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Novel Cholesterol-Based Cationic Lipids for Gene Delivery

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Gene therapy based on gene delivery is a promising strategy for the treatment of human disease. Here we present data on structure/biological activity of new biodegradable cholesterol-based cationic lipids with various heterocyclic cationic head groups and linker types. Enhanced accumulation of nucleic acids in the cells mediated by the lipids was demonstrated by fluorescent microscopy and flow cytometry. Light scattering and atomic force microscopy were used to find structure/transfection activity correlations for the lipids. We found that the ability of the lipids to stimulate intracellular accumulation of the oligodeoxyribonucleotides and plasmid DNA correlates well with their ability to form in solution lipid/NA complexes of sizes that do not exceed 100 nm. Screening of the lipids revealed the most promising transfection agents both in terms of low toxicity and efficient delivery: cholesterol-based lipids with positively charged pyridine and methyl imidazole head groups and either the ester or carbamate linker.

Introduction

Methods of gene therapy are based on delivery into cells of various nucleic acids (plasmid DNA (pDNA), antisense oligonucleotides (ONs), and small interfering RNAs (siRNAs)[1−4] able to down- or upregulate the expression of target genes. Improvement of the delivery efficiency of nucleic acids into different cells is needed for the development of safe and practical protocols for gene therapy.[5] Therefore, the search for the agents providing an efficient gene delivery and protection from the enzymatic degradation is of the highest importance. Nonviral vectors and synthetic transfection agents are used for transfection of mammalian cells and considered as potential vectors for in vivo application.

Cationic lipids represent the largest family of the synthetic gene delivery systems. In comparison with the other transfection agents, cationic lipids possess a number of advantages, namely they do not induce an immune response, are easy to prepare, and stable in storage.[6,7] The complexes of cationic lipids with nucleic acids, lipoplexes, are formed spontaneously due to electrostatic interactions between the cationic head groups of lipids and the negatively charged phosphate groups of nucleic acids.[8,9] Varying the properties of lipoplexes, such as composition, charge ratio, and structure of the cationic lipids, it is possible to regulate the transfection efficiency.

The application of cationic lipids as gene delivery agents is limited by their toxicity and moderate transfection efficiency.[10] Development of biodegradable cationic lipids based on natural compounds can yield transfection agents with improved properties.

In this paper, we attempted to optimize the structure of cationic lipids in order to decrease the toxicity and increase gene transfer activity. For this purpose, we synthesized and studied new biodegradable cholesterol-based cationic lipids CvPB,[a] CvMI, CvII, CcHPB, CmPB, and CmMB (**1**−**6**) built of cholesterol (known for its high transfection potential[11]) and positively charged pyridine, *N*-methylmorpholine, or methyl imidazole headgroup connected by an ester, ether, or carbamate linker. Figure 1 shows the structures of the synthesized cationic lipids containing nitrogenous heterocyclic bases.

Results

Design and Synthesis of the Cationic Lipids 1−6 (Figure 1). The structures of cationic lipid molecules can include a hydrophobic component, hydrophilic cationic domain, and a linker connecting these two. A direct functionalization of each of the domains gives rise to a series of amphiphiles, which can be used to study the correlation between lipid structure and transfection efficiency.[12−16]

There are two major types of hydrophobic domains, namely aliphatic chains and cholesterol-based derivatives. Cholesterol as a lipid anchor domain attracts practical and scientific interest because of its biocompatibility and ability to stabilize membranes, form rigid liposomes, and promote fusion process.

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[a] Abbreviations: DC-Chol, cholesteryl 3β-*N*-(dimethylaminoethyl)-carbamate hydrochloride; CvPB, *N*-[4-(cholest-5-en-3β-yloxycarbonyl)butyl]pyridinium bromide; CvMI, *N*-methyl-*N*-[4-(cholest-5-en-3β-yloxycarbonyl)butyl]morpholinium iodide; CvII, *N*-methyl-*N*′-[4-(cholest-5-en-3β-yloxycarbonyl)butyl]imidazolium iodide; CcHPB, *N*-[6-(cholest-5-en-3β-yloxycarbonylamino)hexyl]pyridinium bromide; CmPB, cholest-5-en-3β-yloxymethylpyridinium bromide; CmMB, *N*-(cholest-5-en-3β-yloxymethyl)-*N*-methylmorpholinium bromide.

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A hydroxyl group at C-3 atom of steroid skeleton can be easily functionalized. For example, a cholesterol containing monocationic lipid, cholesteryl 3β-*N*-(dimethylaminoethyl)carbamate hydrochloride (DC-Chol), and a polycationic lipid, N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN), show a high transfection efficiency and have been used for DNA or ONs delivery in tumor therapy or artificial immunization.[17,18]

It is known that both cationic and linker groups are the major components of cationic lipid that influence transfection efficiency. It has been recently shown that heterocyclic cationic lipids, e.g., pyridinium and imidazolinium, can be used to transfect eukaryotic cells.[19−26] Earlier, we elaborated the methods for synthesis of glycerol- and cholesterol-based positively charged heterocyclic amphiphiles.[27−29] The linker group determines the chemical stability and biodegradability of the lipid in both cell environment and biological fluids.[15] The hydrophobic and polar domains of cationic lipids can be linked via carbamate, amide, ester, or ether bonds. It has been noticed[30−32] that the ether-linked lipids are more chemically stable but more cytotoxic than the ester or urethane lipids. On the contrary, it has been found that using an ether linkage rather than a more hydrolytically unstable ester- or urethane-based linkage offers a significant advantage in improving gene transfer.[33,34]

Taking into account the results of our previous research,[29] we synthesized the cholesterol derivatives **1**−**3** (Figure 1)

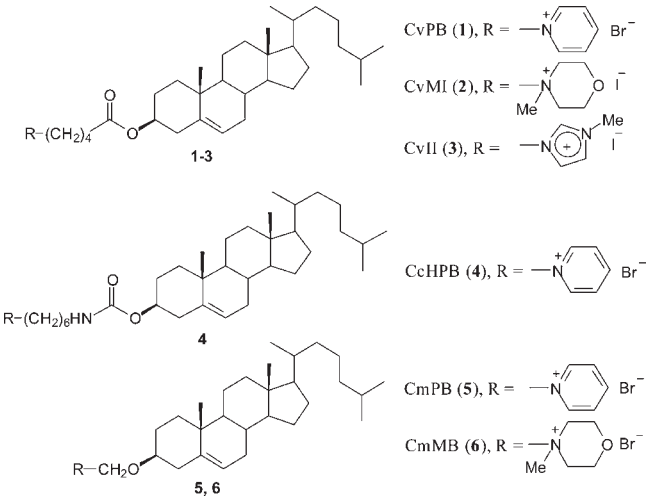


Figure 1. Structure of the cationic lipids **1**−**6**.

with a positively charged group linked to the 3β-position of steroid skeleton via an ester linkage. Pentanoic acid was attached to the cholesterol molecule by treating with the corresponding 5-bromopentanoyl chloride. Two approaches were used to link the cationic group to cholesteryl 5-bromopentanoate, namely one-step quaternization of pyridine and a two-step procedure comprising preparation of tertiary cyclic amine by the alkylation of imidazole and morpholine followed by the quaternization. In the first approach, cholesterol 5-bromopentonoate reacted with pyridine to produce cationic pyridinium salt **1** with 91% yield. To prepare *N*-methylimidazolium **2** and *N*-methylmorpholinium **3** derivatives, imidazole or morpholine was alkylated with cholesteryl 5-bromopentanoate to furnish tertiary amines in practically quantitative yields. In the final stage, the corresponding tertiary amines were subject to quaternization with methyl iodide to produce the target cationic lipids **2** and **3** with 80% yields.

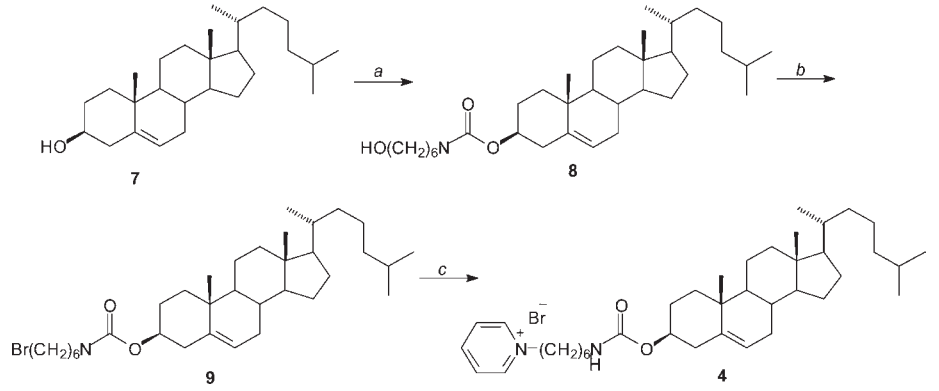
To synthesize the pyridinium cholesterol derivative **4** with a carbamate linker group (Scheme 1), cholesterol (**7**) was treated with 1,1′-carbonyldiimidazole to obtain imidazolide, which, in turn, was converted without purification into carbamate **8** with 90% yield in two stages.

Nucleophilic substitution of the hydroxyl group with bromine by treatment of **8** with carbon tetrabromide in the presence of PPh_3 gave bromide **9**. The final quaternization of pyridine by 6-(cholest-5-ene-3β-yloxycarbamino)hexylbromide (**9**) yielded the key compound **4** as a slightly yellow solid with 90% yield.

It is known that the acidity of endosomal environment after endocytosis is decreased from the physiological pH 7.4 to 6.5−6.0 in the endosome and to pH 5.0 in the primary or secondary lysosomes.[35] Incorporation of acid-labile bonds in the cationic lipid structure facilitates the release of nucleic acids from the endosomal compartment, thereby elevating the transfection efficiency.[36,37] We have prepared the cationic amphiphiles **5** and **6** in which cholesterol is linked with the positively charged heterocyclic base by an acid-labile ether linker (*N*,*O*-acetal) (Scheme 2). It is known that *N*,*O*-acetals readily undergo hydrolysis under acidic conditions.[38,39]

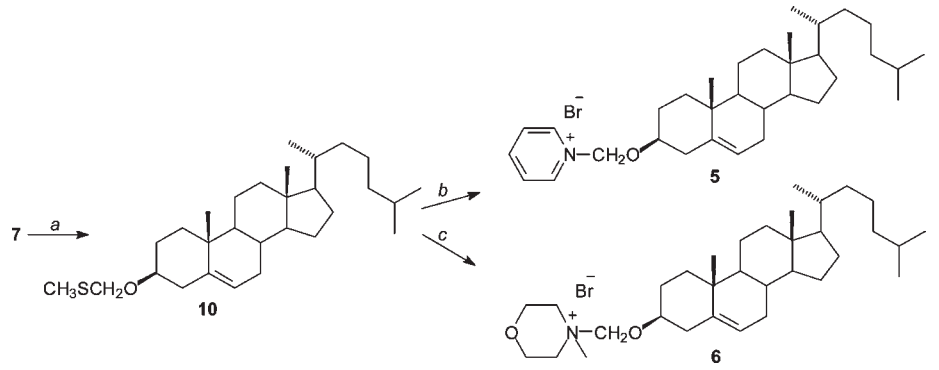
Cholesterol (**7**) was converted into the corresponding methylthiomethyl ether **10** by treatment with a DMSO/acetic anhydride/acetic acid mixture (6.5:3.4:1 mol) for 3 days to obtain methylthiomethyl ether **10** with 75% yield after chromatographic purification. Methylthiomethyl ethers are nonsymmetrical *O*,*S*-acetals and readily react with bromine

Scheme 1. Synthesis of the Carbamate Linked Pyridinium Cationic Lipid **4**[a]



[a] Reagents and conditions: (a) CDI, TEA, 50°C, 16 h, then 6-aminohexanol, DCM, 50°C, 18 h, yield 90%; (b) CBr_4, Ph_3P, 24°C, 1 h, yield 84%; (c) pyridine, 80°C, 3 h, yield 90%.

Scheme 2. Synthesis of the Cationic Lipids **5** and **6** with an Ether Linker[a]



[a] Reagents and conditions: (a) DMSO-Ac_2O-AcOH, benzene, 24°C, 3 days, yield 70%; (b) pyridine, NBS, DCE, 24°C, 15 min, yield 67%; (c) *N*-methylmorpholine, NBS, DCE, 30 min, yield 55%.

Table 1. Critical Micelle Concentration (CMC) Values for the Studied Cationic Lipids **1**−**6**

	1	**2**	**3**	**4**	**5**	**6**
CMC, μM[a]	55	32	51	32	45	48

[a] CMC values were determined by light scattering at 37 °C in HEPES−KOH buffer (pH 7.4) containing 0.1 M NaCl, 0.1 M KCl, and 10 mM $MgCl_2$. Experimental error did not exceed 10% of CMC value.

to yield highly reactive α-bromoethers. The latter can interact with various nucleophilic reagents, namely nitrogen bases. To avoid bromination of the cholesterol double bond, we used *N*-bromosuccinimide (NBS) instead of bromine. Thus, the amphiphiles **5** and **6** were synthesized with 55−67% yields by reaction of **10** with NBS followed by treatment with the corresponding heterocyclic base in anhydrous dichloroethane.

Critical Micelle Concentration (CMC) of the New Cationic Lipids. It is known that cationic lipids can form complexes with nucleic acids due to electrostatic and hydrophobic interactions between cationic lipids and nucleic acids.[40] Cationic lipids are amphiphiles and can spontaneously form micellar structures in aqueous solutions above a certain concentration that is the critical micelle concentration (CMC). It is well known that complexes between DNA and cationic lipids are formed at concentrations below CMC.[41] This is why CMC of the studied cationic lipids, but not CAC (critical aggregation concentration), was determined by static light scattering (LS) in HEPES buffer (pH 7.4, 37 °C) containing 0.1 M NaCl, 0.1 M KCl, and 10 mM $MgCl_2$ using a VA Instruments Co Ltd. LS-01 apparatus (St. Petersburg, Russia). The CMC values of the lipids are located at the intersection of the lines representing the concentration dependence of the normalized scattering intensity at the scattering angle $\theta = 90°$ that is given by the ratio I_{90}/I_0, where I_0 is the primary beam intensity and I_{90} is the corresponding scattering intensity at the scattering angle $\theta = 90°$. The first line corresponds to the intensity of LS from individual lipid molecules at low lipid concentrations and the other line from the micelles of cationic lipid formed at increasing lipid concentrations.[42−44] CMC values of the cationic lipids **1**−**6** are listed in Table 1. It is seen that the cationic lipids form micelles at similar concentrations in the range of 30−60 μM.

Cytotoxicity of the Cationic Lipids. Cytotoxicity of the cationic lipids was estimated by the MTT test[45] using various cell lines: HeLa (human cervical carcinoma), HEK 293 (human embryo kidney), and BHK (baby hamster kidney) cell lines. Cells were incubated in the presence of the cationic lipids at concentrations ranging from 1 to 90 μM for 24 h and 10% FBS. Under serum-free conditions, cells were incubated with the lipids or with lipid/DNA formulations at the same concentrations for 4 h, then DMEM/FBS mixture was added to give 10% FBS concentration in the medium and cells were incubated for additional 20 h. In these experiments, well-known commercially available cholesterol-based lipid cholesteryl 3β-*N*-(dimethylaminoethyl)carbamate hydrochloride (DC-Chol, Sigma) was used for comparison. IC_{50} values (concentration of the lipids providing 50% cell viability) are listed in Table 2.

Table 2. Cytotoxicity (IC_{50} Values) of the Cationic Lipids

	IC_{50}, μM[a]					
	HeLa		HEK 293		BHK	
lipids	−FBS	+FBS	−FBS	+FBS	−FBS	+FBS
1	18 (15)	14	35 (38)	36	23 (28)	32
2	15 (15)	14	27 (28)	31	20 (22)	33
3	13 (13)	13	28 (28)	26	20 (19)	26
4	15 (15)	18	35 (38)	30	17 (16)	24
5	18 (18)	13	31 (34)	27	17 (25)	15
6	17 (17)	10	30 (35)	30	22 (17)	24
DC-Chol	13 (10)	15	18 (14)	25	18 (18)	28

[a] IC_{50} value − lipid concentration corresponding to viability of 50% of the cells after incubation for 24 h in the absence (-FBS) or in the presence of 10% fetal bovine serum (+FBS). Figures in the parentheses correspond to cell viability observed in the presence of pEGFP DNA (2 μg/mL)/lipid complexes. Experimental error of the test did not exceed 10% of the IC_{50} value. For details see Experimental section.

These results show that variation of the linker types or heterocyclic groups do not significantly influence the cytotoxicity of the studied lipids. HeLa cells exhibited highest sensitivity to the cationic lipids; for these cells, IC_{50} values are within the range of 10−20 μM both in the presence and in the absence of serum. For other cell lines, the IC_{50} values are at least 2-fold higher. Note that both DNA and 10% serum had only a minor effect on the toxicity of the lipids. These results demonstrate that the studied cationic lipids **1**−**6** have a low toxicity and can be used for the delivery of nucleic acid both in the presence of serum and under serum-free conditions.

It is important that CMC values of the cationic lipids noticeably exceed their IC_{50} concentrations, as the cationic lipids in the form of micelles can be more toxic for the cell lines. Therefore, the size of the lipoplexes and the

transfection activity of the lipids in respect to different nucleic acids and cell lines were determined at lipid concentrations below their CMC values.

Ability of the Cationic Lipids to Stimulate Cellular Accumulation of Nucleic Acids. Cellular accumulation of nucleic acids stimulated by the cationic lipids was studied by fluorescent microscopy and flow cytometry. In these experiments, cells were incubated with fluorescein isothiocyanate (FITC) labeled oligonucleotide or plasmid DNA in complexes with the cationic lipids under study. It is known that cellular accumulation of nucleic acids in complexes with cationic vectors can significantly decrease in the presence of serum.[46] Therefore, the nucleic acid delivery by the cationic lipids under serum-free conditions was compared with that in the presence of 10% PBS in the growth medium.

FITC-Labeled Oligonucleotide Accumulation in the Cell Mediated by the Cationic Lipids. Cationic lipid mediated accumulation of a 25-mer 5′-FITC-labeled oligonucleotide (hereinafter, FITC-ON) in different cell lines (HeLa, HEK 293, and BHK) was monitored by flow cytometry. The results of these experiments shown as a percentage of the FITC-positive cells in a sample and an average fluorescence of cells in population are presented in Figure 2. The cells incubated with FITC-ON (5 μM) in the absence of the lipids were used as a control. In the latter case, the percentage of FITC-positive cells in the sample did not exceed the experimental error. Cellular accumulation of the FITC-ON mediated by Lipofectamine 2000 (Invitrogene) and DC-Chol were used as positive control and for comparison of the lipids under the study with well-known formulations. Under serum-free conditions (gray bars in Figure 2), the cationic lipids (except for lipid **6**) significantly enhanced the accumulation of FITC-ON in the cells of different origins. Accumulation efficiency (number of FITC-positive cells in population) was different for different cell lines; an efficient accumulation of FITC-ON by the lipids was observed in HEK 293 and BHK but not in HeLa cells.

The presence of serum in the medium considerably decreased the number of FITC-positive HEK 293 and BHK cells, and delivery of FITC-ON into HeLa cells was the most affected. The least efficient in the oligonucleotide accumulation was the cationic lipid **6**, composed of the *N*-methylmorpholine cationic head and ether linker; the other lipids exhibited a similar ability to deliver FITC-ON.

Comparison of the cationic lipids under the study with Lipofectamine 2000 and DC-Chol shows that some of the lipids (**1**, **2**, and **4**) stimulate accumulation of FITC-ON with the efficiency (number of FITC-positive cells in population) close to that of Lipofectamine 2000, while DC-Chol used in these experiments without a lipid helper displays moderate levels of FITC-ON cellular accumulation.

Obtained data on average fluorescence of the cell population (Figure 2, right panel) show that in the case of HeLa and HEK293 cells, only lipid **4** provide for FITC-ON accumulation levels comparable to Lipofectamine 2000. Interestingly, in the presence of serum for **4**, at least 2-fold reduction of the number of FITC-positive cells in population is observed, while average cell fluorescence remained unaffected by the presence of serum. It obviously means that in the presence of serum, lipid **4** stimulates more efficient cellular accumulation of FITC-ON but in the least number of cells.

Localization of FITC-ON in compartments of the HeLa cells transfected with the most efficient lipids is shown in Figure 3. The green signal of FITC-ON is seen as granules in the perinuclear cytoplasm, which is apparently related to accumulation of FITC-ON in the vesicular compartments inside the HeLa cells. Possibly, an increase in the incubation time leads to redistribution of the green signal inside the cells due to release of part of FITC-ON from endosomes and accumulation in the nucleus.[47]

Cationic Lipid Mediated Transgene Expression in the Cell. To evaluate the ability of the cationic lipids **1**−**6** to mediate plasmid DNA accumulation in the cells, pEGFP-C_2 plasmid, encoding EGFP, was used. The percentage of EGFP-positive cells and average fluorescence of cell population were estimated by flow cytometry 24 h after the incubation of HEK 293 cells with the corresponding cationic lipid/pEGFP-C_2 complexes. In these experiments, 40 μM concentration of lipids and 2 μg/mL concentration of pEGFP were used. At this lipid concentration, only a minor toxic effect (primary data not shown) and reliable transfection efficiency were achieved (Figure 4). Concentration of DC-Chol was set at 20 μM due to high cytotoxic effect of the lipid on HEK293 cells at concentration 40 μM.

These results show that the cationic lipid **6** was the least efficient agent for the plasmid DNA delivery among the tested cationic lipids. The highest number of the transgene positive cells and levels of transgene expression (average fluorescence of cell population) were observed for the lipids **3** and **4**. The presence of serum in the medium noticeably decreased the number of EGFP-positive cells. It is worth noting that levels of transgene expression determined by flow cytometry correlate well with those measures as ration of EGFP fluorescence of the lysate of HEK293 cell to total protein concentration in it (primary data not shown).

Determination of the Size of the Cationic Lipid/Nucleic Acid Complexes. An important characteristic influencing the transfection efficiency of cationic lipids is the size of their complexes with nucleic acids.[48]

We evaluated the sizes of particles formed in solution by the lipids alone and in the presence of 20-mer oligodeoxyribonucleotides using dynamic light scattering (LS). In these experiments, lipids at a concentration of 10 μM in HEPES buffer (pH 7.4) supplemented with salts were incubated at 37 °C for 30 min in the absence or presence of 20-mer oligonucleotide (5 μM) prior to the LS measurements. The concentration of the cationic lipids was 10 μM, as this concentration was used for the FITC-ON delivery into cells. As it was mentioned earlier, this concentration of the lipids is far below their CMC values and is not toxic for cells. However, it is known that aggregates of lipids are formed in solution even at concentrations below the CMC value.[49]

The size distribution of lipid particles formed in solution under these conditions (Figure 5, open bars) is multimodal. The particle size does not exceed 300 nm except for lipid **3**, which formed particles of 670 nm. No apparent correlation between the particle sizes and the structure of the cationic lipids was observed.

In the presence of oligonucleotide (5 μM), the particle sizes redistributed. In general, the oligonucleotide induced formation of bigger particles with sizes up to 700 nm (as for **4**) (Figure 5, filled bars). Apparently, the oligonucleotide added to the solution of cationic lipid play the role of a structure-forming unit. For evaluation of the effect of oligonucleotide sequence on the size of the formed complexes, we performed the experiments with three different oligonucleotides. We found that the oligonucleotide sequences (the GC content) did not influence the sizes of formed complexes (data not

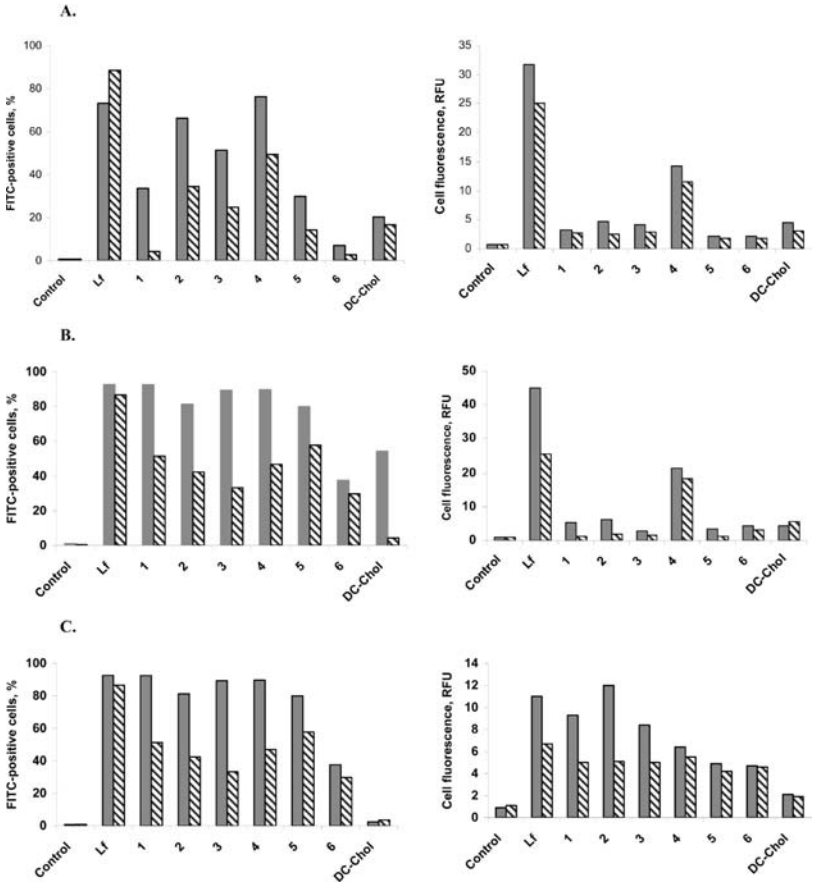


Figure 2. Accumulation of the complexes composed of the cationic lipids (10 μM) and FITC-labeled oligonucleotide (5 μM) in HeLa (A), HEK 293 (B), and BHK cells (C) in the absence (gray bars) and presence (hatched bars) of 10% FBS. The percentage of FITC-positive cells and average fluorescence of cells in population were determined by FACS analysis after 4 h incubation with the corresponding cationic lipid/FITC-ON complex.

shown). Note that the observed formation of relatively large lipoplexes can improve the transfection efficiency.[50]

The size and shape of the complexes between the cationic lipids and plasmid DNA were estimated by atomic force microscopy (AFM). Dynamic LS measurements (in this case) were less precise and informative than AFM analysis because LS is more sensitive to large particles and cannot precisely estimate the size and number of small particles in the presence of large ones.

Topographic AFM images showed the particles formed by the cationic lipids themselves (40 μM, as in the experiments with plasmid DNA delivery into cells) with a predominately spherical shape 20−100 nm in diameter. Figure 6B shows a typical AFM image of the lipid alone.

Complexes formed by different cationic lipids with plasmid DNA significantly differ in the size and shape. The complexes of lipid **4** with plasmid DNA are very small granules with a diameter of 20−30 nm (Figure 6C). The other lipids formed complexes with wider size distribution as compared with the former case. The lipids **1** and **3** formed with DNA the complexes of similar size and shape with a diameter of 30−120 nm (Figure 6D), the complexes of lipids **5** and **2** with DNA have similar images and gave particles with a diameter about 50−200 nm (Figure 6E), and the complexes of **6** had a diameter of 80−220 nm and a low compactness (Figure 6F). It is important that the DNA complexes with **1** and **3** are small granules inferior to 50 nm as compared with the lipids **2**, **5**, and **6**.

Discussion

Currently, only a few cationic agents, such as Lipofectamine, Lipofectin, Oligofectamine, and TransfecACE, are commonly used for in vitro applications; however, the use of these synthetic cationic agents in vivo is limited mostly by their high toxicity and/or significant decrease in the transfection activities in the presence of serum.[51] That is why intensive development and studies of the new nontoxic and efficient cationic lipids for gene delivery are in the progress.[52−54]

It is known that the ability of cationic lipid−nucleic acid complexes to destabilize endosomes and release nucleic acids into the cytosol is necessary for successful gene transfection. Thus, biodegradable cationic lipids with acid-labile linkers are promising agents for transfection due to their abilities to rapidly degrade into nontoxic molecules in the cell after entering endosomes and inducing nucleic acids release with subsequent elevation in the level of gene transfection.

In this study, we synthesized with high yields several new biodegradable cholesterol-based cationic lipids with various positively charged nitrogenous heterocyclic heads (pyridine, *N*-methylmorpholine, and *N*-methylimidazole) and acid-labile linkers (ester, ether, or carbamate). Our results show that the structure of these lipids only slightly if at all influences their ability to form micelles and cytotoxicity but noticeably changes the efficiency of cellular accumulation of nucleic acids.

The transfection activity of these lipids was assessed as an ability to stimulate accumulation in the mammalian cells FITC-labeled oligonucleotide and plasmid DNA. FITC-ON

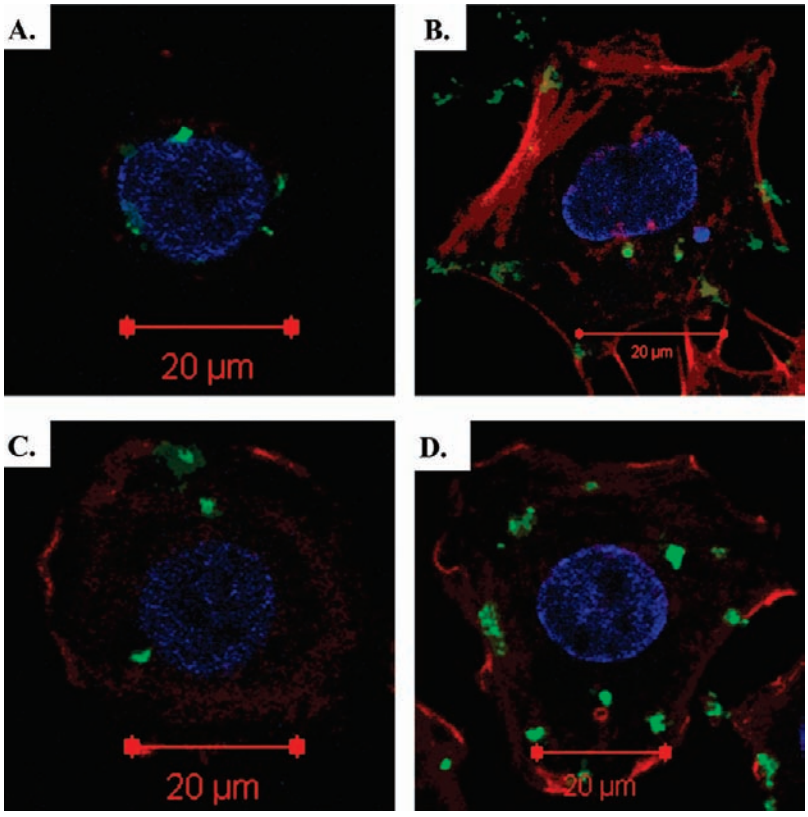


Figure 3. Localization of FITC-labeled oligonucleotide (5 μM)/cationic lipid (10 μM) complexes in HeLa cells after 4 h incubation under the serum-free conditions using confocal fluorescent microscopy. (A) lipid **1**, (B) lipid **2**, (C) lipid **3**, and (D) lipid **4**. Green signal corresponds to the FITC-labeled oligonucleotide, blue signal to the nuclei stained with DAPI, and red signal to the cytoplasm stained with phalloidin-FITC.

is efficiently accumulated by the lipids in different cell types (HeLa, HEK 293, and BHK). As this takes place, cationic lipid **6**, built of *N*-methylmorpholine cationic head and ether linker lipid, is the least efficient in oligonucleotide accumulation and inappropriate as a transfection agent. Note that the ability of other lipids to stimulate accumulation of FITC-ON under serum-free conditions is comparable with that of Lipofectamine 2000, used as a positive control.

Plasmid DNA delivery by the cationic lipids demonstrates that among the tested lipids, those having the structure that combines the positively charged pyridine and methyl imidazole head groups with either ester or carbamate linkers (**1**, **3**, and **4**) display a higher transfection activity as compared with the cationic lipids with either the *N*-methylmorpholine head groups and/or the ether linker (**2**, **5**, and **6**). The ability of lipid **4** with charged pyridine head and carbamate linker to deliver plasmid DNA is comparable with that of Lipofectamine 2000 (26−28% of EGFP-positive cells after incubation of the cells with the Lipofectamine/pEGFP-C_2 complex) under serum-free conditions.

We studied the correlation between the size of the cationic lipid/nucleic acid complexes and their transfection activity. It has been found that the ability of these cationic lipids to accumulate FITC-ON only slightly differs between the lipids (except for **6**) and depends to a considerably degree on the cell type and presence of serum in cell culture medium. On the contrary, the ability of the cationic lipids to deliver plasmid DNA depends on the size of the lipids/DNA complexes formed in solution, which is consistent with the fact that the maximum endocytosis by nonspecialized cells requires that the particle size is below 100 nm.[55]

The lipid **4** formed the smallest complexes with plasmid DNA with a narrow size distribution; this lipid is the most efficient transfection agent. The lipids **1** and **3** formed the complexes with plasmid DNA with a wide size distribution and a large fraction of small particles inferior to 50 nm; these lipids display moderate transfection activity. The lipids **2**, **5**, and **6** with plasmid DNA mainly formed the particles with a diameter of about 200 nm and a low content of small particles inferior to 50−100 nm; correspondingly, these lipids are poor transfection agents. The AFM data comply with the measurements of size distribution of the lipid/oligonucleotide complexes estimated using dynamic LS. The size distributions of the cationic lipid/oligonucleotide complexes are similar as well as the levels of FITC-ON accumulation in HEK 293 and BHK cells achieved in the presence of lipids with the only exception of the lipid **6**, displaying the lowest levels of FITC-ON accumulation.

Thus, the main characteristic of the studied cationic cholesterol-based lipids that influenced their transfection activity is the size of the formed complexes of the cationic lipids with nucleic acids. In addition, a low efficiency of the lipids containing an ether linker (**5** and **6**) is possibly related to the fact that acid-labile ether linker (*N*,*O*-acetal) is more stable as compared with ester and carbamate linkers in an acidic endosomal environment.

It is important to note that the tested cationic lipids offer the following advantages over Lipofectamine 2000: low toxicity (IC_{50} of Lipofectamine was 7 μM for HeLa and 22 μM for HEK 293) and the simplicity of the carrier system formulation, i.e., the formulation without both colipids and liposome formation. It is known that Lipofectamine 2000 is

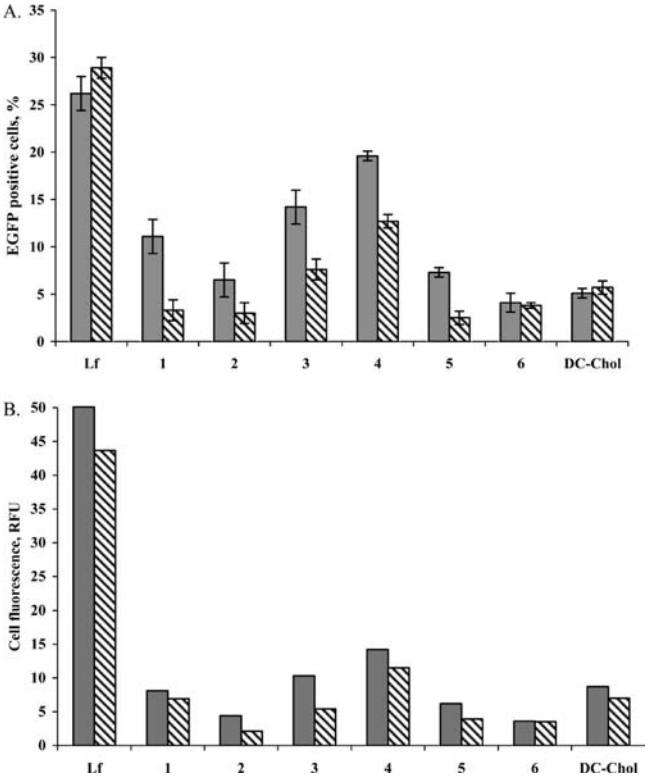


Figure 4. Expression of EGFP-transgene in HEK 293 cells. Cells were incubated with the complexes composed of the cationic lipids (40 μM) and pEGFP-C2 plasmid (2 μg/mL) in the absence (gray bars) and presence (hatched bars) of 10% FBS. The percentage of EGFP-positive cells (A) and average fluorescence of cell population (B) were estimated by FACS analysis after 24 h incubation of the cells with the corresponding cationic lipid/pEGFP-C_2 complex.

the liposome formed by cationic lipid and colipid DOPE, able to facilitate the membrane fusion and aid destabilization of the endosome, in contrast to the tested agents requiring no colipids.

Thus, a series of new biodegradable cholesterol-based cationic lipids for in vitro nucleic acid delivery was synthesized. The cationic lipids based on the cholesterol derivatives with positively charged pyridine and methyl imidazole head groups and either ester or carbamate linkers (**1**, **3**, and **4**) display a low toxicity and can stimulate cellular accumulation of both short oligonucleotides and plasmid DNA into various cell lines under serum-free conditions. These cationic lipids are promising transfection agents but need optimization of the formulation or/and transfection protocol.

Further studies could deal with the design of new cationic lipids with the proposed head groups (positively charged pyridine and methyl imidazole) and linkers (ester or carbamate) and varying hydrophobic parts of the molecules. These structural units can provide for decreasing the cytotoxicity of lipids with retention of their transfection activity.

Experimental Section

General Procedures and Materials. Cholesterol, 6-aminohexanol (Fluka), carbonyldiimidazole (CDI) (Aldrich), methyl iodide, 5-bromopentanoic acid, and *N*-bromosuccinimide (Merck) were used in the synthesis of cationic lipids. Dichloromethane (DCM), dichloroethane (DCE), diethyl ether, triethylamine, and heterocyclic bases were purified and dried prior to use by distillation over calcium hydride. Ethyl methyl ketone was refluxed with potassium carbonate and distilled. All solvents for column chromatography were distilled before using. Thin layer chromatography was performed using precoated aluminum plates (Kieselgel 60 F_{254}, Merck), which were visualized with the phosphor molybdate−ceric sulfate reagent.[56] Flash column chromatography (FC) was performed on Kieselgel 60 (40−63 μm, Merck). Melting points were determined with a Boetius apparatus (Germany). ^{1}H NMR spectra were recorded on Bruker MLS-200 and DPX-300 AMX-400 spectrometers in $CDCl_3$ as a solvent unless otherwise stated. The signals of $SiMe_4$ (δ = 0.00 ppm) was used as the internal references. *J* values are given in Hz. The mass spectra were recorded on a Finnigan MAT 900XL-TRAP mass spectrometer (San Jose, CA) with ESI ionization.

Purity of lipid **4** confirmed by elemental analyses (C, H, N), and of lipids **1**−**3**, **5**, and **6** confirmed by analytical HPLC (Nucleosil C_{18} analytical column 250 mm × 4 mm, Macherey-Nagel, Germany, and methanol−H_2O 10:1 used as a mobile phase) was ≥95%.

General Procedure for Synthesis of the Ester-Linked Lipids 1−3. The cationic lipids *N*-[4-(cholest-5-en-3β-yloxycarbonyl)butyl]pyridinium bromide (**1**), *N*-methyl-*N*-[4-(cholest-5-en-3β-yloxycarbonyl)butyl]morpholinium iodide (**2**), and *N*-methyl-*N*′-[4-(cholest-5-en-3β-yloxycarbonyl)butyl]imidazolium iodide (**3**) were prepared as previously described with some modifications[57] (see Supporting Information).

Synthesis of *N*-[6-(cholest-5-en-3β-yloxycarbonylamino)hexyl]-pyridinium bromide (4). Bromide (**9**) (97.6 mg, 0.165 mmol) and pyridine (0.5 mL) in methyl ethyl ketone (1 mL) were kept at 80 °C for 3 h. After removing organic solvent, the residue was purified by FC (chloroform−methanol−H_2O, 4: 1: 0.1) to yield (**7**) as a slightly yellow solid (95.7 mg, 90%); mp = 86−88 °C. ^{1}H NMR (300 MHz): 0.60 (s, 3 H, 18-CH_3), 0.79 (d, 3 H, *J* = 6.5, 27-CH_3), 0.80 (d, 3 H, *J* = 6.5, 26-CH_3), 0.84 (d, 3 H, *J* = 6.5, 21-CH_3), 0.93 (s, 3 H, 19-CH_3), 0.95−1.57 (m, 29 H, protons of cholesterol, $(CH_2)_4$), 1.69−2.08 (m, 7 H, Chol), 2.15−2.31 (m, 2 H, 4-CH_2), 3.05 (br.t, 2 H, *J* = 6.4, CH_2NH), 4.32−4.45 (m, 1 H, 3-CH), 4.97 (t, 2 H, *J* = 7.3, CH_2N^+), 5.26−5.31 (m, 1 H, 6-CH), 8.02−8.19 (m, 2 H), 8.41−8.48 (m, 1 H) and 9.45−9.52 (m, 2 H, $C_6H_5N^+$). Anal. Calcd for $C_{39}H_{63}BrN_2O_2 \cdot {}^1/_2H_2O$: C, 68.80; H, 9.47; N 4.11. Found: C, 69.17; H, 9.80; N, 4.37%.

Synthesis of the Cationic Lipids 5 and 6 with Ether Linker. Step a: Synthesis of 3β-(methylthiomethoxy)-cholest-5-en (10). Dimethyl sulfoxide (30 mL) and a mixture of AcOH (2.9 mL) and Ac_2O (20.3 mL) were added to cholesterol solution (3.18 g, 9.612 mmol) in anhydrous toluene (10 mL). The reaction mixture was kept for 3 days at 25 °C, supplemented with saturated solution of sodium carbonate (200 mL), and stirred for 2 h. The product was extracted with petrol ether (3 × 60 mL), the combined organic extract was washed with saturated sodium carbonate solution (40 mL) and water (30 mL) and dried over Na_2SO_4, and the organic solvents were removed in vacuum. The residue was purified by silica gel FC (petrol ether−ethyl acetate, 100:1) to yield (**10**) as a white amorphous solid (3.24 g, 75%). ^{1}H NMR (200 MHz): 0.65 (s, 3H, 18-CH_3), 0.86 (d, 6H, *J* = 6.8, 26,27-CH_3), 0.89 (d, 3H, *J* = 6.7, 21-CH_3), 1.01 (s, 3H, 19-CH_3), 1.02−1.60 (m, 21H, Chol), 1.75−2.05 (m, 5H, Chol), 2.16 (s, 3H, SCH_3), 2.28 (m, 2H, 4-CH_2), 3.50 (m, 1H, 3-CH), 4.64 (s, 2H, OCH_2S), 5.25−5.32 (m, 1H, 6-CH); literature data.[18] ^{1}H NMR (τ) 9.34 (18-CH_3), 9.00 (19-CH_3), 7.91 (SCH_3), 6.50 (3-CH), 5.42 (OCH_2S), 4.67 (6-CH). MS (MALDI-TOF): *m*/*z* $[M]^+$ 446.5, $[M\text{-}SCH_3]^+$ 400.1.

Step b: Synthesis of Cholest-5-en-3β-yloxymethylpyridinium Bromide (5). NBS solution (63.2 mg, 0.355 mmol) in anhydrous DCE (2.4 mL) was added to solution of MTM ether (**10**) (151 mg, 0.338 mmol), anhydrous pyridine (63 μL, 0.777 mmol) in anhydrous DCE (1.5 mL), and the mixture was stirred for 15 min. The residue obtained after removal of the solvent was purified by FC on silica gel (chloroform−methanol, 10:1 → 8:1) to yield (**5**) as a white amorphous solid (126 mg, 67%). ^{1}H NMR (200 MHz): 0.60 (s, 3H, 18-CH_3), 0.78 (d, 6H, *J* 6.6, 26,27-CH_3),

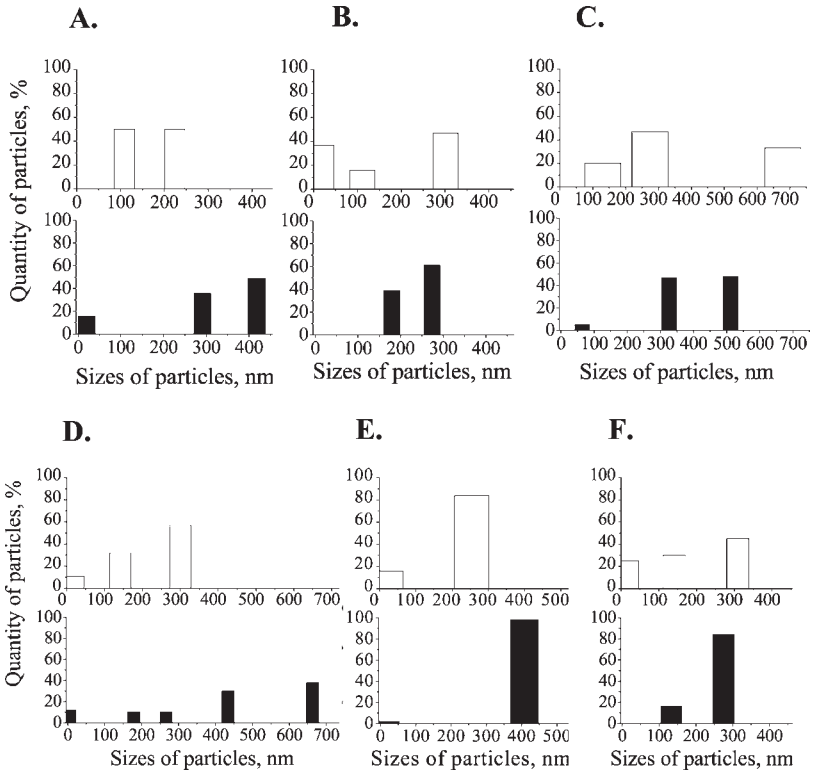


Figure 5. Light scattering measurements of the size of particles formed by the cationic lipids (10 μM) (open bars) and complexes of the cationic lipids (10 μM) with 20-mer oligonucleotide (5 μM) (filled bars). Experimental conditions: 37 °C in HEPES−KOH buffer (pH 7.4) containing 0.1 M NaCl, 0.1 M KCl, 10 mM $MgCl_2$. (A−E) Lipids **1**−**6**.

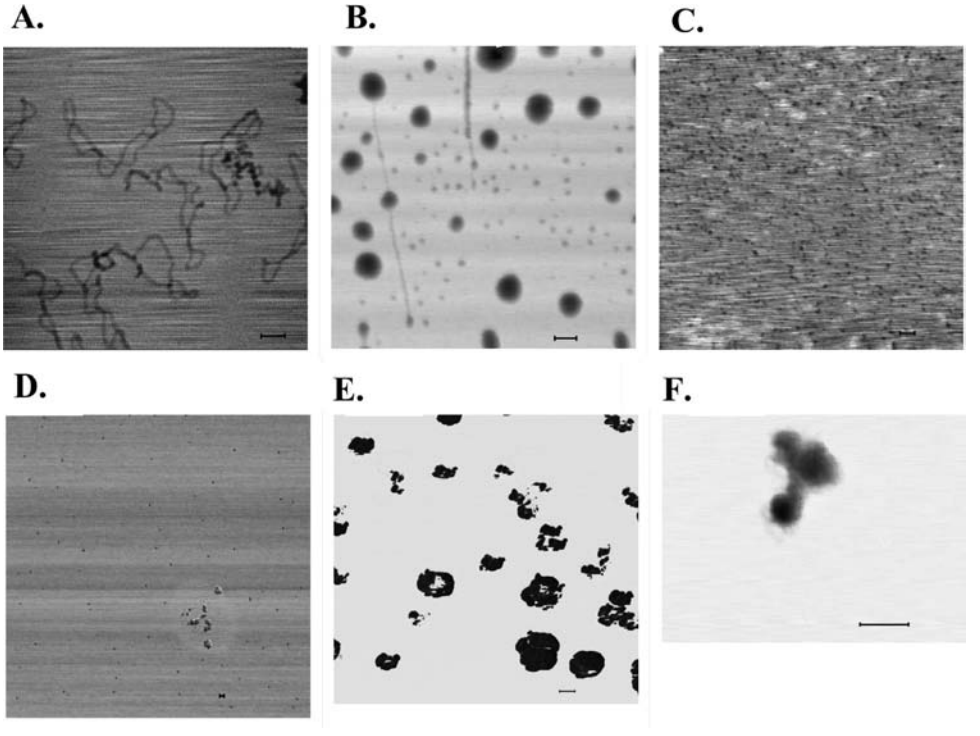


Figure 6. Atomic force microscopy images of (A) free plasmid DNA, (B) free cationic lipid **4** (40 μM), complexes formed by plasmid DNA (5.5 μg/mL) and cationic lipids (40 μM), (C) lipid **4**, (D) lipid **1**, (E) lipid **5**, and (F) lipid **6**. AFM analysis was performed in 20 mM Tris−HCl buffer (pH 7.0) containing 5 mM $MgCl_2$. Bar size is 100 nm.

0.84 (d,3H, *J* 6.6, 21-CH_3), 0.93 (s, 3H, 19-CH_3), 1.00−1.60 (m, 21H, Chol), 1.68−2.01 (m, 5H, Chol), 2.20−2.34 (m, 2H, 4-CH_2), 3.35−3.54 (m, 1H, 3-CH), 5.25−5.33 (m, 1H, 6-CH), 6.04 (s, 2H, OCH_2N^+), 8.06−8.18 (m, 2H), 8.48−8.62 (m, 1H), and 9.06−9.17 (m, 2H, $C_5H_5N^+$). MS (MALDI-TOF): *m*/*z* $[M - Br]^+$ 478.8.

Step c: Synthesis of *N*-(cholest-5-en-3*β*-yloxymethyl)-*N*-methylmorpholinium Bromide (6). Cationic lipid **6** was prepared by the

procedure described for (**5**) using (**10**) (202 mg, 0.452 mmol), *N*-methylmorpholine (269 μL, 2.26 mmol), and NBS (95.6 mg, 0.5424 mmol). The product was isolated by FC (chloroform−methanol, 10:1) to yield (**9**) as a slightly beige solid (144 mg, 55%). ^{1}H NMR (400 MHz): 0.60 (s, 3 H, 18-CH_3), 0.78 (d, 6 H, J = 6.5, 26,27-CH_3), 0.83 (d, 3 H, J = 6.5, 21-CH_3), 0.93 (s, 3 H, 19-CH_3), 0.96−1.57 (m, 21 H, Chol), 1.70−1.97 (m, 5 H, Chol), 2.21−2.37 (m, 2 H, 4-CH_2), 3.22 (s, 3 H, N^+CH_3), 3.34−3.42 (m, 2H, CH_2O), 3.48−3.56 (m, 2H, CH_2O), 3.54−3.62 (m, 1 H, 3-CH), 3.89−3.96 (m, 4 H, $CH_2N^+CH_2$), 4.90 (d, 1 H, J = 7.5, $OCHaN^+$), 4.97 (d, 1 H, J = 7.5, $OCHbN^+$), and 5.31−5.35 (m, 1 H, 6-CH). MS (MALDI-TOF): m/z $[M - Br]^+$ 500.4.

Alcoholic solutions of the lipids used in this study were prepared as follows: the weighed samples of the lipids were supplemented with ethyl alcohol, and the mixtures were intensively vortexed and sonicated in an ultrasonic bath (Cole-Parmer, USA) until clarification of solution. Stock solutions of the lipids in ethyl alcohol (1 mM) were stored at −20 °C.

Oligonucleotides and Plasmid. The 20-mer oligodeoxyribonucleotides with arbitrary sequences $-^{5'}$TGA GGG CAC AAG AAG CCC CT$^{3',5'}$−ACC CCC ACT GAA AAA GAT GA$^{3'}$−, and $-^{5'}$ATC TTC AAA CCT CCA TGA TG$^{3'}-$ and the oligodeoxyribonucleotide with a 3′-amino-hexyls linker $-^{5'}$ TAC AGT GGA ATT GTA TGC CTA TTA T$^{3'}-$ were synthesized by phosphoramidite method and purified by HPLC (Institute of Chemical Biology and Fundamental Medicine, SB RAS, Russia). Purity of the oligonucleotides as analyzed by electrophoresis in 20% PAAM/8 M Urea gel was 95−98%. FITC labeling of the 3′-aminohexyl linker containing oligonucleotide was performed as earlier described.[58] FITC-ON was isolated by conventional HPLC (Alliance, Waters, USA) using a Waters XTerra column and acetonitrile concentration gradient. The purity of FITC-ON samples as analyzed by electrophoresis in 20% PAAM/8 M Urea gel was 95−98%. Concentrations of oligonucleotides were measured in a BioMate 3 spectrophotometer (Thermo Electron Corporation, USA).

For the plasmid DNA transfection experiments, pEGFP-C2 plasmid (Clontech, Heidelberg, Germany) was used.

Preparation of the Cationic Lipid/Nucleic Acid Complexes. Prior to use, the cationic lipid/nucleic acid complexes were formed in a serum free Opti-MEM medium (Invitrogen, USA) by vigorous mixing of 25 μL of lipid solution and 25 μL of nucleic acid solution at an appropriate concentration (see below) followed by incubation for 20 min at a room temperature.

Cell Line and Culture Conditions. HeLa (human cervical carcinoma), HEK 293 (human embryo kidney), and BHK (baby hamster kidney) cell lines were grown in DMEM medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were plated in 96-well culture plates (0.03×10^5 cells per well for HeLa and BHK cell lines and 0.1×10^5 cells per well for HEK 293) or 24-well culture plates (0.2×10^5 cells per well for HeLa and BHK cell lines and 2×10^5 cells per well for HEK 293) and allowed to adhere overnight.

Cell Viability Test. The cells were plated in 96-well culture plates in 100 μL of DMEM with 10% FBS as described above. In the experiments with the presence of 10% FBS, the cells were incubated with the cationic lipids (1−40 μM) in DMEM with 10% FBS in the absence of antibiotics for 24 h. In experiments carried out under serum-free conditions, the medium with serum was removed prior to lipid addition and replaced with 50 μL of serum-free DMEM medium. Then cells were incubated with the cationic lipids (1−40 μM) in DMEM in the absence of antibiotics. After 4 h incubation, 50 μL of the DMEM with triple excess of FBS was added to cells in order to get 10% FBS concentration in the medium and the cells were incubated for additional 20 h.

The viable cells were counted at the end of the incubation period using a colorimetric assay based on reduction of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) by living cells.[45] Absorbance of samples was measured at 570 nm using Multiscan RC (Labsystems). The results were expressed as percentage of living cells. Results are expressed as mean. SD was below 5%.

Cellular Accumulation Experiments. FITC-ON Cellular Accumulation Experiments. 1×10^5 HeLa or BHK cells per well and 2×10^5 HEK 293 cells per well were seeded in 24-well plates. The cells were grown in 500 μL of DMEM supplemented with 10% FBS in an incubator at 37 °C and 5% CO_2 and allowed to adhere overnight. Before transfection, the medium was substituted for 200 μL of DMEM medium with 10% FBS or for 200 μL of serum-free DMEM medium. The complexes composed of cationic lipids (10 μM) and FITC-ON (5 μM) in 50 μL of serum free Opti-MEM were added to the cells and incubated for 4 h.

Plasmid DNA Transfection Experiments. 2×10^5 HEK 293 cells were seeded in 24-well plates and grown as described above. The complexes composed of the cationic lipids (40 μM) and plasmid DNA (2 μg/mL) in 50 μL of the serum-free Opti-MEM were added to the cells and incubated for 24 h.

For flow cytometry, the cells after incubation were washed with PBS and trypsinized. The obtained cell suspension was placed in 1.5 mL Eppendorf microcentrifuge tubes and spun for 10 min at 1000 rpm, the cell pellet was washed twice with PBS, and cells were fixed in 500 μL of 2% formaldehyde in PBS. The obtained samples were analyzed using fluorescence activated cell sorter (BD FACSAria, Becton Dickinson).

Laser Confocal Microscopy. The experiments were performed at the Center for Microscopic Analysis with the Institute of Cytology and Genetics, SB RAS. The cells were seeded in 24-well plates containing 10 mm square glass coverslips. The glass coverslips with adherent cells 4 h after transfection were gently washed with PBS, prefixed in PBS/0.4% formaldehyde solution, and fixed in PBS/4% formaldehyde. Then the coverslips with cells were sealed upside down on object plates in 2 μL of Vectashield Hardset mounting medium with DAPI (Vector Laboratories, UK). The samples were examined using a Zeiss confocal microscope LSM 510 META (Carl Zeiss, Germany) equipped with an HBO 50 W mercury lamp (Germany) and CCD camera (Photometrics, Tucson, AZ) interfaced to an Apple Power Macintosh 8100/80 with IPLab Spectrum version 3.1 (Signal Analytics, Vienna, VA). The filter set for the detection of fluorescein consisted of a 420−480 nm and 505−570 nm excitation filter, 510 nm dichroic mirror, and 520 nm long-pass detection filter (Zeiss). Images of 512 × 512 pixels were taken with a 16 × 0.5 NA Plan-NeoFluar oil immersion lens (Zeiss) and 2 × 2 binning on the CCD-chip.

FACS Analysis. Flow cytometry was performed using a fluorescence activated cell sorter (BD FACSAria, Becton Dickinson) equipped for enhanced green fluorescent protein (EGFP) and fluorescein isothiocyanate (FITC) detections at an excitation wavelength of 488 nm (a coherent sapphire laser, 20 mW). The fluorescence intensity of individual cells was measured in relative fluorescence units (RFU). The fluorescence from approximately 10000 individual cells was collected as the list-mode data, which consisted of the forward and side scattering and was analyzed by FACSDiVa, Becton-Dickinson. The percentage of GFP- and FITC-positive cells was calculated by determining their fluorescence emission at PMT equipped with a 502 LP dichroic mirror and a wide wavelength optical filter 530/30 nm.

Light Scattering Assay. The critical micelle concentration of cationic lipids in the HEPES buffer pH 7.4 containing 0.1 M NaCl, 0.1 M KCl, and 10 mM $MgCl_2$ at 37 °C was determined by static light scattering (LS) using an VA Instruments Co Ltd. LS-01 apparatus (St. Petersburg, Russia) calibrated with a dust-free benzene ($R_{90} = 11.84 \times 10^{-6}$ cm^{-1}). The LS intensity (I_{90}) was measured using vertically polarized light (633 nm) at a scattering angle $\theta = 90°$. The CMC was determined by linear least-squares fitting of the ratio (I_{90}/I_0), which is the intensity of

light scattering normalized to the intensity of the incident light. A sharp increase in the value of the ratio I_{90}/I_0 was observed upon micelle formation.

The particles sizes of the cationic lipids and their complexes with oligonucleotides correspond to the doubled values of the hydrodynamic radius R_h,[59] which was determined using dynamic LS. The time correlation function of the scattering intensity was measured at the scattering angle $\theta = 90°$ with vertically polarized light (633 nm). The values of the hydrodynamic radius R_h shown in this work are averaged over 10 replicates for each measurement. The error in R_h determination was estimate as ±10%. To determine the hydrodynamic radius from the time correlation function, a special program was used (DYNALS release 1.5, all rights reserved by A. Golding and N. Sidorenko, VA Instruments Co. Ltd., St. Petersburg, Russia). The measurements were performed after incubation of the cationic lipids solutions (10 μM) in the abovementioned buffer for 30 min at 37 °C. To determine the sizes of the complexes, the cationic lipids solutions (10 μM) with 20-mer oligonucleotides (5 μM) in the same buffer were vortexed and incubated for 30 min at 37 °C before the light scattering measurements.

Atomic Force Microscopy Experiments. The cationic lipid/nucleic acid complexes were formed in 20 mM Tris−HCl buffer pH 7.0 containing 5 mM $MgCl_2$, plasmid DNA at concentration of 5.5 μg/mL, and one of the cationic lipids at a concentration of 40 μM at a room temperature for 20 min.

AFM samples were prepared by placing 15 μL of the solution of cationic lipids or cationic lipids/pDNA complexes on the surface of freshly cleaved mica. After 1 min (the time of sample/complex sorption on the surface), the excess liquid was removed by washing with Milli-Q water and the mica was dried at a room temperature under a flow of argon. Each AFM sample was prepared three times to ensure reproducibility of results.

An atomic force microscope (Solver P-47H, NT-MDT) was adapted to perform the experiments with DNA/lipid complexes on the surface under ambient conditions. To protect the micromechanical and electronic parts of the microscope from mechanical and electromagnetic influences, the additional strokes were prevented for the relined visualization surface relief with a subangstrom resolution. In particular, AFM was fixed at shut metallic grounded tank, which was placed on rubber stoppers and heavily laden. The chamber allows a controlled changing and fixing of air humidity and temperature during experimental diagnostic operations.

The AFM-images were obtained in a semicontact mode by silicon cantilever with 150 kHz resonance frequency using standard (10 nm) and ultrasharp (~1 nm) tips.

A typical height of the ultrasmall lipid or DNA/lipid particles on the surface is few angstroms (< 10 nm). This fact demands a significant decrease in the initial surface roughness, which should be far less than the size of the measured particles. The average roughness of the obtained surface was measured by AFM as less than 1 Å.

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Supporting Information Available: Synthesis for the cationic lipids **1**−**3** and cholesterol derivatives **8** and **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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