007; Safinya et al., 2011).

Broadly speaking, complexes made with cationic lipids and NAs may have two typical structures, namely the (multi)lamellar phase (L_α^C) (**Figure 6A**) and the hexagonal phase (H_I^C, H_{II}^C) (**Figures 6B** and **6C**).

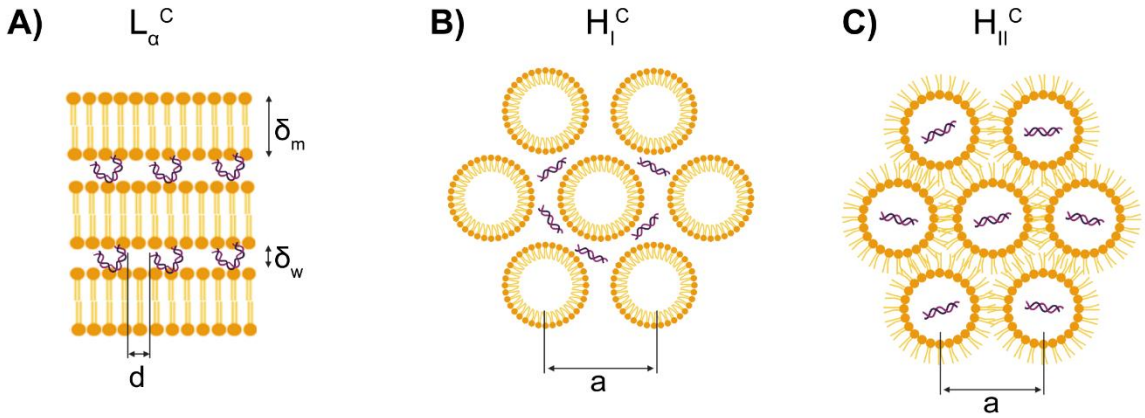

Figure 6. Schematic representation of the supramolecular structures of lipoplexes: A) lamellar phase (L_α^C); B) hexagonal phase (H_I^C) and C) inverted hexagonal phase (H_{II}^C).

L_α^C phase (**Figure 6A**) typically occurs when using cylindrical-shaped lipids (**Figure 5B**). It is composed of different lipid bilayers, with NAs intercalated among them. Studies carried out by means of cryo–transmission electron microscopy (cryo-TEM) revealed that the complexation of lipids and NAs into multilamellar assemblies starts with the absorption of NAs on pre-formed liposomes, followed by the clustering of different unilamellar structures. The high deformation and packing pressure arising from the electrostatic interaction of one side of the bilayer with NAs ultimately lead to the rearrangement of the bilayer itself and the formation of multi-lamellar assemblies (Huebner et al., 1999). The NA strands present in the interlamellar gaps lie parallel from each other, and at a repeatable distance *d* that depends upon cationic lipids and the eventual presence of helper lipids. The interlayer spacings are defined by the membrane thickness (δ_m) and the water gap (δ_w), which are about 5 nm and 2.5 nm, respectively. Depending on the packing level, which is dictated by the NA content, the distance *d* between NA molecules is from 2.5 nm and 5 nm. Of note, even if such bilayers have a quasi-zero curvature, some buckling may occur when interacting with NAs (Safinya, 2001; Safinya et al., 2011). Lipoplexes displaying L_α^C phase have been found stable in both the intracellular and in the extracellular environments, that is, they are little prone to fuse with cellular membranes, such that their transfection efficiency is generally low (Ma et al., 2007).

In the hexagonal phase H_I^C (**Figure 6B**), the DNA molecules are embedded in the aqueous voids of the hexagonal lipid matrix. Due to the constraints of lipid chain packing, lipoplexes adopting a H_I^C phase are usually composed of cone-like lipids (**Figure 5A**) that form a honeycomb-like structure made up of micelles with a constant distance *a* of about 8.15 nm between the centres (Kang et al., 2016).

Conversely, the inverted hexagonal phase H_{II}^C (**Figure 6C**) consists of DNA rods coated with a lipid monolayer arranged on a hexagonal lattice. While the hydrophobic portions of the inverted micelles interact with each other, the positively charged headgroups engage with NAs. With respect to H_I^C, the distance between the centres *a* is lower (*a* = 6.74 nm) (Kang et al., 2016). Besides, this phase is obtained as the result of the arrangement of inverted cone cationic lipids (**Figure 5C**), and it is favored in the presence of the helper lipid DOPE (Wasungu and Hoekstra, 2006) that gives rise to packing constraints (Dan, 2018; Gruner, 1989). Lipid/NA complexes featuring H_{II}^C phase are less stable. Indeed, they have high fusogenicity, that is, NAs are more easily released inside the cell and the transfection efficiency is increased as a result (Ewert et al., 2005; Giacca, 2010; Koynova et al., 2006; Lin et al., 2000; Ma et al., 2007).

Despite the lamellar and hexagonal phases are the most common and researched supramolecular assemblies, recent studies revealed that lipoplexes may also take a cubic phase (Q_{II}^C). Such metastable cubic mesophase confers lipoplexes some fusogenic behavior, which impacts the overall transfection efficiency (Dittrich et al., 2018; Leal et al., 2010; Mcloughlin and Impøror-clerc, 2004).

It is worthy of note that the information provided herein above are specific to the use of DNA. Because siRNAs are obviously shorter, less rigid, and have higher rotational and translational degrees of freedom than large DNA molecules, siRNA-bearing lipoplexes take less time to reach the equilibrium, and feature more fluid bilayers (Bouxsein et al., 2007; Kang et al., 2016).

4.2. Shaping lipoplexes: factors affecting their behavior

The stability and transfection performances of lipoplexes are strongly affected by a variety of formulation factors. Therefore, in order to improve the effectiveness of lipoplexes, some key parameters have to be taken into account, such as i) the lipid-to-NA ratio (i.e., in other words, the CR), which was found to affect the charge density of the complexes, their dimensions, shape and overall colloidal stability; ii) the environmental conditions, that is, the temperature, the ionic strength and the pH of the aqueous solution in which lipoplexes are prepared. These impact the complexation kinetic as well.

4.2.1. Lipid-to-NA ratio (charge ratio)

The charge ratio (CR) represents the mole ratio between cationic moieties, such as the N atoms, of the lipid headgroup and the negative charges brought by the phosphate groups of the NAs to be delivered. This means that at CR 1 there should be a complete charge neutralization such that NAs are fully complexed. Although theoretically reasonable, this seldom happens in practice because of some steric hindrance between NAs and cationic lipids, and because of some other geometrical constraints. A slight-to-moderate excess of positive charges is thus needed in order to get NAs completely buried within lipoplexes (Elouahabi and Ruysschaert, 2005). In light of this, it is apparent that the CR is probably the most prominent factor that allows to fine-tune the effectiveness of lipoplexes through the modulation of their colloidal stability. In order to emphasise how the CR may affect the formation of lipoplexes with different behavior, Garidel and Funari compared lipoplexes made with the cationic lipid DC-Chol and DNA at different lipid: DNA weight-to-weight (w/w) ratios. Of note, only in the presence of a large excess of cationic lipid with respect to NAs, stable non-lamellar structures were achieved (Garidel and Funari, 2006).

The CR is also known to impact the complexation kinetics: while lipoplexes at high CR are known to reach quickly a steady-state equilibrium, those formed at lower CR require longer

time to rearrange and reach stability (Dan, 2015). Besides, although the complexation is carried out faithfully following the general scheme mentioned above, even subtle changes in the experimental protocol adopted have a strong impact on the lipoplex formation (Sennato et al., 2005; Zuzzi et al., 2007) and behavior (Rakhmanova et al., 2004). The process of lipoplex formation has been thoroughly investigated so far. It has been shown that when anionic NAs are added to the cationic lipids, the latter rapidly bind to the former molecules due to counterion release (Harries et al., 2013). On the other hand, if we add cationic lipids to NAs, instead of *vice versa*, the complexation takes longer to happen and leads to the wrapping of NAs by preformed lipid aggregates, thus limiting somewhat the intimate lipid mixing and the structural rearrangements that may happen within the lipoplex (Wasungu and Hoekstra, 2006).

The CR also affects the physico-chemical features of the particles, namely their size (hydrodynamic diameter, D_H), and their surface charge (zeta-potential, ζ_P). Of note, since the cellular uptake of particles is a size- and charge-dependent process (Foroozandeh and Aziz, 2018), small and positive complexes were reported to be the most stable and effective in transfection experiments (Tros de Ilarduya et al., 2010). As a general rule, the higher the CR, the greater the ζ_P, and the lower the size of lipoplexes (Buck et al., 2019). This means that lipoplexes show specific colloidal stability depending on the CR, as schematically depicted by the three-zones colloidal stability model reported in **Figure 7** (Barteau et al., 2008; Pitard, 2002; Sainlos et al., 2007; Tranchant et al., 2004). In more detail, *zone A* (i.e., low lipid concentration) is comprised of colloidally stable but ineffective complexes that are negatively charged because there are still some uncondensed NA molecules surrounding the lipoplexes. The electrostatic repulsive forces prevent lipoplexes from aggregation. *Zone B* consists of large and colloidally unstable lipoplexes with a barely neutral surface charge. Lipoplexes obtained at CRs close to the isoelectric point (**Figure 7**) (i.e., it is where the opposite charges of the polyelectrolyte (NA) and the cationic surfactant (lipid) become neutralized and the overall charge of the assembly is neutral) may interact with each other and form larger aggregates (Faneca et al., 2002). By increasing the lipid concentration (i.e., the CR), the "re-entrant condensation" and overcharging phenomena take place (Bordi et al., 2009; Grosberg et al., 2002; Sennato et al., 2005), so that *zone C* includes colloidally stable and effective suspensions constituted by positively charged, small lipoplexes that strongly repel each other. This implies that small variations in the amount of cationic lipids used to prepare lipoplexes have a tremendous effect on their colloidal stability, and thus on their ultimately effectiveness as gene delivery vectors.

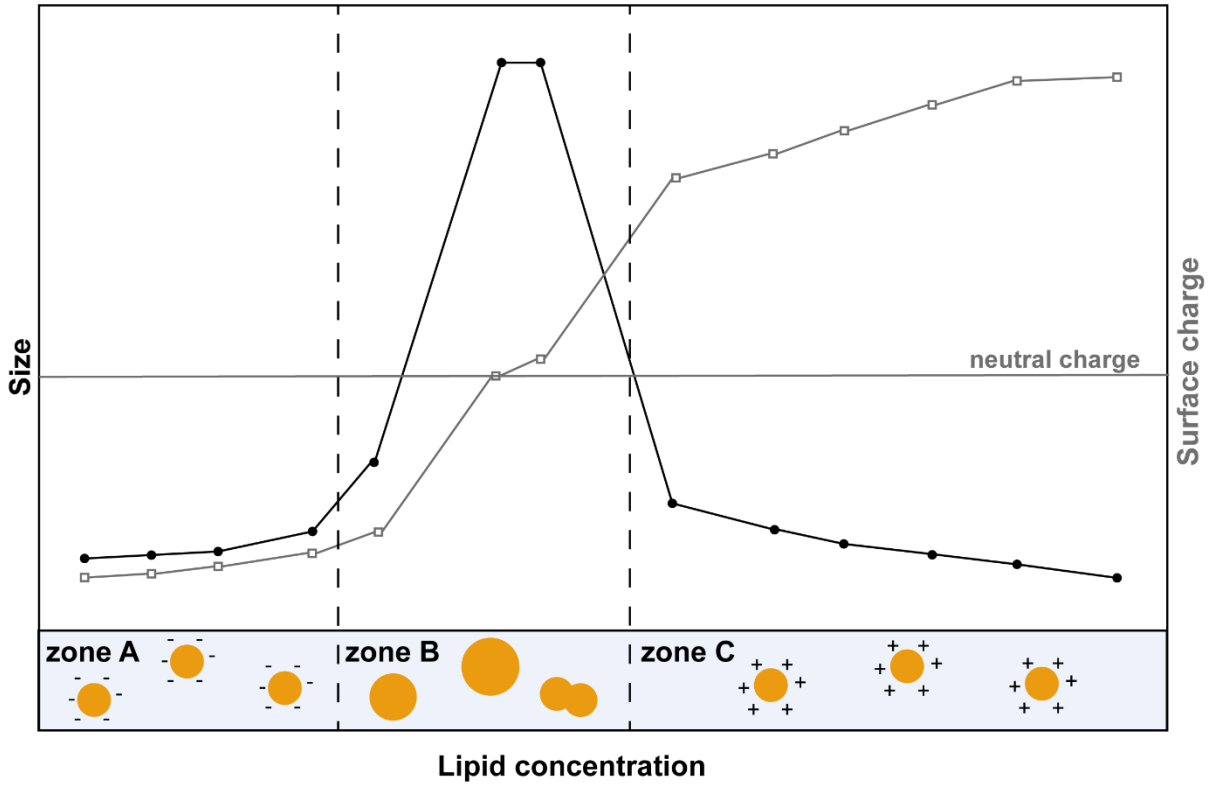

Figure 7. Schematic representation of the colloidal stability model of lipoplexes, which depends on their size (black full dots) and overall surface charge (grey empty squares) as a function of the lipid concentration. Specifically, *zone A* refers to stable lipoplexes with uncomplexed NAs, and thus negative surface charge and small size; lipoplexes in *zone B* are neutral and colloidally unstable, such that they tend to aggregate in cluster-like particles with bigger size; complexes in *zone C* are stable, positively charged, and small due to 're-entrant condensation' and overcharging phenomena. The latter are the most effective in transfection.

4.2.2. Environmental conditions

Being complexation a thermodynamically spontaneous process, environmental cues, such as the temperature and the salt concentration, have a dramatic influence on the cationic lipids-NAs interactions (Muthukumar, 1986). Some evidences showed how a rise in temperature could induce a looser packing of lipoplexes (Silva et al., 2012). A mechanistic explanation of this phenomenon relies on the different conformations that the NAs can take as a function of the temperature. In practice, the double stranded DNA is more relaxed at temperature ≈ 40 °C than the conventional super-coiled conformation found at lower temperatures (Tse-Dinh et al., 1997). Moreover, the strength of the interaction between NAs and lipids proved to be inversely related with temperature, such that when the temperature rises the binding is weaker (Matulis et al., 2002).

Likewise, temperature variations also affect the phase behavior of lipoplexes (Dan and Danino, 2014). Because the transition from L_{α}^{C} to H_{II}^{C} was found to be a temperature-favored process (Scarzello et al., 2005b), inverted hexagonal H_{II}^{C} structures are more likely to occur when heating lipoplexes because δ_m and δ_w of the lamellar conformation consistently decrease (Pozzi et al., 2006).

The pH and the ionic strength of the medium in which complexation takes place are just as important. It has been reported that a neutral-to-slightly acidic environment is usually preferred to deprotonate NAs and protonate lipids at once (Buck et al., 2019). On the other hand, the ionic strength of the complexation medium, in other words, the concentration and the kinds of ions competing for absorption play a role in the final assembly of lipoplexes. Indeed, both NAs and lipids have to be charged and need to exchange counterions with the

solution (e.g., 0.1 M NaCl) in order that complexation takes place (Harries et al., 2013). Nonetheless, a further increase in salt concentration (i.e., ≈ 0.4 M NaCl) may hamper the counterion release, and complexation becomes impaired (Sennato et al., 2016; Tranchant et al., 2004; Zuidam and Barenholz, 1998).

Moreover, an increase in the ionic strength has also been found to trigger L_{α}^{C} to H_{II}^{C} phase transition (Scarzello et al., 2005a) and results in the aggregation of lipoplexes (Bordi et al., 2009).

4.3. Cell-lipoplex interactions and trafficking

The successful delivery of NAs within the cells by means of non-viral vectors is hampered by a wide number of rate-limiting steps, namely the interaction of lipoplexes with the cell surface, their internalization, the release of the genetic cargo from endosomes and, when the delivery involves the DNA, its transfer into the nucleus (**Figure 8**) (Pezzoli and Candiani, 2013).

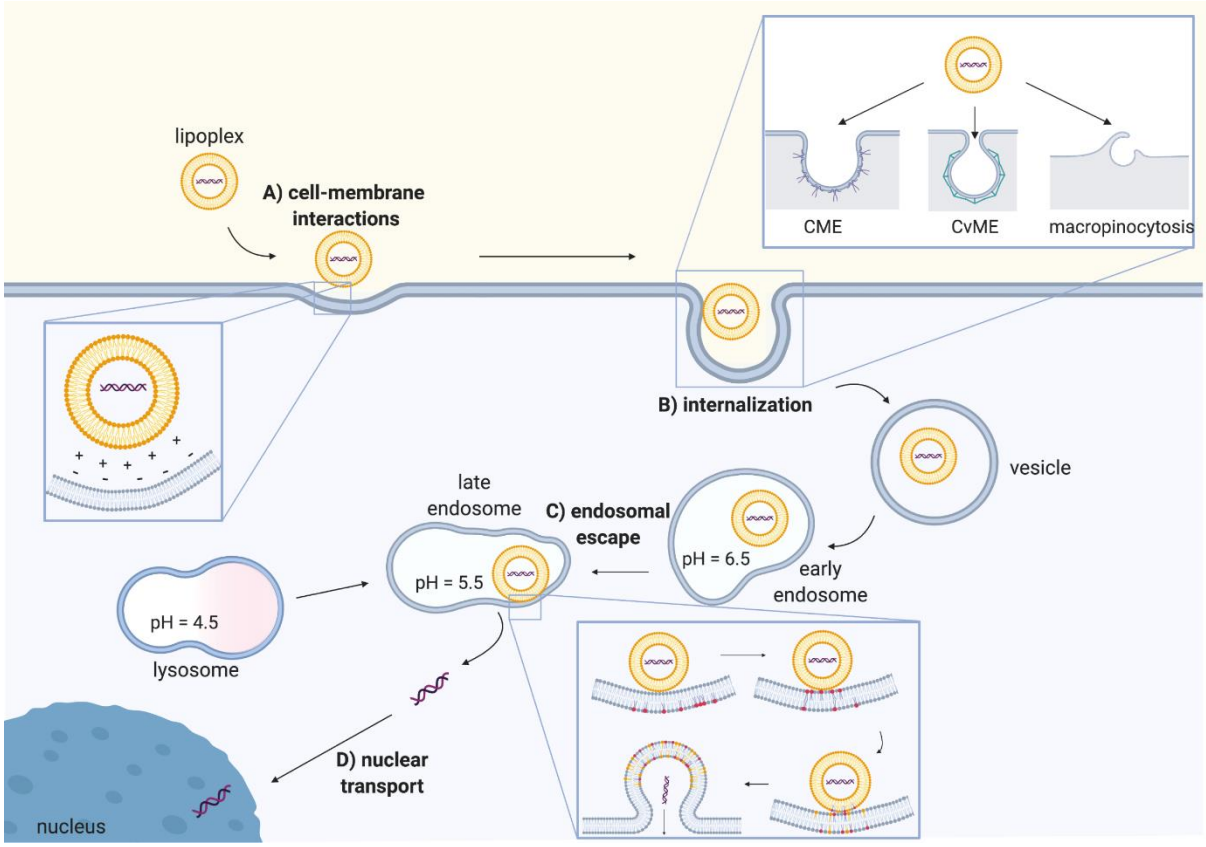

Figure 8. Schematic representation of NAs delivery into cells mediated by cationic lipids. A) First, lipoplexes bind to the cell membrane through electrostatic interactions. B) Depending on their physico-chemical features, lipoplexes are internalized by means of different endocytic pathways, namely clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) or macropinocytosis. C) Once trapped within the endosome, lipoplexes are able to interact and fuse with anionic membrane lipids, ultimately leading to the release of the NAs in the cytosol. D) In case of DNA delivery, the delivery pathway ends with the transport of DNA molecules within the nucleus.

Therefore, the first hurdle that stands in the way of transfection is the binding of complexes to cell membrane (**Figure 8**, step A). It is generally believed that cationic lipoplexes interact with the anionic plasma membrane by means of electrostatic interactions. Although a net positive charge of lipoplexes was shown to enable their binding with the cell membrane, it is

just as true that the cationicity of lipoplexes is also responsible for their interaction with extracellular macromolecules, such as serum proteins (Zelphati et al., 1998; Zhang and Anchordoquy, 2004). In fact, serum proteins interact instantaneously and closely with lipoplexes (Simberg et al., 2005) and form the so-called protein corona. It has been shown that the effects of such a rich protein layer(s) on the ultimate stability and efficacy of lipoplexes are dependent on the serum protein content (Quagliarini et al., 2020): the presence of protein corona formed at low protein concentrations allows overcoming the electrostatic repulsion between lipoplexes, thus promoting their aggregation (Caracciolo et al., 2010). In turn, this was shown to lead to high transfection efficiency and low cytotoxicity (Masotti et al., 2009; Pezzoli et al., 2017; Rejman et al., 2004). On the other hand, protein corona formed at high protein concentrations induces the neutralization of the cationic charge of the lipoplexes. This resulted in the minimization of the electrostatic interactions with plasma membrane and/or the early release of the genetic cargo because of lipoplex destabilization (Maiolo et al., 2018). While most of the investigators agree that the lipoplex stability and functions are adversely affected by the presence of serum, little attention has been paid as to how the adsorption of specific proteins might be affected by some formulation parameters. In this light, the presence of neutral Chol in lipid formulations has been found to loosen the electrostatic interactions between cationic lipids and anionic serum proteins, thus improving their stability in the extracellular fluids, cellular uptake and transfection efficiency (Faneca et al., 2002; Zhang and Anchordoquy, 2004).

Once bound to the cell surface, lipoplexes must enter the cell (**Figure 8**, step B). Basically, this must be accomplished by passive or active (e.g., receptor-mediated) transport throughout the cell membrane. In the first and simplest case, lipoplexes fusion with the plasma membrane was suggested as a way to deliver NAs directly into the cytoplasm. It has been shown that a way to facilitate lipoplex-cell membrane fusion is through the use of helper lipids, such as Chol and DOPE (Buck et al., 2019; Zuhorn et al., 2005). Still, some experimental evidence supports the idea that the vast majority of lipoplexes are taken up by cells through endocytic pathways. Endocytosis, that is, the way cells internalize macromolecules and solutes by means of membrane-bound vesicles, is the main mechanism responsible for the internalization of non-viral vectors into cells (Cooper, 2000; Khalil et al., 2006). Notably, endocytosis can be classified in i) macropinocytosis, ii) clathrin-mediated endocytosis (CME) and iii) caveolae-mediated endocytosis (CvME) (Conner and Schmid, 2003; Lamaze and Schmid, 1995). After initiation of the endocytic site, the cargo is recruited, the membrane undergoes reshaping and scission (Peetla et al., 2015). Generally speaking, endocytic pathways differ in the composition of the coat (if any), in the size of the vesicles, and in the fate of the internalized particles (for further information concerning the uptake mechanisms please refer to (Khalil et al., 2006)). The internalization route of lipoplexes strongly depends on the size of the complexes (Jones et al., 2013; Rejman et al., 2006). As a rule of thumb, particles with dimensions of ~ 200 nm are typically internalized via clathrin-coated pits (CME), while larger particles with a size ~ 500 nm undergo CvME. By using inhibitors of specific endocytic routes, Rejman *et al.* showed that the uptake of DOTAP-containing lipoplexes occurred solely by CME (Rejman et al., 2005). In sharp contrast, Lazebnik *et al.* reported that the specific cationic lipid/siRNA particles they used were mostly internalized through macropinocytosis (Lazebnik et al., 2016).

Each and every endocytic pathway converges at the endo-lysosomal system (**Figure 8**, step C). Indeed, lipoplexes that are internalized through CME are finally trapped in the endosomes. The content next undergoes enzymatic attack in the endo-lysosomes, such that the NA cargo has little or no access to target. This implies that the lipoplex escape from the endosomes is essential for efficient transfection. This can be achieved by adding the co-lipid

DOPE to cationic lipids and/or using a multivalent cationic lipid (Walsh et al., 2013). On the other hand, lipoplexes that are internalized through the CvME pathway associate first with the cell membrane, become trapped into relatively stationary caveolae characterized by the presence of caveolin, and subsequently are taken up into caveosomes (Durymanov and Reineke, 2018). Although it was originally hypothesized that lipoplexes internalized through the caveolae-mediated pathway do not end up in the lysosomes (Rejman et al., 2005), later studies highlighted that the internalized cargo just goes into the lysosomes (Engel et al., 2011). In order to induce the endo-lysosomal escape of lipoplexes, the vast majority of scientists have pointed to the decoration of lipoplexes with peculiar features which can be conveniently exploited in the acidic environment of the endosome. To this aim, lipoplexes able to undergo L_{α}^{C} to H_{II}^{C} phase transition when trapped within the endosomal vesicles is by far the most beaten path (Rehman et al., 2013; Torchilin, 2006). In this regard, transition to non-bilayer phases is known to induce some thermodynamic instability, which, in practice, means that fusion of the cationic lipids with the anionic membrane phospholipids takes place (Caracciolo and Amenitsch, 2012; Koynova et al., 2006). Even more specifically, some phase transitions occurring at lipoplex level destabilize the vesicle membrane by means of flip-flop transitions of the outer anionic phospholipids to the inner leaflet, and subsequent ion-pairing with the cationic lipids that gives rise to the release of intra-vesicular cargo (Xu and Szoka, 1996). However, it has not been fully elucidated yet whether this process truly consists in a fusion mechanism, an endosomal membrane destabilization or an endosomal rupture (Jones et al., 2013; Zuhorn et al., 2007). Recent findings indicated that the endosomal destabilization process operated by cationic lipids is entropy-driven just as complexation, and the release of counterions from opposite-charged lipids has been assumed to trigger the disruption of endosomal vesicles (Avital et al., 2016). Besides, Rehman *et al.* observed that, at the time of endosomal escape, neither complete endosome rupture nor release of intact lipoplexes into the cytosol occurred (Rehman et al., 2013). Rather, they observed the formation of multiple and transient pores studding the endosomal membrane, and through which the genetic cargo was slowly transferred to the cytosol.

Even though endo-lysosomal escape is believed to be the most critical step standing in the way of efficient NAs delivery, the specific site where NAs elicit their functions deserves some attention as well (**Figure 8**, step D). In this regard, whether and once NAs are released from the endo-lysosomes, while RNAs exploit their function into the cytosol, DNA molecules have to enter the nucleus. Therefore, the spatio-temporal factors involved in the intracellular transport of NAs have to be taken into account (Nguyen and Szoka, 2012). As suggested by others (Cardarelli et al., 2016), intracellular trafficking, endosomal escape and lysosomal degradation can be viewed as interdependent phenomena, in such a way that they appear as a single barrier on the route for efficient transfection.

5. *Concluding remarks*

Over the last decades, great effort has been devoted to the development of more and more efficient systems enabling the delivery of NAs into a wide variety of cells. In this context, lipid-based non-viral vectors have emerged as the most promising delivery systems, such that few of them have entered clinical trials. Despite early enthusiasm for the use of these vectors, there is still a long way to go in order to meet the increased expectations. A rational design of lipoplex formulations is thus required to obtain functional particles with precise and reproducible physico-chemical features and improved biological activity. In this light, one must keep in mind each and every factor affecting the performances of lipoplexes. Unfortunately, there is still no general consensus on the features that a lipidic vector should have to be

effective. Rather, different cationic lipids and formulations work better for specific cell types and applications. Though there have been some examples of real improvements in the design of lipid delivery vectors, results escaped general consensus because somewhat inconsistent and patchy. It turns out that improvement in the design of transfectants is perceived as cumbersome and frustrating. Our belief is that constant improvements in lipofection will be made possible through more comprehensive mechanistic investigations and SAR studies. This is the main challenge that chemists, materials scientists, bioengineers and pharmacologists shall strive for.

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