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Cationic liposomes as delivery systems for nucleic acids

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Objectives. *Gene therapy is based on the introduction of genetic material into cells, tissues, or organs for the treatment of hereditary or acquired diseases. A key factor in the success of gene therapy is the development of delivery systems that can efficiently transfer genetic material to the place of their therapeutic action without causing any associated side effects. Over the past 10 years, significant effort has been directed toward creating more efficient and biocompatible vectors capable of transferring nucleic acids (NAs) into cells without inducing an immune response. Cationic liposomes are among the most versatile tools for delivering NAs into cells; however, the use of liposomes for gene therapy is limited by their low specificity. This is due to the presence of various biological barriers to the complex of liposomes with NA, including instability in biological fluids, interaction with serum proteins, plasma and nuclear membranes, and endosomal degradation. This review summarizes the results of research in recent years on the development of cationic liposomes that are effective in vitro and in vivo. Particular attention is paid to the individual structural elements of cationic liposomes that determine the transfection efficiency and cytotoxicity. The purpose of this review was to provide a theoretical justification of the most promising choice of cationic liposomes for the delivery of NAs into eukaryotic cells and study the effect of the composition of cationic lipids (CLs) on the transfection efficiency in vitro.*

Results. *As a result of the analysis of the related literature, it can be argued that one of the most promising delivery systems of NAs is CL based on cholesterol and spermine with the addition of a helper lipid DOPE. In addition, it was found that varying the composition of cationic liposomes, the ratio of CL to NA, or the size and zeta potential of liposomes has a significant effect on the transfection efficiency.*

Conclusions. *Further studies in this direction should include optimization of the conditions for obtaining cationic liposomes, taking into account the physicochemical properties and established laws. It is necessary to identify mechanisms that increase the efficiency of NA delivery in vitro by searching for optimal structures of cationic liposomes, determining the ratio of lipoplex components, and studying the delivery efficiency and properties of multicomponent liposomes.*

Keywords: *liposomes, nucleic acids, gene therapy, lipids, delivery.*

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Катионные липосомы как средства доставки нуклеиновых кислот

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Цели. *Генная терапия основана на введении генетического материала в клетки, ткани или органы с целью лечения наследственных или приобретенных заболеваний. Ключевым фактором успеха генной терапии является развитие систем доставки, способных эффективно переносить генетический материал к месту их терапевтического действия, не вызывая каких-либо связанных с ними побочных эффектов. За последнее десять лет много усилий было направлено на создание более эффективных и биосовместимых векторов, способных переносить нуклеиновые кислоты в клетки, не вызывая иммунного ответа. Катионные липосомы являются одним из самых универсальных инструментов для доставки нуклеиновых кислот в клетки, однако применение липосом для целей генной терапии ограничено неспецифичностью такой доставки. Это связано с наличием различных биологических барьеров на пути комплекса липосом с нуклеиновыми кислотами; например, с нестабильностью в биологических жидкостях; взаимодействиями с белками сыворотки крови, плазматической и ядерной мембранами; а также с эндосомной деградацией. В этом обзоре обобщены результаты исследований за последние годы по разработкам катионных липосом, эффективных in vitro и in vivo. Особое внимание уделено отдельным структурным элементам катионных липосом, определяющим эффективность трансфекции и цитотоксичность. Целью данного обзора являлось теоретическое обоснование выбора катионных липосом, наиболее перспективных для доставки нуклеиновых кислот в эукариотические клетки, а также изучение влияния состава катионных липидов на эффективность трансфекции in vitro.*

Результаты. *В результате проведенного анализа литературы можно утверждать, что одними из наиболее перспективных систем доставки нуклеиновых кислот являются катионные липиды на основе холестерина и спермина с добавлением липида-хелпера DOPE. Кроме того, было установлено, что варьирование состава катионных липосом, соотношения катионных липидов и нуклеиновых кислот, а также размера и дзета-потенциала липосом оказывают значительное влияние на эффективность трансфекции.*

Выводы. *Дальнейшие исследования в данном направлении должны включать в себя оптимизацию условий получения катионных липосом с учетом установленных закономерностей, а также физико-химических свойств. Необходимо исследовать возможности повышения эффективности доставки нуклеиновых кислот путем поиска оптимальных структур катионных липосом, определения соотношения компонентов липоплексов и изучения свойств и эффективности доставки многокомпонентных липосом in vitro.*

Ключевые слова: *липосомы, нуклеиновые кислоты, генная терапия, липиды, доставка.*

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INTRODUCTION

Gene therapy is one of the more promising methods for the treatment of a wide range of diseases. This method is based on the administration of therapeutic nucleic acids (NA) into the organism, which results in either the expression of the genetic construction or the partial/complete suppression of the defective gene [1]. Unlike drug compounds of low molecular weight whose therapeutic effect is based on binding to target proteins, therapeutic NAs could regulate the expression of specific genes by controlling the expression levels of functional proteins.

Therapeutic NAs include small interfering RNAs (siRNA), antisense, antigenic or immunostimulatory oligodeoxyribonucleotides (ODNs), plasmid DNA

(pDNA), and ribozymes. One of the first NAs considered as an object of cargo delivery was pDNA [2].

The low efficiency of NA delivery into target cells and the need to create conditions for their long-term functioning are the main challenges associated with gene therapy [1–3]. The therapeutic effect of NA molecules is determined by their physicochemical properties to a much greater extent than that of low molecular weight compounds. Thus, NAs are negatively charged, can be degraded by serum nucleases, and are rapidly excreted by the kidneys [4–6]. Eukaryotic cells do not have a specialized pathway for the uptake of NA. In this regard, there are many limitations to the use of NA as drugs, including low stability, short half-life, and low delivery efficiency. To address these issues, the development of effective systems for the delivery of NA into eukaryotic cells is essential.

The vast majority of studies on the systemic delivery of NA into cells use viral vectors (retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses), which are characterized by a high transfection efficiency (TE) of cells and provide a high level of gene expression [7–9]. However, these vectors have a number of disadvantages: carcinogenicity [10], immunogenicity [11], tropism for a wide range of cells [12], and difficult production [13]. In addition, some viral systems are rapidly cleared from the organism [14].

The use of nonviral vectors, such as liposomes, polymers, and dendrimers, for the delivery of NA addresses some of the problems mentioned above because they have low immunogenicity and usually do not cause an immune response [15]. The absence of limitations on the size of transferred therapeutic NA, the simplicity of synthesis, and the possibility of modifying the structure make nonviral vectors a promising NA delivery system [16, 17]. The biggest limitation to the use of nonviral vectors is the low TE. One potential solution to this problem is the use of liposomes as nonviral vectors because of their diverse morphology, composition, and ability to include various therapeutic biomolecules.

Liposomes based on cationic lipids (CLs) have attracted particular attention as nonviral delivery systems. By adjusting the surface charge of liposomes by changing the lipid composition, one can control the degree of interaction of liposomes with negatively charged NAs. Liposomes consisting of CLs are biodegradable because endogenous enzymes are capable of breaking down the lipid components of liposomes after being administered into the body. Furthermore, the surface of cationic liposomes can be modified by the addition of polyethylene glycol residues or targeted ligands [18, 19], and the incorporation of lipophilic chemotherapeutic drugs in the lipid bilayer can ensure the co-delivery of the drug and therapeutic NA [20, 21].

The formation of lipoplexes, which are complexes of negatively charged NAs and positively charged lipids/liposomes, occurs as a result of electrostatic interactions. The size of the formed lipoplexes mainly depends on the type of CL used and the quantitative ratio of positively charged CL nitrogen atoms and negatively charged NA phosphate groups (N/P ratio). By modifying the conditions for producing lipoplexes (NA concentration, pH, and the composition of the buffer solution), their size can also be controlled. As a rule, lipoplexes are formed, with a slight excess of a positive charge so that they can interact with negatively charged components of the cell membrane.

EXTRACELLULAR AND INTRACELLULAR BARRIERS

Most lipoplexes undergo structural changes and can be broken down by endogenous factors *in vivo* due to the presence of a number of extracellular (interactions with blood components, endothelial barriers, and cell membrane) and intracellular (cell uptake, release from endosomes, intracellular transfer, and delivery to the nucleus) barriers [22, 23].

The interaction of lipoplexes with blood components plays a significant role in their biological distribution [24–26] and can provoke the rapid elimination of lipoplexes from the bloodstream due to the reticuloendothelial system of the body and being captured by tissue macrophages [27]. It is known that serum proteins, such as albumin and high- and low-density lipoproteins, can bind to the surface of lipoplexes. In addition, the balance of adsorbed opsonins, which are differently recognized by receptors on the surface of macrophages, affects the clearance of lipoplexes [28–30]. The positive charge of lipoplexes activates the complement system, which accelerates the process of their elimination [31]. In general, larger lipoplexes are more rapidly excreted from the body compared with smaller particles [32].

It was shown that the absorption of cationic liposome–siRNA complexes can lead to the activation of innate immunity [33–35] by acting on RNA-sensitive Toll-like receptors and inducing inflammatory cytokines; in this case, a response occurs in the interferon production [35]. An immune response can also be caused by cationic liposomes themselves, even in the absence of siRNAs [36].

Lipoplexes that avoid clearance are delivered to the target tissue, but an endothelial barrier arises in their path. The endothelial barrier is a dense network of intracellular matrix that blocks the diffusion of liposome–NA complexes into target cells [37].

It is generally accepted that the penetration of lipoplexes through the cell membrane is due to the electrostatic interaction between the CL of lipoplexes and the negatively charged surface of the cell [38]. Endocytosis is the most common pathway for lipoplex

uptake and includes many mechanisms, such as clathrin- and caveolin-mediated endocytosis, macropinocytosis, and clathrin- and caveolin-independent pathways. An additional mechanism of lipoplex uptake, phagocytosis, only occurs in specialized cells, such as macrophages and dendritic cells [38]. The "choice" of the uptake mechanism is determined by the size of the absorbed lipoplexes, the type of transfected cells, and the composition of cationic liposomes.

After penetration into the cells, an important step in the transport pathway of lipoplexes is their escape from endosomes. There are several mechanisms for the release of NAs. Advantageously, monocationic lipid-based liposomes release their contents into the cytosol via lipid mixing. The basis of this mechanism is the fusion of lipoplex and endosome membranes, while CLs stimulate the translocation of negatively charged phospholipids of the membrane to the inner surface of endosomes. As a result, endosomal membrane destabilization and NA release into the cytoplasm occur [39]. Of note, the presence of special helper lipids in the composition of liposomes (see Section "Helper Lipids") promotes the fusion of lipoplexes with the endosomal membrane and its destabilization.

The second mechanism of NA release from lipoplexes is called the "proton sponge effect." It is characteristic of CLs containing a large number of secondary or tertiary amino groups, which have pKa values between physiological and lysosomal pH (usually from 5.5 to 6). When the medium is acidified inside the endosomes, the amino groups of CLs are protonated, which contributes to an additional influx of unbound chloride anions. To compensate for the increase in anions, additional water molecules enter the endosomes, causing osmotic swelling and rupture [39].

After their release from endosomes and entering the cytoplasm, NAs need to be delivered to the target compartment of the cell to achieve the desired biological effect. For NAs whose activity is in the cytoplasm, such as ODNs and siRNAs, this barrier is not important, but for pDNA, the target compartment is the nucleus. The mobility of large molecules, such as pDNA, in the cytoplasm is extremely low, which makes them susceptible to degradation by cytoplasmic nucleases [40].

The determining factors for the movement speed of pDNA through the cytoplasm are the size and structure of the pDNA molecule; cyclic pDNA moves faster than linear pDNA [41]. The effect of pDNA packing density on delivery efficiency is not well understood. However, tight packing increases the mobility and resistance of pDNA to cytoplasmic nucleases.

Finally, the expression of pDNA requires overcoming the last intracellular barrier, which is the nuclear membrane. During cell division, pDNA can penetrate the nucleus due to a transient loss of the integrity of the nuclear membrane; however, in nondividing cells, pDNAs pass through the membrane through a nuclear pore complex that can transfer molecules up to 9 nm in size and weighing less than 40 kDa by free diffusion [42]. The incorporation of peptide sequences termed nuclear localization signals into the composition of lipoplexes promotes a more efficient transfer of pDNA across the nuclear membrane [39].

Thus, when transfecting cells with lipoplexes, it is necessary to take into account not only their characteristics but also the existence of extracellular and intracellular barriers.

CATIONIC LIPIDS

Among nonviral vectors, the use of cationic liposomes as an NA delivery system has attracted the attention of drug developers owing to their obvious advantages, such as their ease of preparation, reproducibility, biodegradability, and commercial availability [43]. The main structural components of liposomes are CLs, which are amphiphilic molecules that can be easily obtained during chemical synthesis and used to study the relationship between structure and TE.

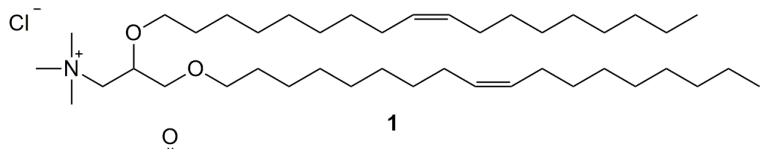
Among CLs for the construction of cationic liposomes, the most widely used are *N*-(1-(2,3-dioleyloxy)propyl)-*N,N,N*-trimethylammonium chloride (**1**, DOTMA) [17], *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (**2**, DOTAP) [17], 3β-[*N*-(*N′,N′*-dimethylaminoethyl)carbamoyl]cholesterol hydrochloride (**3**, DC-Chol) [8], dioctadecylamidoglycyl spermine (**4**, DOGS) [3], and 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propylammonium tetrafluoroacetate (**5**, DOSPA) [3].

CLs consist of four main structural units:

- Hydrophilic cationic group (HCG);
- Spacer group (SG);
- Linker group (LG);
- Hydrophobic domain (HD).

The cationic nature of a lipid is determined by the structure of its hydrophilic group, which is formed by primary, secondary, tertiary amines, quaternary ammonium groups, amino acids, short peptides, or heterocyclic bases [43]. It has been experimentally demonstrated that CLs based on quaternary ammonium salts are more toxic compared with analogs containing tertiary amino groups [44]. For the binding and delivery of NA, CLs containing charged phosphorus or arsenic atoms can be used, which increases the TE and decreases toxicity [45].

The hydrophobic CL domain is most often formed by long-chain hydrocarbon substituents (from 12 to 20 carbon atoms) or cholesterol. It was found that CL with a single hydrocarbon chain exhibited greater



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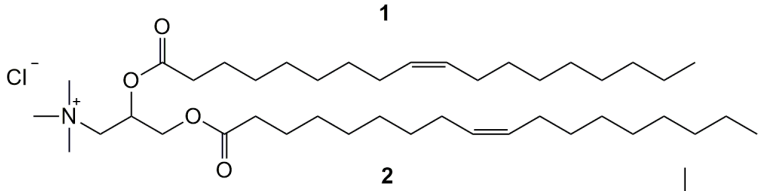

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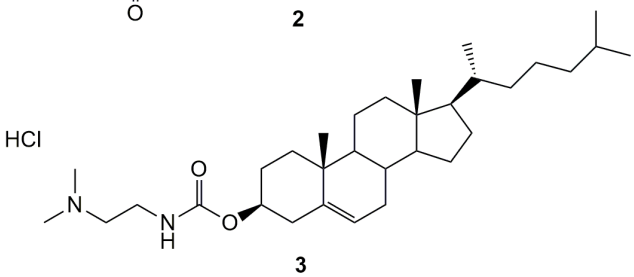

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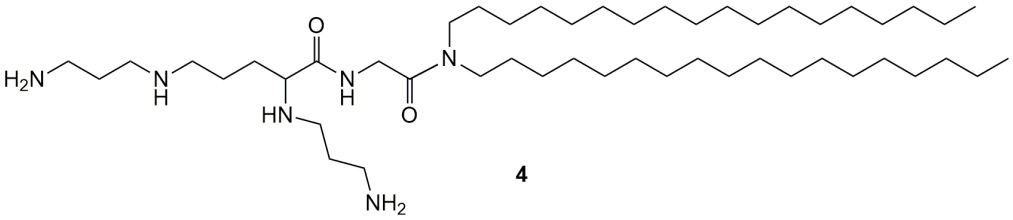

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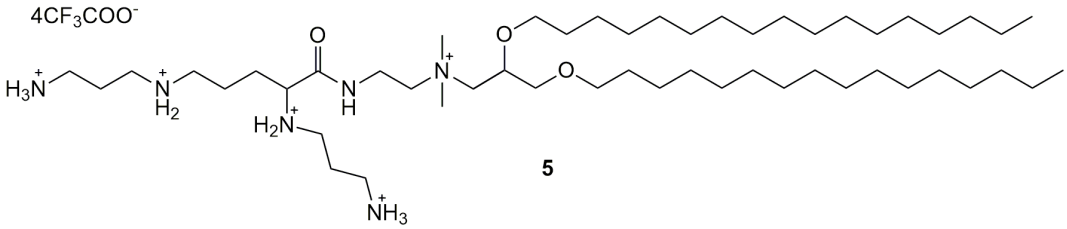

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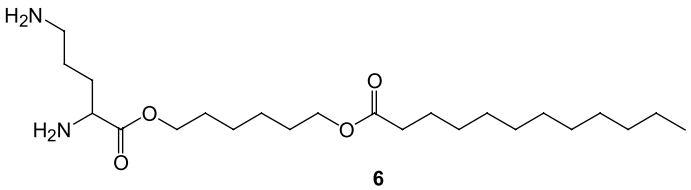
toxicity and a lower TE compared with CL with two chains [46]. However, it was shown that compound **6**, containing one dodecanoic acid residue as the HD, was not only more effective but also less toxic than DOTAP (**2**) [47]. Given the different dependence of TE and CL toxicity, as well as the structure of their HDs, the HD should be specifically selected.



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The LG (ester, ether, carbamoyl, or disulfide) binds to the HD and HCG and determines the stability and biodegradability of CL, which also affects the TE. DOTMA (**1**) with an ether linker exhibits a high TE but is too stable to be biodegraded in the body, which explains its high toxicity. Lipids with ester linkers, such as compound **2**, are more readily biodegradable after systemic administration [48]. When using a carbamoyl bond as a linker (lipid **3**), a decrease in pH will trigger the separation of the hydrophobic and hydrophilic parts of CL and, thus, promote the release of NA after penetration into the cell and endosomal uptake [49, 50]. Compounds containing disulfide bonds sensitive to the action of reducing agents as an LG are stable in the bloodstream but are broken down after penetration into the cytosol by the action of glutathione and/or reductases, which can be used to improve the TE [51]. However, the presence of a disulfide bond as an LG in compound **7** led to a complete loss of luciferase gene expression in HepG2 and HeLa cells [52].

The SG (glycerin, amino acids, oligomethylene groups, and polyethylene glycols) separates the HD and HCG. The length of the SG affects the toxicity and TE of cationic liposomes. For example, for CL **8** based on cholesterol, an increase in the length of the SG to three carbon atoms decreases the cytotoxicity of CL *in vitro* and *in vivo* [53].

When using spacers of the same length, but an HCG with a different structure, it was shown that the replacement of the methyl group in compound **9** with the ethyl in compound **10** increased both the toxicity and TE *in vitro* [53].

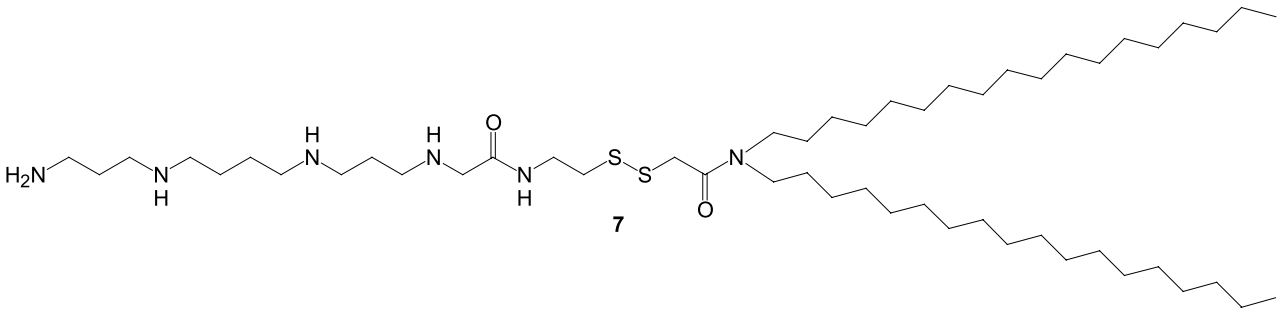
Cationic gemini amphiphiles **11a,b** with short SGs exhibited an increased TE compared with their analogs **11c,d** with long spacers [54].

In addition to length, the hydrophobicity of SGs plays an important role in the delivery of NA. When comparing the TE of CL based liposomes with hydrophobic oligomethylene spacers (n = 3, 5, 7, 11) and a hydrophilic trioxyethylene spacer, the maximum expression level was achieved using lipid **12** with a heptamethylene spacer [55].

HELPER LIPIDS

Cationic liposomes can form only from one CL; however, the addition of a neutral helper lipid to their composition can increase the TE [56]. Among the helper lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (**13a**, DOPE) [19] and phosphatidylcholine (**14**, PC) are the most widely used [57].

Two different helper lipids **13a** (DOPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (**13b**, DPPE) were used as component liposomes to study the efficiency of the endosomal release of NA into the cytosol and its further transport to the nucleus. In the case of helper lipid **13b**, which promotes the formation of lipoplexes with a lamellar structure, the TE was low (25% of transfected cells). However, when using lipid **13a**, which forms an inverted hexagonal phase, the number of transfected cells increased to 75% [58]. In contrast to the lamellar structure, which is a repeating layer of pDNA and CL, the inverted hexagonal structure promotes the condensation of pDNA inside the cylinders [19], which are assembled due to Van der Waals interactions between the lipid tails.



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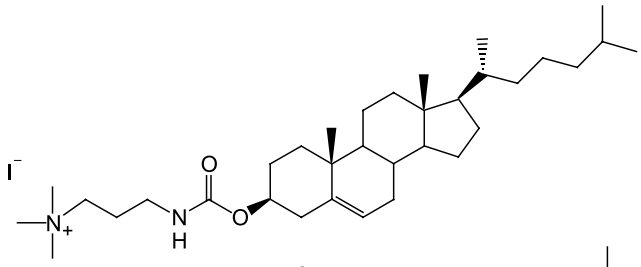

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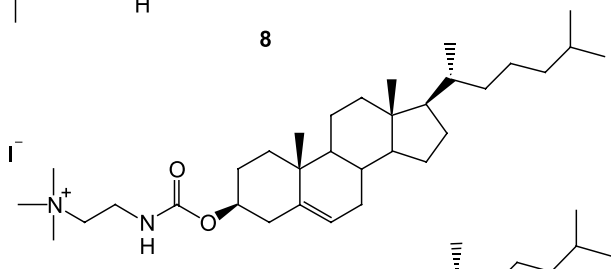

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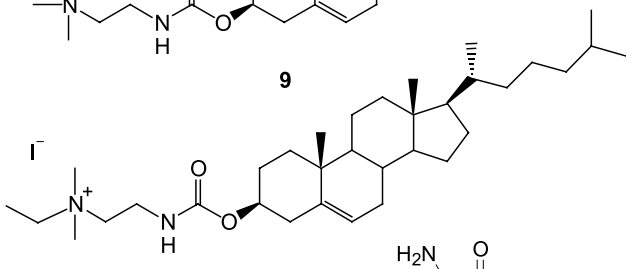

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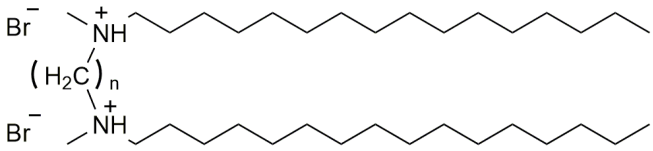
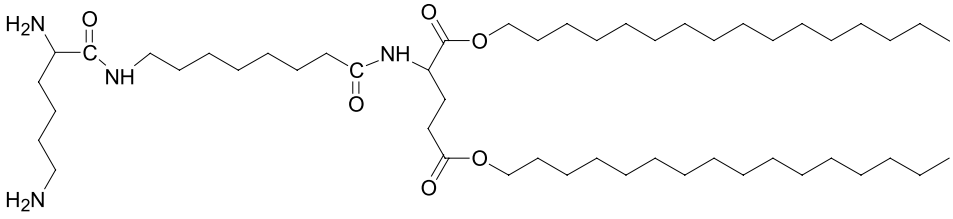


11 a-d

a, n = 2; **b**, n = 3; **c**, n = 5; **d**, n = 12



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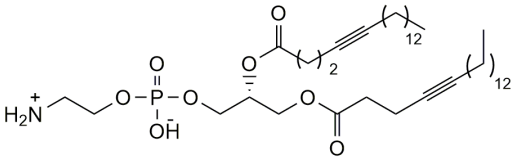
The use of DOPE as a helper lipid in various cationic liposomes increases the TE of numerous cell lines [19, 56, 59], because under the conditions of endosomal acidification, it can form an inverted hexagonal phase with a decrease in pH and thereby destabilize the endosomal membrane. Thus, a study of micelles formed by DOPE revealed that with a decrease in pH from 10.8 to 7.0, the transition of spherical micelles to hexagonal packed cylinders occurred [60]. The optimal range for this transition is a pH value from 9 to 7. This transition is associated with the zwitterionic nature of the DOPE polar head group. At high pH values, the phosphate group is negatively charged, which causes the repulsion of the hydrophilic groups of neighboring molecules. With a decrease in pH, hydrogen bonds form, which, together with the electrostatic interaction, causes the formation of hexagonally packed aggregates.

Liposomes based on CL **2** efficiently formed lipoplexes with pDNA starting from the N/P ratio of 2:1 and higher [59]. The incorporation of DOPE into the liposomal composition and further incubation with pDNA leads to the formation of a negatively charged lipoplex. The formation of salt bridges between the positively charged hydrophilic group of the CL **2** and the phosphate groups of the DOPE lipid allows the primary amino group of DOPE to stabilize near the surface of liposomes and interact more closely with negatively charged phosphate groups of DNA. It should be noted that at the N/P ratio of 6:1 and higher, compact, and homogeneous lipoplexes with a high positive charge were formed. DOPE can also provide the availability of HCG of the CL for DNA binding, thereby decreasing the interaction energy [59].

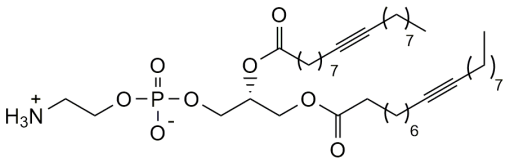
Thus, the use of DOPE provides an efficient release of compacted NA from endosomes by destabilizing the endosomal membrane [58, 61] and favors the more compact packing of DNA [62, 63].

Phosphatidylcholine **14** (PC) is a phospholipid consisting of a choline residue as a hydrophilic group and a phosphatidic acid with various acyl residues as an HD [57]. However, when using PC as a helper lipid, which forms lamellar cationic liposomes, the TE was lower than DOPE [56].

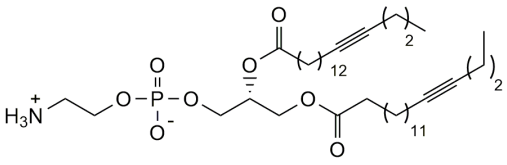
The low-DOPE phase transition temperature (10°C) reduces the stability of cationic liposomes and lipoplexes *in vivo*. One approach to solving this problem is the synthesis of DOPE analogs, the phase transition of which is near the temperature of the human body (~37°C). DOPE analogs (**15a–c**) were synthesized, in which the *cis* double bond in two acyl residues was replaced by a triple bond located at different positions of hydrocarbon substituents [64]. This chemical modification made it possible to form a new intermolecular package, which contributes to an increase in the phase transition temperature under physiological conditions.



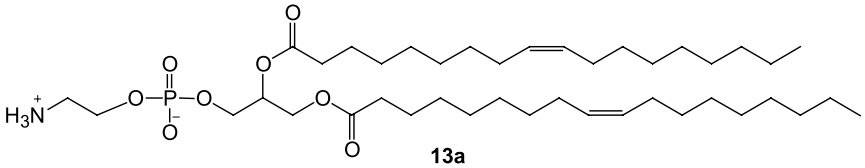
15a



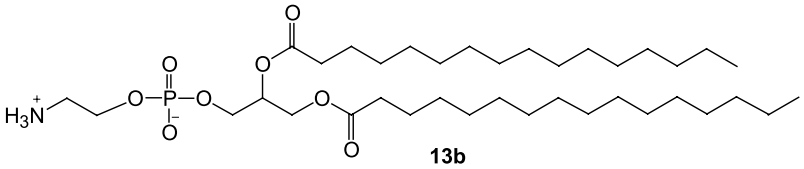
15b



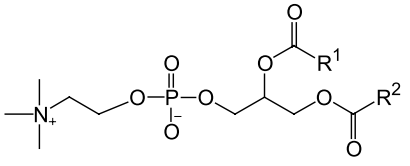
15c



13a

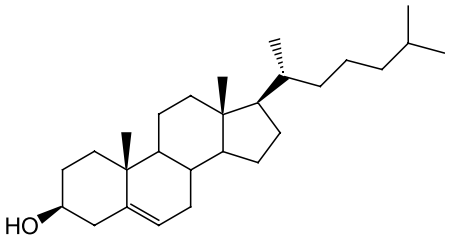


13b



14

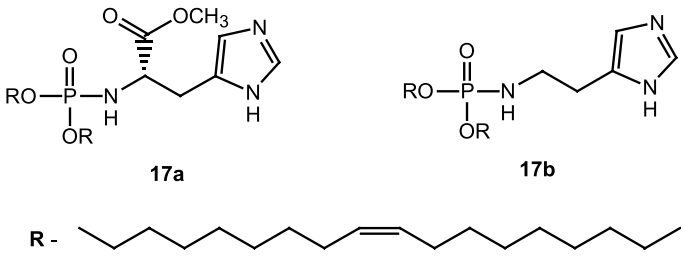
Cholesterol (**16**, Chol) is another common helper lipid [65–72]. An increase in the amount of cholesterol to 66.7% in the composition of liposomes with CL **3** led to the formation of stable particles that resulted in a high TE in the presence of blood serum. Of note, a further increase in the amount of cholesterol up to 80% did not change the TE [66].



16

It was found that some CLs have a high affinity for the helper lipid and are only active in the presence of cholesterol or DOPE. Liposomes containing a helper lipid are often more effective at transfecting cells than those with phospholipids, which may be related to the endogenous nature of cholesterol [73].

Another approach to identifying more efficient helper lipids was the synthesis of new lipids **17a** and **17b** with an imidazole-based polar group, which are not charged at a physiological pH. Their protonation in endosomes induces the fusion of lipoplexes with the endosomal membrane and promotes the release of NA into the cytosol. Of note, adding the new helper lipids **17a** and **17b** to the composition of cationic liposomes can improve transfection by 100 times compared with DOPE [74].



17a

17b

Thus, the efficiency of NA delivery can be optimized by adding helper lipids to the composition of cationic liposomes.

COMMERCIAL TRANSFECTANTS BASED ON CL

To date, there are a number of commercial transfectants based on CL: Lipofectamine (Lipofectamine 2000, Lipofectamine 3000, Lipofectamine RNAiMAX, Lipofectamine MessengerMAX, Lipofectamine CRISPRMAX, Lipofectamine LTX, Lipofectamine Stem), Lipofectin, LipofectACE, Transfect, Trans, Transfectam, Cellfectin, Cellfectin II, and others [75–79].

Since it was launched into large-scale production in 1993, lipofectamine and its analogs have been mainly used for cell transfection [80]. Achieving a high TE of a wide range of cell lines and the ability to transfer various types of NA, lipofectamines are considered the "gold standard" among transfection reagents and are most often used for the comparative evaluation of efficacy both in the development of new CLs and alternative transfection methods.

Lipofectamine 2000 (Lf 2000) is a mixture of a polycationic lipid **5** and a neutral lipid DOPE at a ratio of 3:1 mol [78]. When choosing the appropriate transfection agent, it is necessary to consider the type of NA being delivered. Universal reagents, such as Lf 2000 and Lf 3000, are used for both DNA and RNA delivery. Lipofectamine RNAiMAX [81, 82] and Lipofectamine MessengerMAX™ were developed specifically for the transfection of siRNA and miRNA cells, respectively.

Lf 2000 successfully transfects neonate hamster kidney cells (BHK-21), mouse embryonic fibroblasts (NIH 3T3), African green monkey cells (COS-1), human colonic epithelial cells (HT-29), human diploid cells (MRC-5), and breast cancer cells (SK-BR3). Lf 3000 is an improved version of Lf 2000 and successfully transfects a wide variety of biologically relevant cell types [78, 83–85]. Its distinguishing feature is its high TE in the presence of blood serum, and therefore, there is no need to change the culture medium after transfection, as well as the presence of the second component, which is only used for the delivery of pDNA. Lipofectamine LTX can effectively transfect cells that are usually difficult to transfect, sensitive cells, and primary cell cultures. In turn, Lipofectamine Stem was developed specifically for the transfection of stem cells. Invivofectamine 3.0 is suitable for the *in vivo* delivery of NA [86–88] and, in particular, for the delivery of siRNA and duplex miRNA in mouse liver cells by the injection of lipoplexes into the tail vein.

The transfection agent Lipofectin is a mixture of lipid **1** and DOPE at a ratio of 1:1 mol and is used for the transfection of a wide range of cells [89, 90]. It is believed that DNA spontaneously interacts with lipid **1** according to the same principle as with lipid **5**, while 100% of the DNA binds into lipoplexes.

Based on lipopolyamine **4**, the commercial transfectant Transfectam was created, which effectively delivers NAs into corticotropic tumor cells (AtT20) and NIH 3T3 cells [91, 92].

A study based on identifying the simplest surfactants for the transfection of eukaryotic cells revealed that liposomes based on dimethyldioctadecylammonium bromide (**18**, DDAB) and DOPE were more effective than Lipofectin [93, 94], and therefore, TransfectACE (or LipofectACE) containing compound **18** and DOPE in a ratio of 1:2.5 mol was patented.

Transfecting agents Cellfectin and Cellfectin II are a mixture of *N,N,N,N*-tetramethyl-*N,N,N,N*-tetra(hexadecyl)spermine (**19**, TM-TPS) and DOPE at a ratio of 1:1.5 mol that are suitable for the transfection of both mammalian and insect cells (Sf9, Sf21, and S2) [78, 79, 94].

The DMRIE-C transfection agent, consisting of 1,2-di(tetradecyloxy)propyl-3-*N*-2-hydroxyethyl-*N,N*-dimethylammonium bromide **20** and cholesterol in a ratio of 1:1 mol, is suitable for the transfection of eukaryotic cells and is especially effective for the transfection of suspension cultures (human T-lymphoblastic leukemia cells or Jurkat cells), as well as other cell lines derived from lymphoid cells [95, 96].

Oligofectamine is a proprietary composition for ODN and siRNA delivery into eukaryotic cells. Due to the formation of stable complexes with ODNs, it efficiently transfects eukaryotic cells, including Chinese hamster ovary cells, human kidney embryo cells (HEK 293), NIH 3T3, and human cervical cancer cells (HeLa) [97].

Despite the wide variety of existing transfection agents, the development of new delivery systems that can efficiently transfer various types of NA *in vitro* and *in vivo* without having a toxic effect on cell viability will lead to the appearance of more and more liposomes.

LIPOSOMES BASED ON NEW SYNTHETIC CL

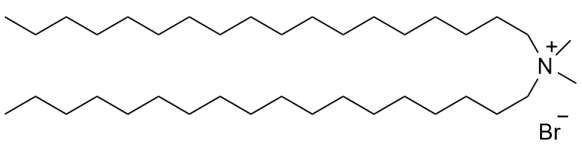
Over the past two decades, a large number of studies have been published, in which cationic liposomes are used as carriers for NA delivery *in vitro* and *in vivo*. The formation of liposome–NA complexes (lipoplexes) and their ability to transfect eukaryotic cells depends on both the composition of liposomes and the structural features of CL. In addition, the ratio of the lipoplex components (defined by the N/P ratio) and their physicochemical characteristics (size and zeta potential) also determine the efficiency of cell transfection.

Recently, it has been suggested that the penetration of lipoplexes into the cell is mediated by special cholesterol transporters. The cholesterol presented in cell membranes disrupts the dense packing of phospholipids and reduces their membrane fluidity, as well as their permeability to small water-soluble molecules. Since the energy barrier for the flip-flop translocation of cholesterol molecules is low, its redistribution between layers occurs quickly, which affects the cellular uptake of complexes, as well as the formation and degradation of endosomes [98].

The use of cholesterol-containing CL or cholesterol as a helper lipid increases the TE [66]. This may be due to the formation of cholesterol nanodomains in lipoplexes, which mediate endocytosis, and/or intracellular transport of complexes. In addition, cholesterol in liposomes protects NA from degradation by nucleases in the body and reduces the binding of lipoplexes to serum proteins, thereby improving the delivery of NA. Thus, the use of cholesterol-based CL can significantly increase the efficiency of NA delivery and expression [59, 66, 67, 99–105].

One of the most widely used cholesterol-based CL (**3**, DC-Chol) effectively delivered NAs either alone or in combination with other lipids into various eukaryotic cells [59, 66, 68, 99, 100]. In addition, other cholesterol-based CLs (**21–26**) were synthesized (Table 1) for the delivery of various types of NA.

A comparative study of the effectiveness of liposomes consisting of monomeric **21** or dimeric CL **22a–e** and DOPE, which was conducted both in the presence (+FBS) and absence of serum (–FBS) (Table 1), revealed that in the absence of serum, CL **21** transfected about 70% of cells with a mean fluorescence intensity (MFI) of 20 relative units, whereas CL **22a–e** transfected 45–70% of cells with an AFI of 20–75 relative units. In the presence of blood serum, the TE of monomeric CL and dimeric CL decreased to ~20 and 40%, respectively. Liposomes based on CL **22d–e** were toxic in the absence of serum in the culture medium [101].



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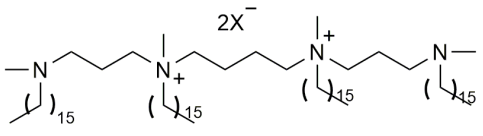

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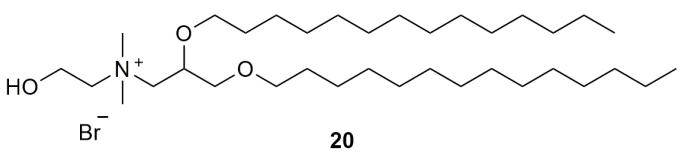


20

Table 1. CL based on cholesterol

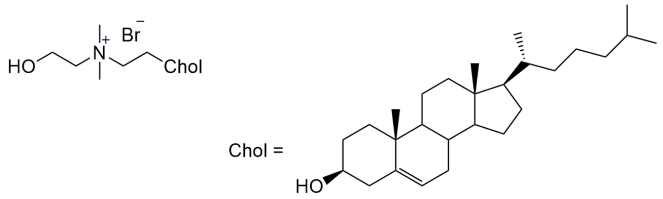
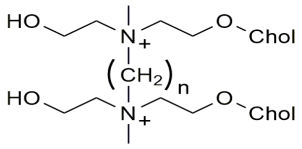
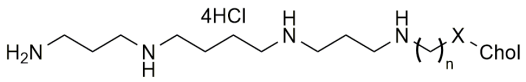
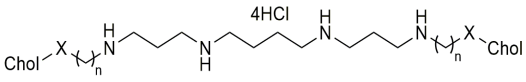
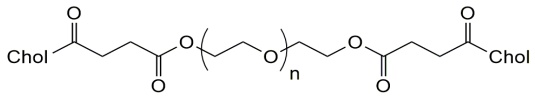
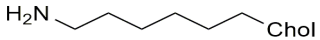
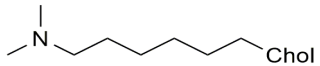
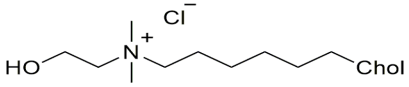
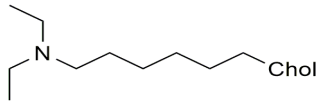
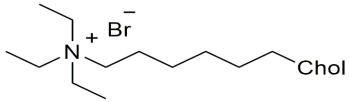
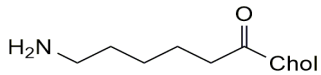
	CL structure		CL:helper lipid, molar ratio	Transferred NA	Cell type	IC_{50}[1], μM	TC[2], %	MFI[3], rel. unit	Source
						FBS (−) / FBS (+)[4]			
21			**21**-DOPE, 1:1	pEGFP-C3[5]	HeLa[6]	>80 / >80	70 / 20	20 / 20	[101]
22a		n = 3	**22a**-DOPE, 1:1				60 / 40	20 / 30	
22b		n = 4	**22b**-DOPE, 1:1				70 / 70	25 / 30	
22c		n = 5	**22c**-DOPE, 1:2				70 / 50	75 / 60	
22d		n = 6	**22d**-DOPE, 1:2			40 / >80	55 / 45	55 / 55	
22e		n = 12	**22e**-DOPE, 1:2				45 / 40	30 / 55	
23a		X = C(O), n = 4	**23a**-DOPE, 1:1	pEGFP-C2	HEK 293[7]	36.0 / 35.0	55 / 29.6	30 / 21.4	[59]
23b		X = NHC(O), n = 4	**23b**-DOPE, 1:1			37.2 / 37.3	68 / 44.2	55 / 42.1	
23c		X = NHC(O), n = 6	**23c**-DOPE, 1:1			38.8 / 35.5	45 / 18.9	18 / 9.1	
24a		X = C(O), n = 4	**24a**-DOPE, 1:1	pEGFP-C2	HEK 293	>80	35 / 21.5	12 / 5.9	[59]
24b		X = NHC(O), n = 4	**24b**-DOPE, 1:1			>80 / 71.9	35 / 21.8	10 / 3.3	
24c		X = NHC(O), n = 6	**24c**-DOPE, 1:1			40.5 / 37.1	70 / 75.7	29 / 42	
25a		n = 600	**2-16-25a**	pEGFP	SKOV-3[8]	100 / n.d.[10]	20 / n.d.	n.d.	[102]
					A549[9]	n.d.	17 / n.d.		
25b		n = 1000	**2-16-25b**		SKOV-3	143 / n.d.	7 / n.d.		
					A549	n.d.	15 / n.d.		
25c		n = 2000	**2-16-25c**		SKOV-3	302 / n.d.	9 / n.d.		
					A549	n.d.	12 / n.d.		

Table 1. Continued

	CL structure	CL:helper lipid, molar ratio	Transferred NA	Cell type	IC_{50}[1], μM	TC[2], %	MFI[3], rel. unit	Source
					FBS (−) / FBS (+)[4]			
26a		**26a**-DOPE, 2:1	pEGFP-N1	293T[11]	100 / n.d.	43 / 41	300 / 290	[103]
				HeLa		15 / 16	20 / 23	
26b		**26b**-DOPE, 2:1		293T	50 / n.d.	22 / 25	150 / 155	
26c		**26c**-DOPE, 2:1		293T	30 / n.d.	3 / 5	40 / 50	
26d		**26d**-DOPE, 1:0.5		293T	50 / n.d.	23 / 25	150 / 153	[103]
26e		**26e**-DOPE, 1:0.5		293T	30 / n.d.	5 / 4	3 / 2	
26f		**26f**-DOPE, 1:0.5		293T	100 / n.d.	37 / 53	240 / 250	
				HeLa		80 / 77	110 / 100	

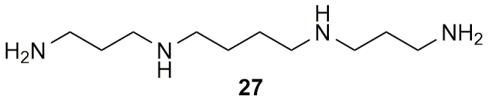
[1]IC_{50} – liposome concentration at which cell growth is inhibited by 50%.
[2]TC – transfected cells.
[3]MFI – mean fluorescence intensity.
[4]FBS (−) / FBS (+) – absence / presence of fetal bovine serum (10%).
[5]EGFP – enhanced green fluorescent protein.
[6]HeLa – cervical cancer cell line.
[7]HEK 293 – human embryonic kidney cells.
[8]SKOV-3 – human ovarian adenocarcinoma cell line.
[9]A549 – human lung carcinoma cells.
[10]n.d. – no data.
[11]293T – human embryonic kidney cells transfected by adenovirus type 5 and SV40 virus.

The transfection activity of liposomes formed by polycationic lipids depends on the type of linker, the length of the spacer, and the amount of cholesterol residues. Liposomes prepared from CL **24c** (Table 1), consisting of two cholesterol residues, a carbamoyl linker, and a hexamethylene SG and DOPE revealed the best *in vitro* TE among other tested analogs with CL **23a–c** and **24a,b** [59]. In the absence of blood serum, an increase in TE was observed with an increase in the N/P ratio for all liposome compositions, whereas the **23a**-DOPE, **23b**-DOPE, or **24c**-DOPE liposomes transferred pDNA efficiently at an N/P ratio of 6:1. Lf 2000 provided less efficient delivery of NA. The presence of blood serum decreased the TE by 20–30% for all cationic liposomes, except for the **24c**-DOPE composition (Table 1).

Cationic liposomes based on CL **2**, helper lipid **16**, and cholesterol-containing PEG derivatives **25a–c** efficiently delivered pDNA encoding a green fluorescent protein into 7–20% of SKOV-3 cells and 12–17% of A549 cells (Table 1) [102].

A number of liposomes based on lipids **26a–f** (Table 1), in which cholesterol was used as an HD, and primary, tertiary, or quaternary amino groups served as hydrophilic cationic heads were also studied. The hydrophobic and hydrophilic domains were linked via ether or ester bonds. Among the six studied compositions, the highest TEs were demonstrated by liposomes with CL **26a** and **26f** containing primary amino groups in their structure. Specifically, they delivered pEGFP pDNA into 293T cells more efficiently than the commercial agent Lf 2000 [103].

Another structural element that has a strong effect on TE is the cationic group, which is necessary for the binding and compaction of NA. It is known that mammalian polyamines, such as spermine (**27**), spermidine (**28**), and putrescine (**29**), not only have the ability to bind NA but also affect the fusion of liposomes with the cell membrane [104]. Liposomes based on CL **30a–c** were used to deliver siRNA into HeLa cells (Table 2) [105]. The maximum number of transfected cells (62%) was achieved using spermine-based liposomes with CL **30a**.



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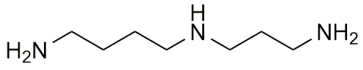

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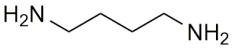


29

The important role of spermine in the formation of lipoplexes has been presented in a number of studies [105–111]. In [106], the prospects of using cationic liposomes consisting of spermine-containing CL **31a–c** with acyl substituents of various lengths and a helper lipid **14** were considered (Table 2). Among them, liposomes containing CL **31a** with a myristoyl substituent revealed the highest level of transfection and the lowest cytotoxicity. The same pattern was observed when using these lipids in the composition of niosomes (nonionic surfactant vesicles) [107].

Liposomes based on N^4,N^9-diacylated spermine derivatives **32a–j** (Table 2) containing two fatty acid residues from 18 to 24 hydrocarbon chains protect NA from nucleases and promote efficient NA transport. In the absence of serum, liposomes based on asymmetric CL **32d** with oleic and arachidonic acid residues were found to transfect the highest number of cells (68%), and CL **32c** with two linoleic acid residues produced the highest MFI (15 relative units) [108]. In the presence of serum, liposomes with CL **32e–j** have demonstrated a TE comparable or superior to Lf 2000 [70, 109]. The most promising CL (**32g**) containing unsaturated oleic and saturated lignoceric acids transfected 85% of HeLa cells with a MFI of 50 relative units.

In [110, 111], the authors studied the transfection ability of liposomes based on spermine-containing CL with different LGs (di(hydroxyethyl)amino (**33**), di(hydroxyethyl)aminocarboxy (**34a–c**), 3-amino-1, 2-dioxipropyl (**35a–c**), and 2-amino-1,3-dioxipropyl (**36a–c**)) and three HDs (lauric, myristic, and palmitic acids). All obtained liposomes and their complexes with pDNA were found to be slightly toxic for cells (Table 2). Among liposomes containing CL **33–36**, lipids **35b** and **35c** with myristoyl and palmitoyl residues were the most effective for cell transfection, although CL **36a**, with a shorter hydrocarbon chain, also facilitated efficient delivery of pDNA in the absence of blood serum. The addition of serum to the culture medium decreased the TE of liposomes based on CL **35b** and **35c** with a 3-amino-1,2-dioxipropyl linker, whereas the efficiency of liposomes based on CL **36a** with a 2-amino-1,3-dioxipropyl linker did not change (93% of cells).

CONCLUSIONS

The development of cationic liposomes capable of efficiently delivering NA to target cells with minimal toxic effects is the ultimate goal of any transfection related research field. Despite numerous studies on the development of optimal systems for the delivery of NA into eukaryotic cells, the question

Table 2. CL based on spermine

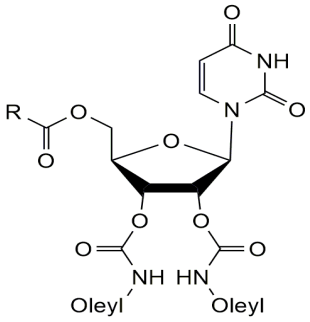
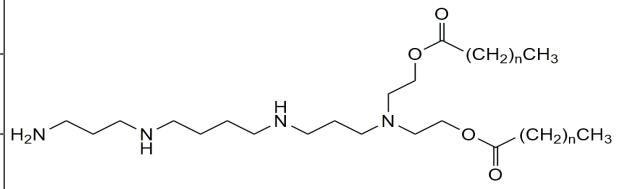
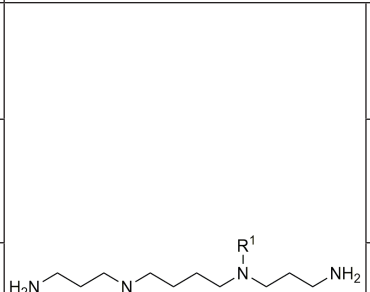
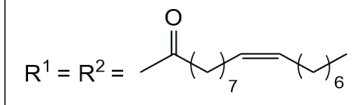
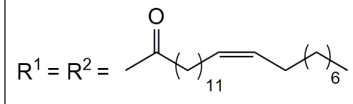
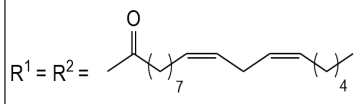
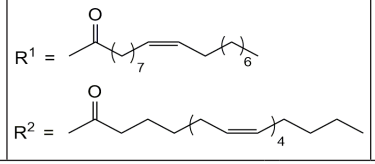
	CL structure	CL:helper lipid, molar ratio	Transferred NA	Cell type	IC_{50}[1], μM	TC[2], %	MFI[3], rel. unit	Source
					FBS (–) / FBS (+)[4]			
30a	 R = **27**	**30a**	siRNA	HeLa[5]	50 / n.d.[6]	62 / n.d.	n.d.	[105]
30b	R = **28**	**30b**				43 / n.d.		
30c	R = **29**	**30c**				15 / n.d.		
31a	 n = 12	**31a–14**, 5:1	pEGFP-C2[7]	HeLa	30 / n.d.	15 / n.d.	n.d.	[106], [107]
31b	n = 14	**31b–14**, 5:1			40 / n.d.	12.5 / n.d.		
31c	n = 16	**31c–14**, 5:1			40 / n.d.	10 / n.d.		
32a	 	**32a**	siEGFP-AF	HeLa	n.d. / 100	50 / n.d.	17 / n.d.	[108]
32b		**32b**				30 / n.d.	10 / n.d.	
32c		**32c**				25 / n.d.	15 / n.d.	
32d		**32d**				68 / n.d.	10 / n.d.	

Table 2. Continued

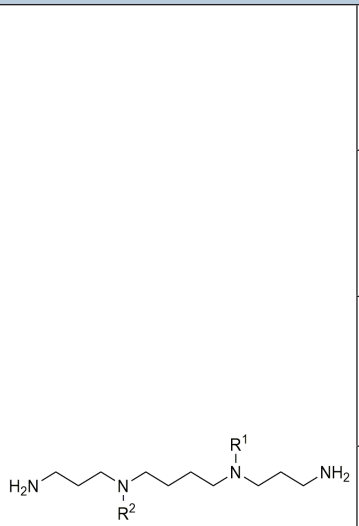
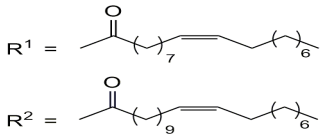
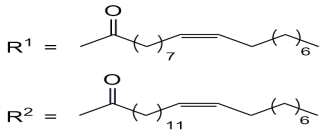
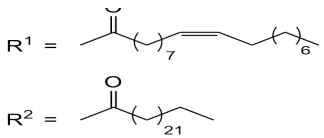
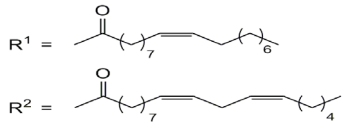
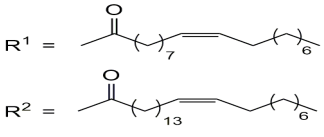
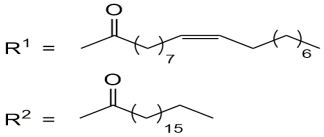
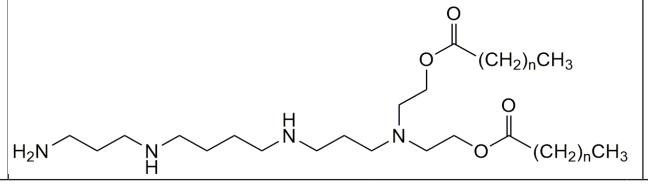
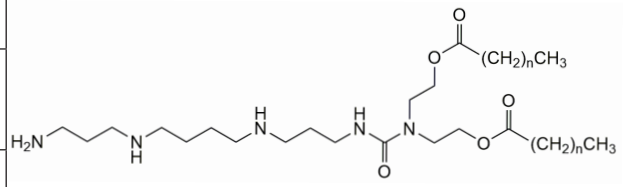
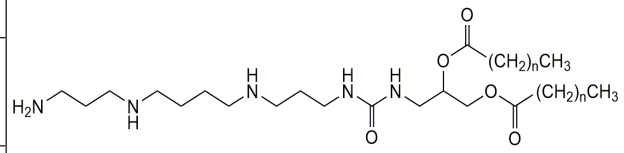
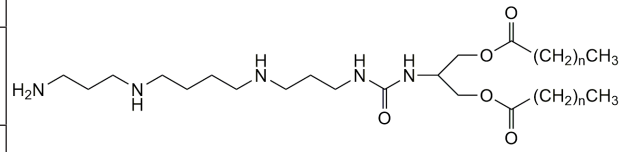
	CL structure		CL:helper lipid, molar ratio	Transferred NA	Cell type	IC_{50}[1], μM	TC[2], %	MFI[3], rel. unit	Source
						FBS (−) / FBS (+)[4]			
32e			**32e**	siEGFP-AF	HeLa	n.d. / 100	n.d. / 75	n.d. / 19	[108]
32f			**32f**				n.d. / 73	n.d. / 35	
32g			**32g**				n.d. / 85	n.d. / 50	[108]
32h			**32h**				n.d. / 57	n.d. / 10	
32i			**32i**				n.d. / 83	n.d. / 23	[70], [109]
32j			**32j**				n.d. / 75 n.d. / 30		
33		n = 10	**33**-DOPE, 1:1	pCMV	HeLa	40 / n.d.	30 / 21	n.d.	[110], [111]

Table 2. Continued

	CL structure		CL:helper lipid, molar ratio	Transferred NA	Cell type	IC_{50}[1], μM	TC[2], %	MFI[3], rel. unit	Source
						FBS (−) / FBS (+)[4]			
34a		n = 10	**34a**-DOPE, 1:1	pCMV	HeLa	40 / n.d.	48 / 24	n.d.	[110], [111]
34b		n = 12	**34b**-DOPE, 1:1				64 / 33		
34c		n = 14	**34c**-DOPE, 1:1				48 / 26		
35a		n = 10	**35a**-DOPE, 1:1				32 / 20		
35b		n = 12	**35b**-DOPE, 1:1				98 / 50		
35c		n = 14	**35c**-DOPE, 1:1				94 / 47		
36a		n = 10	**36a**-DOPE, 1:1				88 / 93		
36b		n = 12	**36b**-DOPE, 1:1				16 / 25		
36c		n = 14	**36c**-DOPE, 1:1				8 / 13		

[1]IC_{50} – liposome concentration at which cell growth is inhibited by 50%.
[2]TC – transfected cells.
[3]MFI – mean fluorescence intensity.
[4]FBS (−) / FBS (+) – absence / presence of fetal bovine serum (10%).
[5]HeLa – cervical cancer cell line.
[6]n.d. – no data.
[7]EGFP – enhanced green fluorescent protein.

of the effectiveness of cationic liposomes remains one of the main factors limiting their use in gene or antisense therapy.

Synthetic and structural studies that aim to identify new CLs and the generation of liposomes on their basis open up new prospects in the development of nonviral systems for the delivery of NA in gene therapy. Among the most promising *in vitro* delivery systems are cholesterol- and spermine-based lipids with variations in composition, spacer length, and the type of linker used. However, in order to optimize the targeted delivery of NA, in addition to composition, it is also necessary to take into account the physicochemical parameters of cationic liposomes and the complexes with NA formed by them. These parameters include the size and surface potential of the complexes, which depend on both the ratio of components and the composition of lipoplexes. Thus, in addition to the search for the optimal structures of CLs, it is necessary to determine the ratio of the component of lipoplexes providing the delivery of NA to target cells.

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The authors declare no conflicts of interest.

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