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Non-Viral Gene Delivery Systems Based on Cholesterol Cationic Lipids: Structure-Activity Relationships

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1. Introduction

Gene therapy as an alternative to conventional medicine implies the elimination of the cause of a disease via the introduction of therapeutic nucleic acids (antisense oligonucleotide, or siRNA, or transgene expressing plasmid DNA, or aptamers, etc) into the cells of the organism (Blau & Springer, 1995; Lv et al., 2006; Karmali & Chaudhuri, 2007; Rayburn & Zhang R., 2008; and references in it). However, efficient delivery of genes at the physiological level into cells of living organism (''transfection'') is always more simple in theory than in practice. Both the therapeutic nucleic acids and cell membrane are negatively charged and therefore the spontaneous entry of naked nucleic acid inside cells is unlikely to be an efficient process. In other words, the development of clinically viable gene-targeted therapeutics and the design of safe and efficient gene delivery reagents (''transfection vectors'') are inseparable problems. (Templeton, Ed. 2010).

Prior to reaching the nucleus, the therapeutic nucleic acids must overcome a number of biological barriers, in particular, the cellular, endosomal, and nuclear membranes. This process is achieved by the utilization of appropriate delivery systems, that protect the genetic material from the destructive action of enzymes and encourage their penetration into the intracellular space, transfer through the nuclear membrane, and further expression in the nucleus (Eliyahu et al., 2005). In addition, these delivery systems should be nontoxic, non-immunogenic and biocompatible.

The administration of genes for therapeutic purposes can be achieved using one of three different approaches. The first approach consists of a direct injection of naked non-protected DNA into the cell resulting in a high level of transgene expression. The simplicity of this approach ensures it can be reasonably applied in a number of experimental protocols (Huang et al., Eds.; 2005). However, therapeutic application of unprotected naked nucleic acids is limited by the easily accessible organs (skin or muscles) for direct injection and is not applicable for systemic delivery due to a number of factors, the most important of which being extracellular nucleases. Gene-modified viruses and virus like particles represent the second approach for the cellular delivery of therapeutic nucleic acids. Viruses are efficient in transducing cells. However, the safety concerns regarding the use of viruses in humans make non-viral delivery systems an attractive alternative.

The third approached is focused upon the use of non-viral vectors. Non-viral vectors are particularly suitable with respect to their simplicity of use, large-scale production and lack of specific immune response. Non-viral vectors can be grouped into three main categories: cationic lipids, cationic polymers, and peptides. In comparison with their viral counterparts, these vectors are currently considerably less efficient, but their well-defined physical and chemical composition coupled with their reduced immunogenicity and toxicity make them promising candidates for gene delivery (El-Aneed, 2004; Dass, 2002; Verma & Weitzman, 2005).

Another important method of gene delivery is lipofection, a method based on the use of cationic lipids/cationic liposomes for gene transfer (Templeton, Ed. 2010; Huang, et al., Eds.; 2005; Karmali & Chaudhuri, 2007; Tseng et al., 2009). Cationic lipids have many potential advantages and have thus been viewed favorably in comparison with other non-viral vectors, including the significant simplicity and ease of production, good repeatability and biodegradability, potential commercial value, and their wide range of clinical application and safety.

The cationic liposomes are formed using cationic lipids, comprising a wide range of chemical compounds with a common structural feature, namely, the presence of both positively charged hydrophilic and hydrophobic domains. Of the most significant interest are biodegradable cationic lipids of natural origin (Lv et al., 2006). Long-chain hydrocarbons, steroids, and diglycerides are used as the hydrophobic domains (Zhi et al., 2010). The cationic hydrophilic domain can be represented by one (monocationic lipids) or more (polycationic lipids) positively charged groups. Monocationic lipids are most often secondary, tertiary or quaternary derivatives of aliphatic or heterocyclic nitrogen bases. In polycationic lipids, natural or synthetic polyamines or amino acids are used as the hydrophilic domains. The stability and toxicity of cationic lipids in biological systems, are determined by the type of bond connecting hydrophobic and hydrophilic domains.

In addition to cationic lipids, the zwitterionic helper lipid has a major impact upon the structure and activity of lipoplexes. A helper lipid can improve the ability of cationic liposomes to transfect cells. *In vitro* studies show that liposomes composed of an equimolar mixture of dioleoylphosphatidylethanolamine (DOPE) and cationic lipids (DOTMA, DOTAP) can mediate higher levels of transfection than those containing only the cationic lipid (Hui et al., 1996; Mok et al, 1997; Kerner et al., 2001). This fact has been attributed to the ability of DOPE tendency to undergo a transition from a bilayer to a hexagonal configuration under acidic pH, possibly facilitating fusion with, or destabilization of target membranes, in particular endosomal membranes (Zuidam & Barenholz, 1998; Zuidam et al., 1999).

Cholesterol initially used as a helper lipid form more stable but less efficient complexes than those containing DOPE *in vitro*. However, cholesterol containing lipoplexes have shown a higher rate of biological activity when compared to lipoplexes with DOPE, when these complexes were utilized *in vivo* (Liu et al., 1997; Sternberg et al., 1998; Smith et al., 1998; Simberg et al., 2003). The significant transfection activity attained was attributed to an improved cell binding and uptake of the lipoplexes promoted by the presence of cholesterol (Crook et al., 1998) and/or better stability of the lipoplex in serum (Simberg et al., 2003).

In 1991, Gao et al. reported the synthesis and application of the cholesterol-based cationic lipid 3-β-[*N*-(*N*′,*N*′-dimethylaminoethyl)carbamoyl]-cholesterol (DC-Chol, **1a**), which was combined with DOPE to transfect mammalian cells (Gao & Huang, 1991). Since then, considerable endeavors have been made in the synthesis of steroidal cationic lipids, due to

their potential applications in gene therapy (Zhdanov et al., 1998; Gao & Hui, 2001; Choi et al., 2001; Geall et al., 2000; Percot et al., 2004; Ding et al., 2008; Medvedeva et al., 2009; Maslov et al., 2010). The cationic amphiphiles containing cholesterol as a hydrophobic residue possess a high transfection activity and a low toxicity, finding use in the studies of both the structure–activity relationships and membrane fusion mechanisms (Noguchi et al, 1998; Kearns et al., 2008). Found within the synthesized cholesterol derivatives are commercially available lipids, (DC-Chol) and *N*1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN). The liposomes prepared with DC-Chol or CDAN and DOPE lipids are widely used to deliver the plasmid DNA for tumor immunotherapy or artificial immunization and are under clinical trials for the therapy of mucoviscidosis.

The efficiency of liposome mediated gene delivery is ascertained not only by the structure of cationic and helper lipids, properties of the nucleic acid, but also by the size and ζ-potential of the lipoplex formed with the nucleic acid. Therefore, the efficiency of the lipid vehicles for nucleic acid delivery is dependent upon the structure of cationic amphiphile whose chemical design requires a tailored approach, taking into account the lipid composition of the membranes, the nature of cell receptors, and the chemical processes in the intracellular environment.

Thus, in this chapter we summarize recent results on the design and structure-activity relationships of cholesterol-based cationic lipids with multiple architectures. We show that some of the designed cationic lipids (liposomal formulations) mediate efficient cellular accumulation, endosomal escape and the biological activity of the delivered nucleic acids (siRNAs, transgene expressing DNA, etc).

2. Cholesterol-containing cationic lipids and polymers

2.1 DC-Chol

Cationic lipid DC-Chol (**1a**) was originally synthesized by Gao et al. (Gao & Huang, 1991) and is available commercially. The DC-Chol was formulated as a cationic liposome with the helper lipid DOPE which promotes the fusion of lipoplexes with the cell membrane resulting in an increase of the DNA transfection efficiency (Zuidam & Barenholz, 1998; Lin et al., 2003).

Cationic liposomes DC-Chol/DOPE have been used extensively both *in vitro* and *in vivo,* displaying high transfection efficiencies (TE) (Litzinger et al., 1996; Song et al., 1997; Porter et al., 1998). There are documented reports that cationic liposomes DC-Chol/DOPE work well in various cell lines e.g. A431 human epithermoid carcinoma cells, A549 human lung carcinoma cells, L929 mouse fibroblast cells, YPT minipig primary endothelial cells (Gao & Huang, 1991), COS-7 cells, CFNPE-9o and 16HBE14o epithelial cell lines (Caplen et al., 1995), SKnSH and the primary rat neuronal cells (Ajmani et al.,1999), glioma cells (Esposito et al., 2003).

An experimental study of DNA compaction with the liposomes DC-Chol/DOPE that covered the whole range of mixed lipid composition and several lipid/DNA charge ratios was published (Rodriguez-Pulido et al., 2008; Munoz-Ubeda et al., 2010). A series of experimental techniques (electrophoretic mobility, SAXS, and fluorescence anisotropy), together with a theoretical aggregation-disaggregation model, has attested to the fact that DC-Chol/ DOPE cationic liposomes, with an average hydrodynamic diameter of (120+/-10) nm, properly condense and compact DNA and the liposomes composition is a key factor pertaining to the properties and structure of the resulting lipoplex.

Commercially available, DC-Chol is widely used for the development of novel approaches for DNA delivery, i.e. as a component of cationic solid lipid nanoparticles (SLNs) (Choi et al., 2008). The SLNs for gene delivery composed of DC-Chol, DOPE, and Tween 80 with TC (tricaprin) as a core in various ratios were produced by the melt homogenization method. The SLNs were bound to the cellular membrane 10 min after transfection, and translocated to the cytosol 60 min later. After 24 h, the SLNs were detectable in the nucleus and cytosol. SLNs showed high transfection efficiency in comparison with commercially available Lipofectin. In fact, PCR analysis indicated that SLNs prolonged the mRNA expression of the plasmid in various organs for up to 5 days. The SLNs-mediated transfection of the p53 gene resulted in the efficient reestablishment of wild-type p53 function in lung cancer cells and restored the apoptotic pathway (Choi et al., 2008).

The use of the most thoroughly investigated cationic lipids DC-Chol, DOTAP, and dimethyldioctadecylammonium (DDA) as vaccine delivery systems to form an antigen depot at the site of injection (SOI) and to induce immunological responses *in vivo* was reported (Henriksen-Lacey et al., 2011). DC-Chol, DOTAP, and DDA liposomes incorporating immunomodulating trehalose dibehenate (TDB) were prepared. DC-Chol/TDB liposomes were stable under storage and were retained at a significantly better degree at the SOI, with nearly 40% of the original dose still detectable 14 days p.i, in comparison with DOTAP/TDB liposomes. With regards to the depot effect at the SOI, the formulations were able to cause antigen retention between the range of 59 and 79% of the antigen dose recovered one day p.i.

The key step in lipid-mediated DNA delivery may be the structural changes of lipid carriers resulting in DNA release (Tarahovsky et al., 2004; Koynova et al., 2006; Hoekstra et al., 2007). In recent times, it was shown that multicomponent lipoplexes are superior in transfection with respect to the binary ones usually employed for gene delivery (Caracciolo et al., 2005a; 2005b; 2006). For instance, the four-component lipid system incorporating cationic lipids DOTAP, DC-Chol and neutral helper lipids dioleoylphosphocholine (DOPC) and DOPE transfer DNA into mouse fibroblast (NIH 3T3) and tumoral myofibroblast-like (A17) cell lines more efficiently than the thoroughly studied DOTAP/DOPC and DC-Chol/DOPE cationic liposomes separately. To answer the question concerning how TE will change with an increasing number of lipid components, the multicomponent lipoplexes were studied incorporating three to six lipid species simultaneously and the TE was then evaluated with respect to mouse fibroblast (NIH 3T3), ovarian (CHO) and tumoral myofibroblast-like (A17) cell lines (Caracciolo et al., 2007). These multicomponent lipoplexes exhibited a much higher TE (about two orders of magnitude) than binary lipoplexes that are more commonly employed for gene delivery. Furthermore, a trend was discovered that the TE increases in correlation with the number of lipid components (with some exceptions as a result of lipid composition). This discovery may be related to the higher fusogenicity and compatibility of vesicles composed of several lipid components with respect to single lipids (Caracciolo et al., 2007). The existence of different regimes of stability was demonstrated for these multicomponent lipoplexes: the most efficient lipoplexes exhibited intermediate 'optimal stability'. To this end, lipoplexes DOTAP/DOPC-DNA were the least resistant mixture to disintegration; DC-Chol/DOPE/DOPC-DNA, the most resistant mixture to disintegration; and DOTAP/DC-Chol/DOPC/DOPE-DNA, the mixture exhibiting an intermediated behavior and characterized by a high TE. The extent of DNA release estimated by electrophoresis was in total concurrence with the structural stability of lipoplexes revealed by SAXS and TE (Caracciolo et al., 2007).

2.2 Monocationic lipids

The basic structure of the cholesterol-based cationic lipids used in gene therapy includes four functional domains: 1) Appositively charged headgroup capable to bind with the negatively charged phosphate group of nucleic acid; 2) a hydrophobic cholesterol anchor, which interacts with the cellular membrane; 3) a spacer group; 4) a linker group, which connects the positively charged head and the hydrophobic domain.

In order to estimate the contribution of each functional domain into the efficacy of DNA delivery and cytotoxicity, various types of cholesterol cationic lipids were synthesized. In the first investigation of the structure-activity relationship, it was revealed that in order to achieve an efficient transfection, the tertiary ammonium group must be connected to the cholesterol by a short spacer *via* the ester or urethane bond. (Farhood et al., 1992).

In further studies the 3-deoxycholesterol cationic derivatives **2a-c** were synthesized, which are shown to be more efficient than DC-Chol (**1a**) or lipid **1b** (Takeuchi et al. 1996). It was observed that introduction of the ethyl or propyl groups into the cationic head results in the decrease of the transfection efficiency (TE). The value of the surface charge (ζ-potential) for the liposomes based on lipids **1a,b, 2a-c, 3a** and their analogs was in positive correlation with TE of the HeLa, COS-7, and NIH 3T3 cells. Cationic liposomes **2a**/DOPE, having the highest ζ-potential, demonstrated the highest TE in all cell lines tested. Furthermore, the activity of the liposomes derived from the compounds **2c** and **3a** having the lowest ζ-potential, was less than 20% of the activity of lipid **2a** (Takeuchi et al., 1996). Moreover, it was found that to achieve an efficient transfection, the size of the complexes should be neither smaller (<400 nm) nor larger (>1.4 μm) (Kawaura et al., 1998).

Lipid **2d,** containing the 2-hydroxyethyl group was found to be more active than the compound **2a** with dimethylamine group, both in the presence and absence of serum (Okayama et al., 1997). Further modifications of the lipid structure by introduction of additional 2-hydroxyethyl groups (compounds **2e-i**) into the cationic head resulted in the collapse of transfection activity. Similarly, the derivative **2f** containing the primary amino group was almost not active. The TE of the lipid **2d** was 2-fold higher in comparison with the activity of lipids **2a** and **2h** (Hasegawa et al, 2002). Based on fluorescence resonance energy transfer (FRET) it was observed that DNA is released differently from the lipoplexes by means of anionic liposomes. Furthermore, both the release rate and the amount of unbound DNA have a positive correlation with the TE. The transfection efficiency of nanoparticles composed of the lipid **2d** was 11.5-fold higher than the TE of DC-Chol (**1a**) and was comparable with Lipofectamine 2000, DMRIE-C and Tfx-20 (Hattori et al., 2007). The size of the nonoplex was 290 nm, and the highest TE *in vitro* was observed for the nitrogen/phosphate (N/P) ratio of 3, when the nanoplex was positively charged. The *in vivo* delivery directly into the tumor demonstrated that the optimum TE corresponds to the N/P ratio of 1; meanwhile the size of nanocomplexes was around 145 nm, and ξ-potential was negative (-16.9 mV). After the intravenous, intramuscular and peroral administration, there was no transgene expression detected in any organ.

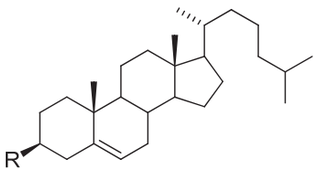
Lipids containing ester (**3b,c**), ether (**3d-f**) and urethane (**1b**) bonds were synthesized (Ghosh et al., 2000; Ghosh et al., 2002) in order to study the influence of linker type on the TE. It was subsequently demonstrated, that the presence of the urethane-based linkage in the compound **1b** led to the 6-fold decrease of the TE, in comparison to the ether lipid **3d**. However, in our opinion, this kind of comparison is fallacious due to presence of the different type of cationic head in the structure of the lipids **1b** and **3d**. Cholesterol lipids

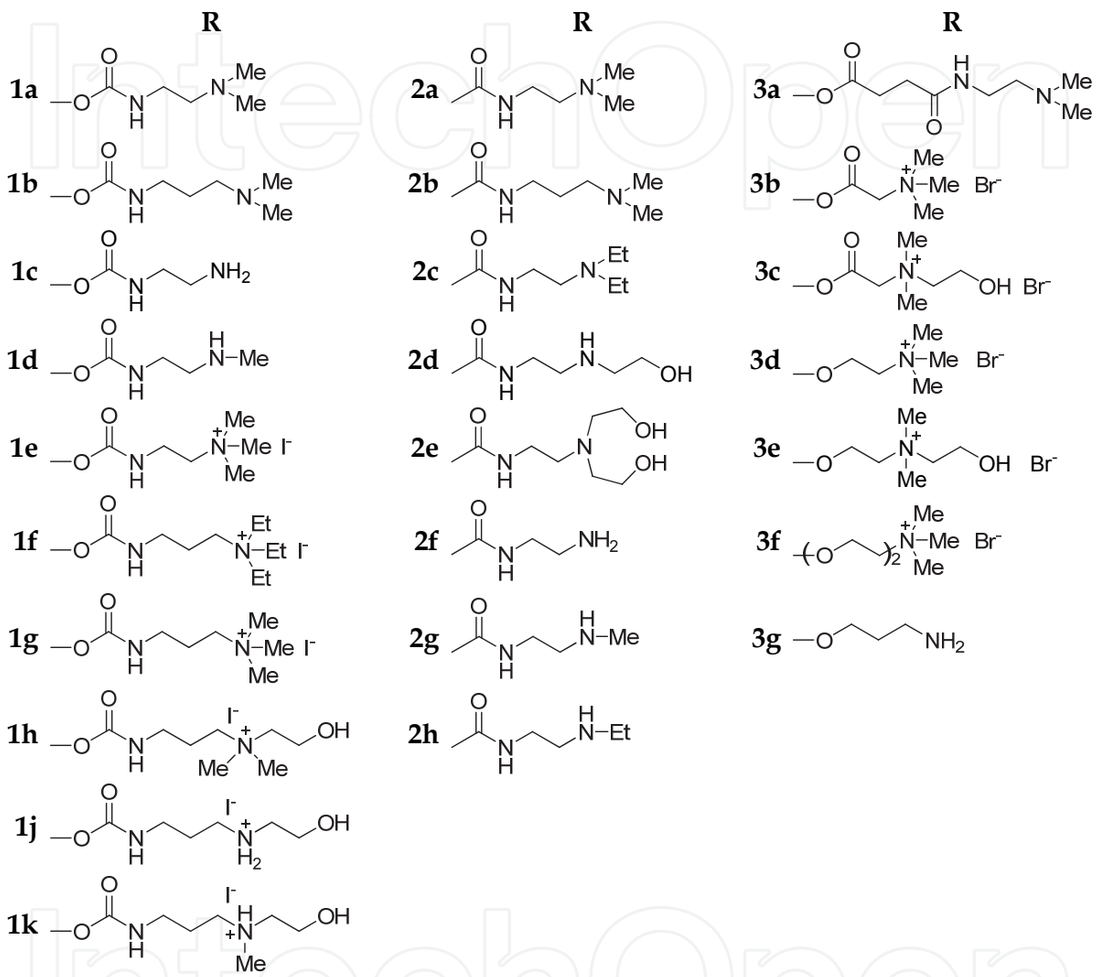
with the ether linkage **3d,e** were significantly more efficient than their analogues **3b,c** with ester bond. The increase of the spacer length by one oxyethylene unit (lipid **3f**) resulted in the decrease of the TE. The efficiency of the **3d**-based liposomes was comparable with the Lipofectamine. Lipid **3e** with 2-hydroxyethyl substituent efficiently transected cells without DOPE. A comparison of the liposome mediated siRNA delivery in the presence of serum was carried out for the lipids with ether (**3g**) and urethane (**1c**) linkage. (Han et al., 2008). The cellular accumulation of the fluorescein-labeled siRNA mediated by liposome **1c**/DOPE was inhibited by serum, while liposomes **3g/**DOPE had the ability to efficiently deliver siRNA into the cell in the presence of serum. Liposomes **3g/**DOPE were successfully applied for silencing survivin and green fluorescent protein (GFP).

A comparative study of the TE of DC-Chol (**1a**), and lipids **1c-e** was performed in order to estimate the effect of the cationic headgroup in the cholesterol lipids containing the urethane linker group (Kearns et al., 2008). The lipids **1c** and **1d,** containing both primary and secondary amino groups, were able to transport DNA into the melanoma B16-F10 cells. These data differ from the results received for the 3-deoxycholesterol derivatives **2a-f**, where the lipid **2f** with primary amino group display extremely low TE in comparison of the analogue **2a** with the tertiary amino group. It was observed that lipids **1c** and **1d** containing primary and secondary amino groups are less toxic, in comparison to the lipids with tertiary (**1a**) and quaternary (**1e**) amino groups. The highest TE achieved for liposomes with lipids **1c** and **1d** could be a result of the ability of these liposomes to penetrate into the cells, to interact with the endosomal membrane and to release the nucleic acid into the cytoplasm.

Cationic liposomes formed by lipid **1f** in combination with DOPE were able to deliver DNA into the cells (Reynier et al., 2002**;** Lesage, 2002**)**. The TE of these liposomes was 2-fold higher in comparison with DC-Chol (**1a)**/DOPE liposomes. What is more, the TE increased in the presence of 4% PEG 8000. Using the liposomes **1e**/DOPE and **1f**/DOPE (1:1) the relocation of DNA within the lipoplexes upon the transfection was monitored in addition to the localization of the plasmid DNA inside the cell nucleus was visualized using immunogold labeling (Briane, et al., 2002).

As mentioned above, the introduction of the 2-hydroxyethyl substituent into the cationic headgroup can increase the TE (Okayama et al., 1997; Hasegawa et al, 2002). The cationic lipid **1h** was synthesized and formed stable liposomes with DOPE with the monomodal size distribution (Percot et al, 2004). *In vivo* direct injection into the tumor of the liposomes **1h/**DOPE and **1g/**DOPE demonstrated that the TE of the 2-hydroxyethyl-containing lipid **1h** was slightly lower than the TE of the lipid **1g** containing trimethylammonium headgroup. In order to study the effect of 2-hydroxyethyl group on the TE more thoroughly, the activity the lipids **1b** and **1h-k** was tested and compared with the activity of DC-Chol (**1a**) and 3-deoxycholesterol derivative **2d** (Ding, et al., 2008). It was discovered that lipids without OH-groups (DC-Chol and **1b**) had the higher TE in comparison with the 2-hydroxyethyl-containing lipids **1h-k**, excluding lipid **2d**. Liposomes **2d**/DOPE possessed the highest TE *in vitro* (comparable with Lipofectamine2000) when N/P ratio was equal to 3, and were characterized by the big sizes of both liposomes itself (400 nm) and lipoplexes. The maximal level of the luciferase gene expression *in vivo* after intratracheal administration of the lipoplexes **1k/**DOPE-pDNA was 2-fold higher than the levels for the **1h/**DOPE, **1j/**DOPE and 4-fold higher than for DC-Chol (**1a**)/DOPE. The liposomes containing lipid **2d** were the least active.



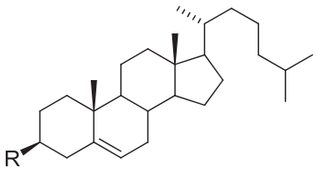


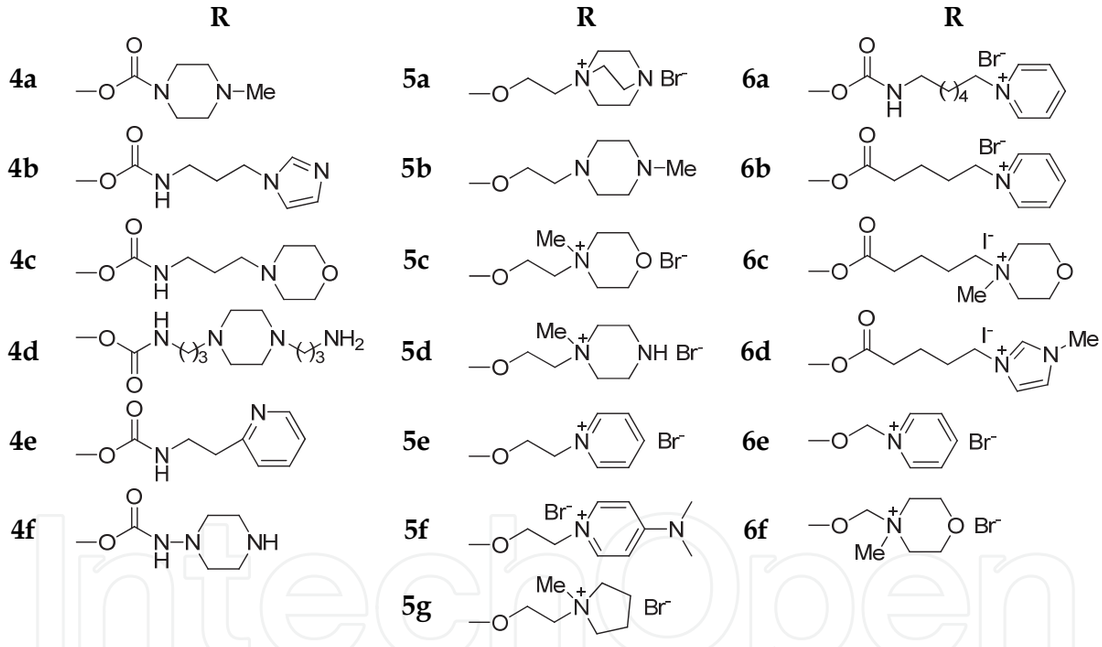
It was found that heterocyclic cationic lipids containing imidazolinium (Solodin et al., 1995) or pyridinium polar heads (Ilies et al., 2006) reveal a higher TE and a reduced level of cytotoxicity in comparison with the classical transfectants. To study the influence of this type of heterocyclic polar head on the TE, the cholesterol-based lipids containing heterocyclic amine connected to the cholesterol residue *via* urethane (**4a-f**, **6a**), ether (**5a-5h**) and ester linkers (**6b-f**) were synthesized (Gao & Hui, 2001; Bajaj et al., 2008c; Medvedeva et al., 2009).

The study of the transfection activity for the lipids **4a-f** revealed that liposomes **4c**/DOPE and **4f**/DOPE displayed the highest TE, which from 3 to 6-fold exceeded the TE of DC-Chol (**1a**)/DOPE and Lipofectamine 2000 (Gao & Hui, 2001). The serum (from 1 to 10%) have no effect on cells transfection mediated by these cationic liposomes at different N/P ratios. It was observed that these liposomes gained a negative charge in the presence of serum. *In*

vivo DNA delivery (direct administration into the spleen) was efficient at low N/P ratios, but as yet, no reasonable explanation for this occurrence has been found.

Liposomes formed from lipids **5a-g** and DOPE were able to transfect 50-80% of cells, and the TE increased when the N/P ratio increases (Bajaj et al., 2008c). The highest TE was observed for lipid **5f** containing *N,N*-dimethylaminopyridinium headgroup. This lipid transfected cells in the presence of serum without any loss of activity. The experiments with sodium dodecylsulphate (SDS)-induced DNA release from the lipoplexes demonstrated that DNA is released from the complex with liposomes 5f/DOPE in an unhurried manner. This could possibly be a result of a more effective lipid shielding of DNA. This is probably a reason for the high TE displayed by liposomes **5f**/DOPE, even in the presence of the serum.



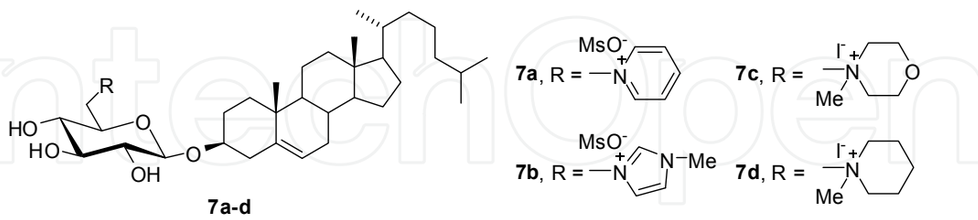


The structure-activity relationships study for the series of lipids **6a-f**, containing different heterocyclic cationic groups and linkers, permitted us to determine that lipids containing pyridinium (**6a,b**) or *N*-methylimidazium (**6d**) heads and ester or urethane linkers are the most promising in terms of transfection (Medvedeva et al., 2009). It was also revealed, that the ability of these lipids to deliver the oligodeoxyribonucleotides and pDNA into cells, correlates positively with their ability to form lipoplexes with the size not exceeding 100 nm.

Cholesterol-based lipids containing heterocyclic polar heads linked *via* biodegradable β-glucosyl spacer were prepared (Maslov et al., 2010).

The study of the biological activity of the cationic glycolipids **7a-d** demonstrated that nucleic acids could be efficiently delivered only by means of cationic liposomes; however this was

not possible using individual lipids. The delivery of fluorescein-labeled oligonucleotide was comparable for the liposomes **7d/**DOPE and Lipofectamine 2000. In the case of the siRNA delivery, the highest TE was observed for the liposomes **7c/**DOPE and the GFP gene-silencing was observed both in the absence and in the presence of serum in the culture media.



7a-d

2.3 Stimuli-responsive cationic lipids

It was revealed that lipoplexes enter the cells *via* nonspecific endocytosis, which occurs after the electrostatic binding of the positively charged lipoplexes to the negatively charged components of the cell membrane (Rejman, et al., 2006; Belting et al., 2005). There are a number of obstacles, hindering the efficient cationic liposome-mediated gene transfection: DNA release from the endosomes, DNA dissociation from the lipoplexes (Escriou et al., 1998; Rolland, 1998; Zabner et al., 1995) as well as insufficient release of DNA from endosomes. The use of stimuli-responsive delivery systems offers a new opportunity for the improvement of the delivery of nucleic acid (Ganta et al., 2008). Therefore, pH and redox microenvironment can be used as biological stimuli to improve the TE of lipoplexes. To achieve a stimuli-responsive release of DNA it is necessary to design cationic lipids containing trigger-groups, which specifically react to the alteration of the pH value, or to the presence of the intracellular reducing agents.

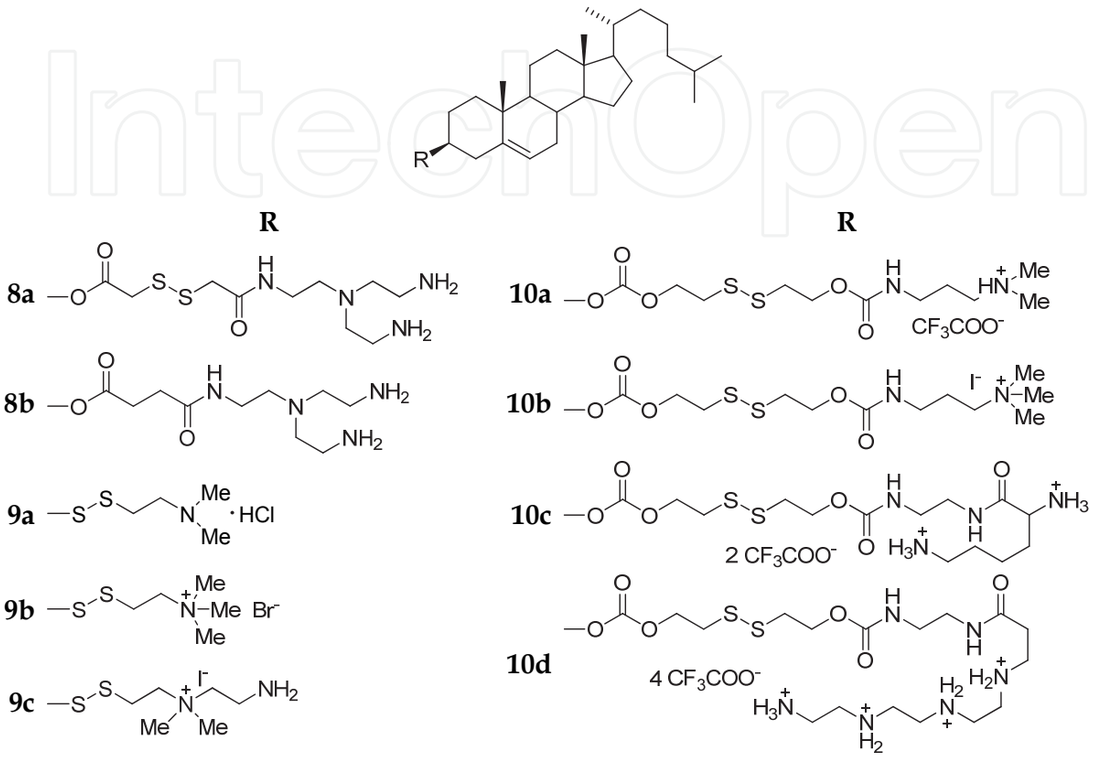
2.3.1 Redox-responsive disulfide cationic lipids

Thiol-disulfide exchange reactions play an important role in the biological functions of living cells; notably in the stabilization of the protein structure and redox cycles. The strong intracellular reductive micro-environment can stimulate the disintegration of the lipoplexes if these compounds contain the disulfide cationic lipids that are stable outside the cells, but could be reduced in the cells by intracellular reductive agents, *e.g.* glutathione (Tang & Hughes, 1998). The reduction of the disulfide bond will enhance the release of DNA from the DNA-liposomes complexes. Previously, it was demonstrated that the transfection of the plasmid DNA by the glycerolipids containing disulfide bonds was higher compared to the transfection activity of its non-disulfide analogue (Tang & Hughes, 1998).

Lipid **8a** containing cholesterol, was synthesized and its activity to mediate DNA transfer was compared with the activity of both non-disulfide analogue **8b** and DC-Chol (**1a**) (Tang & Hughes, 1999). In the presence of glutathione (10 mM) a 50% DNA release from the complex with liposomes **8a/**DOPE was observed, while the lipoplexes formed by lipid **8b** did not release DNA. The TE for the disulfide lipid **8a** was 100-fold higher, in comparison with the DC-Chol (**1a**) and 7-fold higher when compared to the lipid **8b,** in spite of the fact, that the amount of DNA internalized by cells was lower in the case of **8a**.

Lipids **9a-c** based on thiocholesterol (in the mixture with DOPE) were more active in comparison to PEI and DOTAP/DOPE when transfecting CV-1 cells (Huang et al., 2005).

Nanolipoparticles (NLP) were formed from lipids **9a-c** and PEG was added for the steric stabilization. The treatment of NLP with cysteine or glutathione changed the surface charge of the particles; the modification of the NLP surface with Tat-protein resulted in the increase of the TE of the neutral and negatively charged NLP.

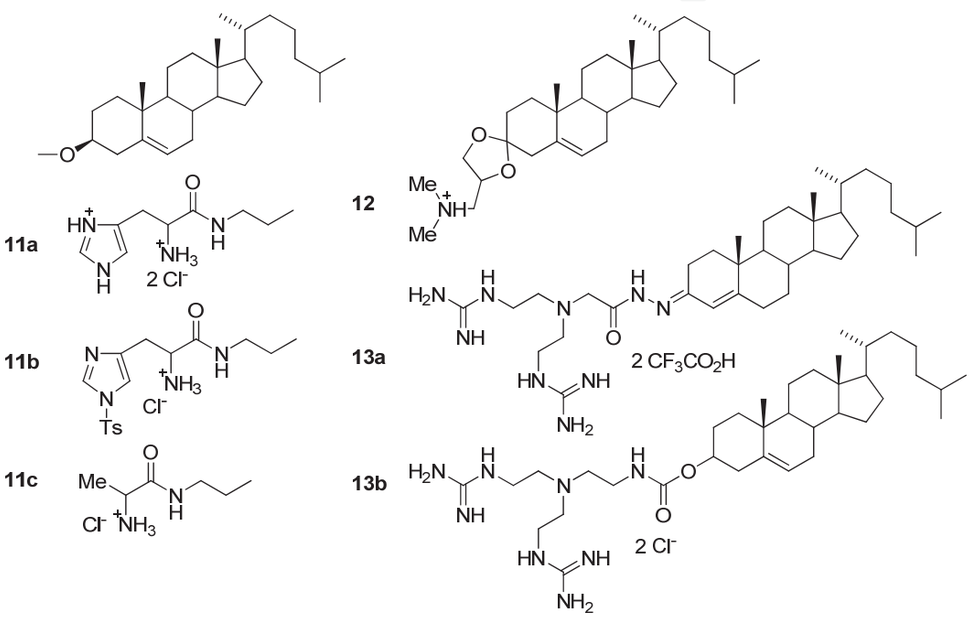


To find new efficient transfectants, the water-soluble low-toxic cholesterol-based lipids **10a-d** were synthesized. The lipids contain the positively-charged headgroups connected to the cholesterol backbone *via* the disulfide and carbonate linkers (Sheng et al., 2011). The atomic force microscopy indicated that mixing the cationic lipids and DNA gave compact, condensed lipoplexes with a size 200-300 nm. The addition of dithiotreitol (10 mM) resulted in the disassembly of these complexes into tiny, irregular-shaped fragments and small sized pieces, confirming the cleavage of disulfide bonds and distortion of the stable lipoplexes. Lipid **10c**, which contained the natural aminoacid lysine, displayed the highest TE in respect to COS-7 cells, both in the presence and absence of serum. The least active was lipid **10b** with the quaternary amino group. It is worthy of note, that lipid **10a** was almost as active as **10c** at low N/P ratio (up to 5). This ratio is characterized by the formation of the small lipoplexes (approximately 250 nm) with the negative ξ-potential.

2.3.2 pH-responsive cationic lipids

It is known that endocytosis is accompanied by a noticeable increase in the environment acidity from the physiological value of pH 7.4 to 6.5–6.0 in endosomes and to 5.0 in primary or secondary lysosomes (Mukherjee et al., 1997). Endocytosed lipoplexes may be digested by acidic hydrolases, active at the acidic pH of the lysosome. In order to protect DNA from

such hydrolytic degradation and enhance the release of DNA from endosome pH-sensitive cationic lipids were synthesized (Budker et al., 1996). These lipids contain weakly basic lysosomotropic imidazole head group which acts as a proton sponge, preventing the acidification of endosomal environment and inhibiting the degradative hydrolysis. Another approach to enhance the DNA release is the incorporation of the chemical trigger-bond into lipid that could be hydrolyzed at a specific pH gradient. Therefore, incorporation of acid-labile bonds into the lipid structure favors the lipoplex destabilization and facilitates the DNA release from the endosomal compartment to the cytoplasm, thus improving the transfection efficiency (Boomer et al., 2002; Guo & Szoka, 2003). Furthermore, the use of biodegradable cationic lipids lowers the cytotoxicity of cationic liposomes, making them promising transfection agents.



Cholesterol-based, endosomal, pH-sensitive, histidylated, cationic lipid (**11a**), its less pH-sensitive analogue with the electron-deficient head group (**11b**) and cationic lipid, which does not contain histidine headgroup (**11c**) were synthesized (Singh, et al., 2004). Lipid **11b** exhibited lower TE than lipid **11a** in relation to 293T7 cells. The activities of both lipids were inhibited in the presence of Bafilomycin A1, demonstrating the involvement of imidazole ring protonation in the endosomal escape of DNA. However, the TE of histidinylated lipid **11a** did not exceed this value for lipid **11c**. The lipid **12** with an acid-sensitive ketal bond was hydrolyzed in acidic medium where an ether analogue remained undegraded (Zhu, et al., 2002). Lipid (**12**) achieved levels of gene delivery similar to DC-Chol (**1a**), but the toxicity was correspondingly low.

In recent time a new series of cationic steroid derivatives, containing guanidinium headgroup connected with the hydrophobic cholesterol *via* the acid-sensitive acylhydrazone linker have been developed (Aissaoui et al., 2004). The lipid **13a** was found to possess a low cytotoxicity and was able to mediate the efficient gene transfection into various mammalian cell lines *in vitro*. The TE of the lipid **13a** was comparable with TE of its analogue **13b**, which did not contain the acid-labile group. Colloidally stable

13a/DOPE-DNA complexes were prepared and administered *via* nasal instillation into the mouse airways. A significant expression of the reporter protein in the lung homogenates was subsequently detected. It should be noted, the TE in the experimental groups was much higher compared to the control group of mice receiving the identical dose of "naked" DNA.

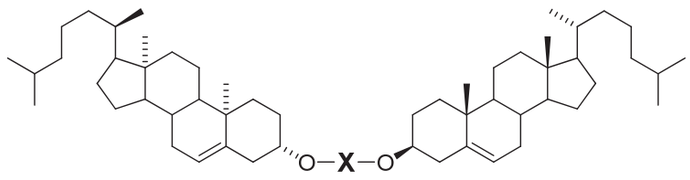
2.4 Cholesterol gemini-surfactants

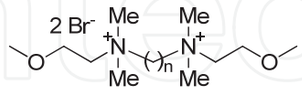
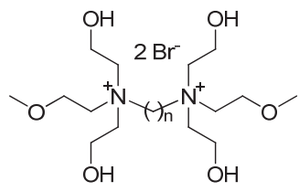
Recently, a new class of surfactants was discovered. These so-called 'dimeric' or 'gemini-surfactants' attract a considerable amount of scientific attention as being of particular importance in biology. For instance, the ability to form "bilayer bridges"was demonstrated for some gemini-surfactants containing long hydrophobic spacers (Moss & Li, 1992). The structure of these gemini-surfactants resembles the structure of lipids found in the membrane of the thermophiles archaebacteria. Other gemini-surfactants were specially synthesized for the usage as nucleic acids carriers into the cells (Kirby et al., 2003;) and the effects of the nature of the spacer, hydrocarbon chains, and headgroups on the transfection activity of these compounds were studied in detail.

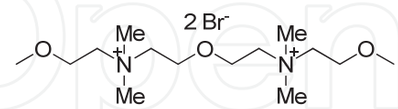
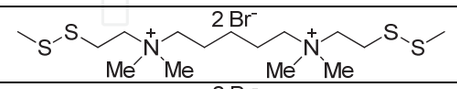
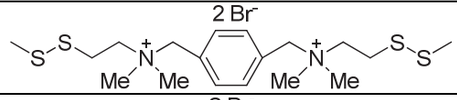
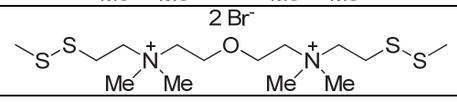
Dimeric lipids **14a-k**, differing in the length of the spacer and the type of the cationic headgroup, were synthesized, and the transfection activity of these compounds was compared with this parameter for the monomeric lipids **3d** and **3e** (Bajaj et al., 2007a; Biswas et al., 2011). Lipids formed different aggregates, depending on the structure of the cationic head. For instance, individual vesicles with sizes from 20 to 160 nm were formed by the compounds **14a-e**. In turn, for lipids **14f-k** formation of vesicular conglomerates of various lengths composed of the individual vesicles was observed. These conglomerates were formed due to the hydrogen bonding interactions imposed by the 2-hydroxyethylated headgroups of the lipids of each vesicle of geminies **14f-k** (Biswas et al., 2011). The maximal TE was observed for the lipid **14c**, containing pentamethylene spacer, and, on the contrary, lipid **14e** was not active in the absence of serum in the culture media. A different morphology of the lipoplexes formed by these lipids and DNA was subjected to further study. Transmission electron microscopy showed the presence of the aggregates with the size range from 100 to 200 nm for the lipoplexes formed by **14c**, while lipid **14e** was found to form lipoplexes of irregular shapes and sizes.

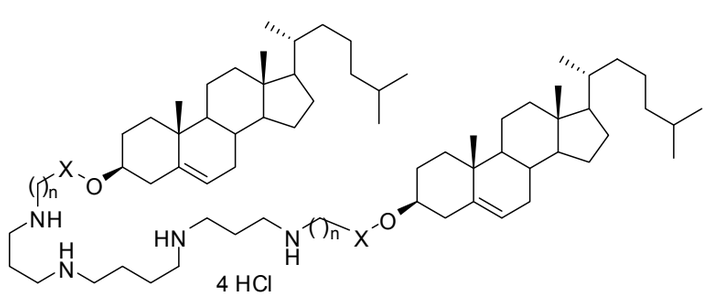
The replacement of the hydrophobic spacer by hydrophilic one led to the new set of dimeric amphiphils **15a-d** possessing low toxicity. The amphiphiles **15a,b** were able to transfect the cells at the low N/P ratio, as well as in the presence of 30 and 50% serum (Bajaj et al., 2007b).

Due to the fact that lipids with ether bond are poorly degraded in the organism, new disulfide bond containing dimeric lipids **16a-c** were synthesized, able to degrade under the influence of intracellular reducing agents (Bajaj et al., 2008a). These lipids contain flexible hydrophobic and hydrophilic spacers, in addition to rigid hydrophobic spacers. The comparative analysis indicated that the TE of the lipids is decreases in the range **16c > 16a > 16b**. Thus, the hydrophilic flexible spacer in the lipid structure is essential for an effective transfection. The serum inhibited the activity of the lipids **16a-c**, as its negatively charged components compete with DNA for the binding to the cationic lipids, which results in the dissociation of the complexes and a decrease of the TE. In recent times, a new method for the synthesis of the dimeric lipids **17a-c** that contain cholesterol and spermine moieties and potentially posses the high TE was described (Petukhov et al., 2010).



#	n	X
14a	3	
14b	4	
14c	5	
14d	6	
14e	12	
14f	3	
14g	4	
14h	5	
14i	6	
14k	12	

#	n	X
15a	1	
15b	2	
15c	3	
15d	5	
16a		
16b		
16c		



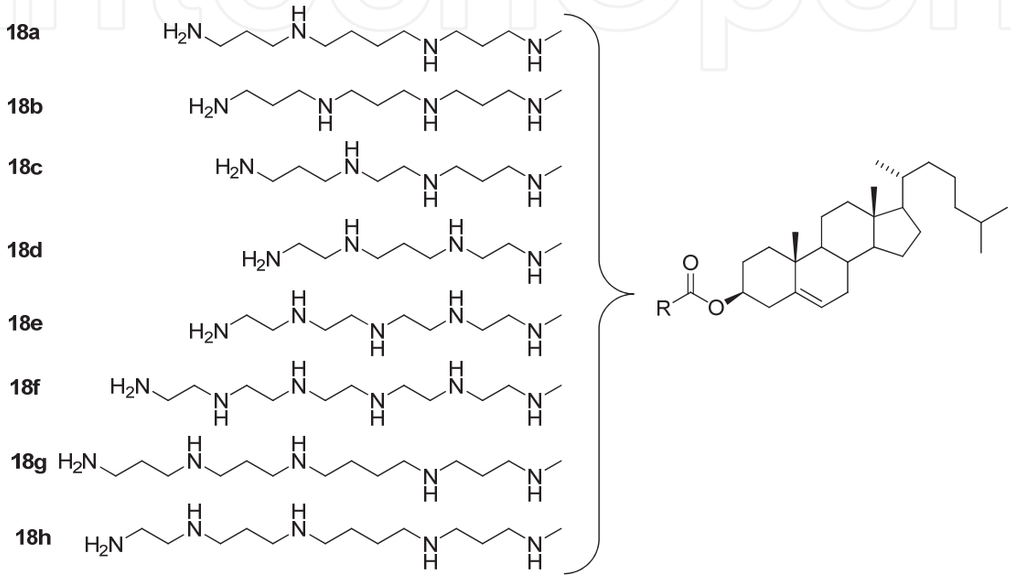
17a, X = C(O), n = 4
17b, X = C(O)NH, n = 4
17c, X = C(O)NH, n = 6

2.5 Polycationic lipids

Polycationic lipids contain a polar head that bears either several or multiple positive charges increasing their affinity to nucleic acids. Polyamines were successfully used as a component of polycationic lipids (Geall et al., 2000; Blagbrough et al., 2003; 2004; Oliver et al., 2004). Polyamines are a class of naturally occurring compounds that display excellent nucleic acid binding and condensing properties. It is well known that the overall positive charge of the lipoplexes is important for initiating cell entry and release of the complexed nucleic acid into the cell cytoplasm. Although the exact mechanism by which polycationic lipids mediate transfection requires more detailed investigation evidence in literature points towards the notion that the success of these reagents arises from a couple of factors: the abnormally low pKa's (pKa <7) of the polyamines, a direct result of the number of amino groups present and the methylene spacings between them (Stewart et al., 2001; Keller et al., 2003; Geall et al., 1999; 2000).

The effects of the regiochemical distribution of positive charges along the polyamine moiety in DNA condensing agents were studied (Geall et al., 2000). DNA condensation is dependent upon the number of positive charges, the regiochemical distribution of charges of

polyamines (determined by the p*K*a of each amino group), and the local salt concentration. A series of polyamine carbamates of cholesterol was prepared where both the charge and its regiochemical distribution have been varied along the polyamine moiety (**18a-f**) (Geall et al., 1999). Lipids **18a-f** were tested for transfection competence at three different N/P ratios (0.5:1, 1:1, 4:1), calculated taking into account the average charge per molecule at pH 7.4. It was found that the spermine based lipid **18a**, incorporating 3-4-3 methylene spacing along the polyamine moiety, has the highest TE, while lipid **18f** display the lowest TE. Geall and co-workers supposed that the four methylene spacing found in spermine, could have significant implications for DNA polyamine association and lipoplex formation.



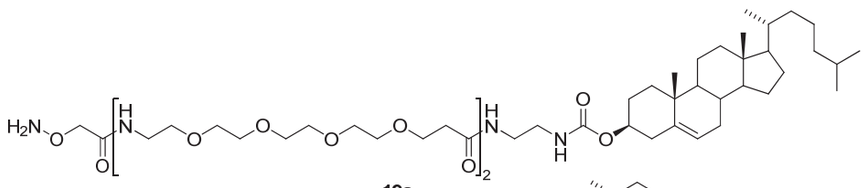
Recently second generation cationic liposomal systems that were formulated from polyamine analogues of DC-Chol and DOPE have been described (CDAN (**18d**), CDAD (**18a**), CTAP (**18g**), CTAH (**18h**)) (Cooper et al., 1998). Among the liposomal formulation tested the formulation from the novel pentaamine amphiphile CTAP (**18g**) and DOPE were shown to be approximately 400 times more efficient at mediating gene delivery to a mouse lung *in vivo* than DC-Chol/DOPE liposomes. CDAN (**18d**) is another cholesterol-based polyamine lipid with an unnaturally occurring 2–3–2 methylene spacing, which, in combination with DOPE forms an exceptionally effective transfection agent. Biophysical analyses show that CTAP/DOPE liposomes are effective *in vivo* because these liposomes are able to efficiently neutralise, condense and encapsulate nucleic acids into lipoplex particles and the unprotonated amine groups (p*K***a** < 8) presented in the polyamine at neutral pH that could have the capacity for endosome buffering, thereby facilitating nucleic acid escape from endosomes into the cytosol, like polyethylenimine (Stewart et al., 2001).

Recently a new solid-phase strategy to synthesize a library of cholesterol-based polyamine lipids in excellent yields (>87%) and purity was set forth (Oliver et al., 2004). The strategy employs 2-chlorotrityl chloride resin as a solid support and protecting group for one primary amine on the starting material, utilizing the high selectivity of 2-acetyldimedone as a protecting group for the second primary amine (Oliver et al., 2004).

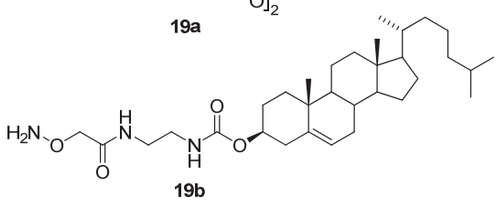
Cationic liposomes CDAN (**18d**)/DOPE were tested as delivery systems for siRNAs (Spagnou et al., 2004). The results show that CDAN and DOPE with and without siRNA

confer low toxicity to mammalian cells. CDAN/DOPE-siRNA complexes exhibited a slower cellular uptake than Lipofectamine2000 based formulations. Intracellularly CDAN/DOPE-siRNA complexes appear to behave in a different fashion, accumulating in distinct but diffuse small non-lysosomal compartments for at least 5 h after transfection (Spagnou et al., 2004).

Recently, a new hepatotropic nontoxic lipid-based vector system for delivering chemically unmodified siRNA to the liver to inhibit Hepatitis B virus (HBV) propagation was described (Carmona et al., 2008). These anti-HBV formulations were created based on synthetic, self-assembly ABCD nanoparticle paradigm (Kostarelos & Miller, 2005). ABCD nanoparticles comprising nucleic acids, such as plasmid DNA (pDNA) or siRNA (A component) which are condensed with cationic liposomes (B component) to form AB core nanoparticles. An important feature of the assembly is the incorporation of an aminoxy cholesteryl lipid into these AB core nanoparticles in order to enable the quantitative chemoselective post-coupling of biocompatibility polymers (C component) and optional tissue-targeting ligands (D component) to the core nanoparticles. (Carmona et al., 2008). AB nanoparticles (70-80 nm in diameter) were initially formulated in an aqueous solution by mixing of siRNAs with cationic liposomes CDAN (**18d**)/DOPE/**19a** (40:50:10 molar ratio) or CDAN/DOPE/**19b** (40:50:10 molar ratio).



19a

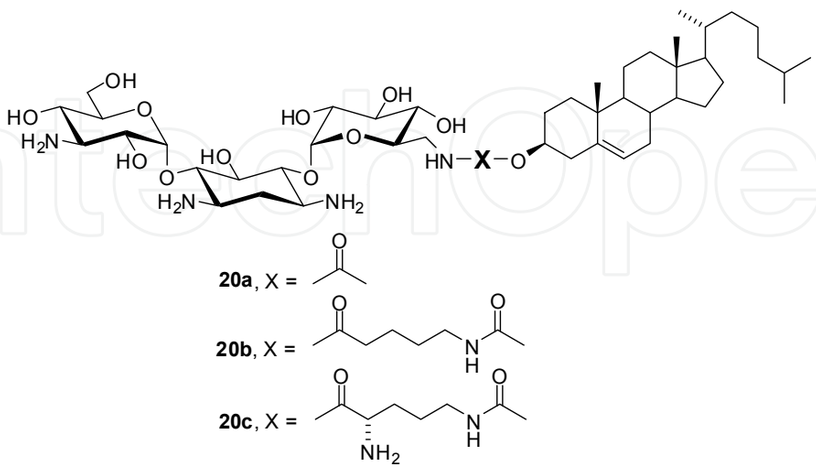


19b

Polyethylene glycol^{2000}-dialdehyde (C component), was coupled to AB particles under aqueous acidic conditions (pH 4). This surface post coupling was facilitated by a rapid, quantitative chemoselective aminoxy-aldehyde conjugation between the aminoxy functional group of aminoxy cholesteryl lipid and of the aldehyde functional groups of the C-component. The resulting oxime linkages are robust at pH 7; however at a level of pH 5.5 and below, they are prone to decomposition. The developed vectors administered intravenously, efficiently deliver unmodified siRNAs to murine livers leading to the strong suppression of HBV replication (Carmona et al., 2008).

The conjugates of guanidinium and cholesterol were synthesized with the yields of up to 61%, with the purpose of further improving the polar domain of cationic lipids (Vigneron et al., 1996). The guanidinium group can form with phosphate anions characteristic pairs stabilized by parallel zwitterionic hydrogen bonds. Moreover, the guanidinium group is also able to develop hydrogen bonding with nucleic bases, especially with guanine. The tertiary amine of compound **13b**, which is situated between two positive guanidinium groups and has probably a lower pKa, could also be able to buffer the acidic environment of late endosomes and of lysosomes, hence protecting the DNA against degradation. The lipid **13b** can be used *in vitro* without DOPE, and this permits the avoidance of the liposomes preparation step. Commonly

used for the transfection **13b**-DNA complexes were found to form ordered aggregates characterized by a fingerprint-like structures, but not the concentric multilamellar vesicles demonstrated for the **13b**/DOPE–DNA complexes (Pitard et al., 1999).



The analysis of transfection of a wide range of cell lines with **13b**/DOPE-DNA complexes revealed the high efficiency of this process, which is comparable with the commercially available transfectants and 10–20-fold exceeds the calcium phosphate precipitation protocol (Vigneron et al., 1996; Ouderhiri et al., 1997). Mediated by guanidinium-cholesterol lipids gene transfection is appropriate for the mammalian airway epithelium (Ouderhiri et al., 1997). The positive results of the transfection into primary human cells *in vitro* and into the mouse airways cells *in vivo* confirm the potential of the cationic lipids in relation to lung-directed gene therapy.

Aminoglycosides, natural polyamines that are known to bind to nucleic acids, represent a favorable scaffold for the synthesis of a variety of cationic lipids. The synthesis of a cationic cholesterol derivative of kanamycin A and its polyguanidinylated derivative was recently described (Belmont et al., 2002). The amino-sugar-based cationic lipid **20a** demonstrated a high level of TE in terms of gene transfection of a variety of mammalian cell lines when used either alone or as a part of a liposomal formulation with helper-lipid. In addition, colloidally stable kanamycin-cholesterol/DOPE lipoplexes were found to be efficient for gene transfection into the mouse airways *in vivo*.

Designed cholesterol-based kanamycin A analogues (**20b,c**) bearing various linkers between the aminoglycoside headgroup and the cholesterol moiety were prepared (Sainlos et al., 2005). It was successfully shown, that the length and nature of the spacer can affect the physicochemical and biological properties of the lipoplexes. The incorporation of a longer spacer into the structures of cholesterol derivatives of kanamycin A can yield the lipoplexes with improved in terms of TE physicochemical properties. The cholesterol derivatives **20b,c** were successfully used for the transfection of mouse airway epithelium *in vivo*. But the beneficial effects of the longer spacers observed *in vitro* with **20b,c** were not found *in vivo*, as only **20c** yielded levels of transgene expression higher than those obtained with **20a**.

2.6 Colesterol-PEI conjugates

Polyethyleneimine (PEI) is polycation widely used for DNA compaction and delivery. The high transfection efficiency of PEI was firstly demonstrated by Behr and co-workers (Boussif

et al., 1995). PEI is well-known for its ability to compact DNA and to facilitate its early endosomal release, preventing the delivered DNA from degradation in the late endosomes (Kichler, 2004). The reason for the good transfection activity of PEI is the presence of the primary, secondary, and tertiary amino groups. These groups have different p*K*a values, which give the PEI a good buffer capacity, therefore not allowing a decrease in the pH value in the early endosomes (Kichler, 2004).

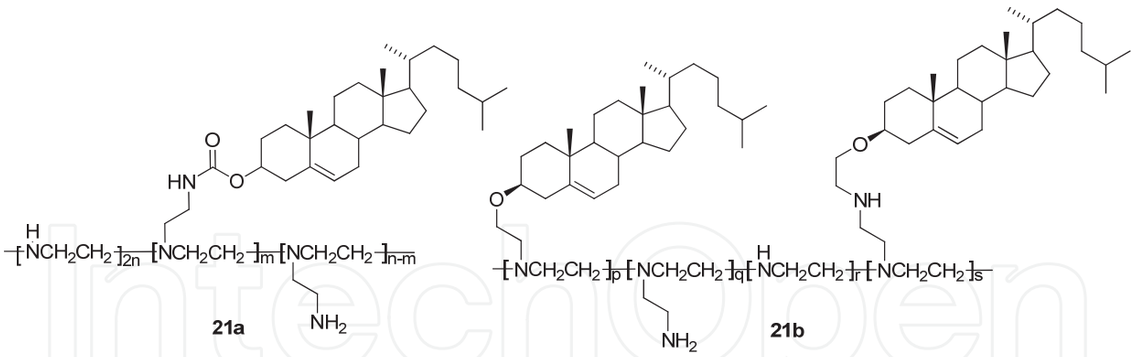
In literature several kinds of the PEI-cholesterol conjugates have been described. Kim and co-workers (Han et al., 2001) synthesized water-soluble lipopolymer (WLSP (**21a**)) in the reaction of the branched PEI (mw 1.8 kDa) and cholesteryl chloroformate. The average molecular weight of WSLP (**21a**) was approximately 2 kDa and the extent of modification with cholesterol was ~ 0.5. The WSLP (**21a**)-pDNA complexes were characterized by low toxicity *in vitro* and they induced the aggregation of erythrocytes to a lesser extent, as compared to PEI 25 kDa. WSLP (**21a**)-pDNA complexes demonstrated higher TE in both CT-26 and 293 T cells compared to PEI 25 kDa- or PEI 1.8 kDa-based formulations. As an experimental model for the estimation *in vivo* of the biological activity the authors used the antitumor activity of IL-12 coded by the pDNA transfected into the tumor cells. As a result of the injections of the WSLP (**21a**)-pDNA complexes into mice tumors a significant decrease in the tumor growth rate (~20% higher, as compared to the administration of the *naked DNA*) and of the level of metastasis formation were observed; resulting in higher survival rates of the animals after treatment (Mahato et al., 2001; Janat-Amsbury et al., 2005*)*. Later data confirming the high efficacy of WSLP (**21a**) for the transfection of siRNA *in vitro* and *in vivo* were obtained (Kim et al., 2007).

The same authors endeavored to design a more effective transfection reagent based on branched PEI (bPEI) using chemically protected primary amine and conjugation of cholesterol at the secondary amino groups of PEI. However, contrary to expectations, the presence of the non-modified primary amino groups only resulted in a slight increase (1.5-fold) of the TE (Wang et al., 2002).

Kim and co-workers obtained partially contradictory data (Kim et al 2001). In this work a simple one-step synthetic procedure was used to yield myristyl and cholesterol derivatives of PEI having molecular mass 2 kDa. According to the obtained data these modified PEI efficiently transfected cell *in vitro* and displayed lower toxicity when compared to bPEI 2 kDa; however the compounds were less tolerated by cells than the parent bPEI 2 kDa.

Fewell and co-workers studied the properties of the PPC conjugates, containing the bPEI 1.8 kDa, cholesterol and PEG, where the PEGylation extent varied from 0.6 to 20 PEG molecules to one bPEI molecule (Fewell et al., 2005*)*. The highest transfection efficiency *in vivo* was observed for the PPC conjugate, where the molar ratio bPEI:cholesterol:PEG was 1:1:2. When using the conjugates containing a large quantity of PEG molecules, a decrease of the reporter gene expression was observed. The variation of the number of the cholesterol residues in the conjugates was not performed: all the tested conjugates contained the bPEI and cholesterol at the ratio of 1:1. In order to study the TE of these molecules *in vivo* the authors used a biological model similar to the one described in (Mahato et al., 2001) and also demonstrated the efficient inhibition of the tumor growth.

Furgeson and co-workers also used linear PEI for the synthesis of new cholesterol-containing conjugates apart from bPEI. The polymer, obtained in the reaction of a low molecular weight lPEI (423 Da) with cholesterol chloroformate, was used for the preparation of the water insoluble liponanoparticles using DOPE as a lipid-helper (Furgeson et al., 2002). The *in vitro* TE of nanoparticle-pDNA complexes was ~4-fold higher, in comparison with bPEI 25 kDa.



T-shaped and L- (linear) shaped PEI 25 kDa conjugates with cholesterol (PEIC) were synthesized and thoroughly studied, revealing the improved transfection and reduced cytotoxic effect, partially a result of the sequestering of charged secondary amines of PEI, in the presence of cholesterol moiety (Furgeson et al., 2003*)*. The modification extent of the PEI carrier in this study ranged from 1 to 2 cholesterol molecules per PEI. Polyplexes, formed by L- and T shapes PEICs with DNA possessed a higher TE *in vitro*, in comparison with the initial linear polymer; as well as the bPEI with the same molecular weight. The highest TE among the compounds tested was observed for L-PEIC: the expression of the reporter gene, delivered into the Renca cells using this conjugate was 32-fold higher in comparison with the expression observed in the presence of bPEI. The authors proposed that the differences in the TE depended on the conformational changes of the PEI molecule in the presence of the hydrophobic substitues. It was shown that the PEIC conjugates penetrated into the cells *via* the interaction with LDL receptors. The high TE of the LPC was confirmed *in vivo* in experiments with systemic and local administration of the polyplexes (Furgeson et al., 2004*)*
Multiple modifications of PEI's amines were commonly avoided, due to the importance of the cationic charge for DNA condensation and buffering capacity of the polymer. Therefore quantitative data characterizing the impact of cholesterol conjugation was not yet available. In connection with this, we attempted to find the optimal extent of modification of the 25 kDa PEI with cholesterol. Conjugates of PEI bearing a different number of cholesterol residues with 0.5 to 20% of amines modified were synthesized. We found that a small number of cholesterols attached to PEI (extent of modification was 0.5 or 1%) significantly increased the TE of the polymer, while extensively modified PEI-cholesterol conjugates demonstrated reduced TE, although possessing lower cytotoxicity (Gusachenko (Simonova) et al, 2009). TE studies were performed using different types of biologically active nucleic acids: single stranded oligonucleotide, plasmid DNA and siRNA duplex. The most promising conjugates in the series were found to be PEIC 0.5 and PEIC 1 demonstrating the best combination of TE with lower cytotoxicity.
The aforementioned PEI-cholesterol conjugates were prepared in the reaction of PEI, having various molecular weights and cholesteryl chloroformate. The PEI-lipid conjugates (**21b**) based on ether-linked cholesterol units were first described by Bhattacharya and co-workers (Bajaj et al., 2008b). Nine PEI-cholesterol-based conjugates having polymer amine backbone linked to the cholesterol unit via the ether link were synthesized. Three low molecular weight PEIs were used for the synthesis of these lipopolymers. The TE studies in HeLa cells showed a high potency and low cytotoxicity of these lipopolymers in comparison with the commercially available PEI. The TE of PEI 25 kDA decreased in the presence of a high percentage of serum, whereas PEI-cholesterol-based lipopolymers were discovered to be

effective, even in the presence of 50% of serum. The TE and cytotoxicity of the lipopolymers were found to depend on the percentage of cholesterol moieties and the molecular weight of PEI used for the synthesis of lipopolymers. Optimized lipopolymer-DOPE formulations exhobited a higher cell viability and high TE, which was unaffected by serum: the TE of the lipopolymers was obviously one of the highest among known non-viral delivery systems.

3. Physico-chemical properties of lipoplexes and their influence on the transfection efficiency

It is commonly known, that the efficiency of liposome-mediated gene delivery is determined not only by the structure of cationic and helper lipids or properties of the transfected plasmids, but also by the size of the lipoplex and its ζ-potential. The structure of the supramolecular DNA–lipid complexes is dependent upon both the external (pH, degree of hydration, temperature, and the presence of doubly charged cations, i.e., Ca^{2+}, Mg^{2+}) and internal factors. The physico-chemical properties can alter the lifetime, distribution, and the TE efficacy of lipoplexes. Thus, in order to shed more light on the mechanism of transfection and to elucidate the structure-activity relationships, it is necessary to investigate a number of physico-chemical parameters of the lipoplexes.

Using a set of physico-chemical methods, it was demonstrated that condensation and compactization of DNA by DC-Chol (**1a**)/DOPE cationic liposomes is a result of a strong entropically-driven surface electrostatic interactions. Fluorescence anisotropy results have revealed that low cationic lipid contents in the liposomes tend to favor more fluid bilayers; which in turn are potential advantages for transfecting cells. DC-Chol/DOPE-DNA lipoplexes are represented by supramolecular complexes with a different morphology: DNA-coated unilamellar lipoplexes, lipoplex nanostructures with thickened, flattened, and deformed walls, and also multilamellar lipoplexes with or without open bilayers (Rodriguez-Pulido et al., 2008). The effects of hydration and temperature on the structure of DC-Chol/DOPE–DNA lamellar lipoplexes were also investigated (Pozzi et al., 2006). The DNA complexation and condensation properties of two established cationic liposome formulations, CDAN (**18d**)/DOPE (50:50, *m*/*m*; Trojene™) and DC-Chol (**1a**)/DOPE (60:40, *m*/*m*), were studied by means of biophysical methods (Keller et al., 2003). The results provide a suitable framework for the understanding of why CDAN/DOPE cationic liposomes are exceptionally efficient, in comparison with other cationic lipid-based systems, at mediating cell transfection. The liposomes CDAN (**18d**)/DOPE formed the metastable lipoplexes, exhibiting greater transfection efficiency *in vitro* in the presence of 10% serum, in comparison to DC-Chol/DOPE liposomes. This metastability may be related to the unusually low p*K*a value of 5.7 of amino groups. In addition, it was supposed that CDAN (**18d**)/DOPE-pDNA particles may have a greater tendency to interact with negatively charged serum components and facilitate the DNA release from endosomes (Keller et al., 2003).

A critical factor in the lipid-mediated gene delivery is the structural and phase evolution of lipoplexes upon interaction and mixing with anionic cellular lipids (Tarahovsky et al., 2004; Koynova et al., 2005, 2006; Koynova & MacDonald, 2007). Such a structural rearrangement is supposed to play a central role in the DNA escape process; i.e. how DNA dissociates from lipoplexes and is released into the cytoplasm. The structural and phase evolution of lipoplexes upon interaction with lipid mixtures similar to real membranes and DNA release

were investigated using liposomal formulations DC-Chol/DOPE, DC-Chol/DMPC, DOTAP/DOPC, DOTAP-DLPC, DOTAP/DOPE, prepared at different molar ratia. (Pozzi et al., 2009). It was shown that the most unstable lipoplexes (DOTAP/DOPC/DNA) rapidly release DNA, while the most stable ones (DC-Chol/DOPE/DNA) exhibit a lower degree of DNA release. Therefore, the results can be generalized as follows: the higher the structural stability, the lower the extent of DNA release. Using the SAXS technique it was demonstrated that the dilution of the DNA lattice takes place upon lipoplex interaction with anionic lipids, which is unequivocal proof of the charge neutralization of cationic lipids by anionic membranes (Banchelli et al., 2008; Lundqvist et al., 2008). Upon further interaction, disintegration of lipoplexes by anionic lipids as well as the formation of nonlamellar phases in lipoplex/anionic lipids mixtures are strongly affected by the shape coupling between lipoplexes and anionic lipids. Furthermore, coupling between the membrane charge densities of lipoplexes and anionic membranes contributes greatly to regulating the evolution of lipoplexes/anionic lipids mixtures and the release of plasmid DNA (Pozzi et al., 2009).

3.1 Influence of size

The data found in literature, describing the influence of the size of lipoplexes on the TE are contradictory. A number of researchers have argued that either size of the formed lipoplexes is not associated with TE, or that TE is not affected by initial lipoplex size (Han et al., 2008; Kearns et al., 2008; Malaekeh-Nikouei et al., 2009). Some researchers posit that large lipoplexes possess higher TE in comparison to the smaller ones. For instance, Ross et al. demonstrated that TE and cell uptake increased with the increase of the size of lipoplexes (Ross & Hui, 1999). The study of the CDAN(**18d**)/DOPE liposomes demonstrated the ability of the liposomes to form large complexes with plasmid DNA, which are characterized by the tendency for the sedimentation on the cell surface resulting in the increase of the TE (Keller et al., 2003). When siRNA was used as cargo for delivery into cells, the lipoplexes with a size between 60 and 400 nm were obtained and no influence of the lipoplex size on the efficacy of gene knockdown was observed (Spagnou et al., 2004). It was previously reported by Kawaura et al. that vesicles of a moderate size (0.4-1.4 micron) exhibit higher TE in terms of gene delivery (Kawaura et al., 1998). The data supporting the higher TE of the large lipoplexes were also reported by other researchers (Ding et al., 2008).

When the formation of complexes has been performed in physiological ionic strength conditions, compared with 40 mM Tris buffer, the size of lipoplexes can be significantly increased (Kearns et al., 2008). In contrast, the presence of serum could slightly decrease the size of the lipoplexes (Han et al., 2008).

Conversely, some researchers have demonstrated that smaller lipoplexes were more efficient (Salvati et al., 2006). We studied the correlation between the size of the cationic lipids **6a-f**/nucleic acid complexes and their TE (Medvedeva, et al., 2009). The ability of the cationic lipids to deliver plasmid DNA is dependent upon the size of the lipids/DNA complexes formed in solution, which is consistent with that the maximum endocytosis by non-specialized cells requires that the particle size is below 100 nm (Chen et al., 2007). The lipid **6a** formed the smallest complexes with the plasmid DNA, characterized by a narrow size distribution; this lipid exhibited the highest TE. The lipids **6b** and **6d** formed with plasmid DNA the complexes with a wide size distribution and a large fraction of small particles inferior to 50 nm; these lipids display moderate TE. A reduction in the size of

liposomes, might facilitate their penetration through the physiological barriers, after the administration *in vivo*, in addition to the possibility of passively targeting the tumor sites, which is facilitated by the enhanced permeability of blood vessels and retention (EPR) effect (Gullotti & Yeo, 2009).
It was demonstrated that the size of latex particles has a significant effect upon the efficiency of cell uptake and the mode of the endocytic pathway (Rejman et al., 2004). Particles that have a size of 500 nm penetrated into the cell via the caveolae-mediated endocytosis. On the contrary, the microspheres having a diameter of 200 nm or less and preferred the clathrin-mediated endocytosis, were found to accumulate in the lysosomal compartment. The precise control of the size of lipoplexes is important for further intracellular fate of lipoplexes that determine by-turn their transfection efficiencies (Rejman et al., 2006).

3.2 Influence of surface charge

The colloidal stability of the lipoplexes is determined by the surface charged of the particles, which can be expressed as zeta potential (ξ-potential). The value of ξ-potential can be changed from negative to positive depending on the N/P ratio (ratio describing the number of the negatively-charged phosphate groups of the nucleic acid to the positively charged groups of the amphiphile). N/P ration contributes significantly to the delivery of nucleic acids into cells. The complexes having a neutral charge are usually characterized by a large size and a low TE, as a result of a tendency to form aggregates and to precipitate (Salvati et al., 2006). A small redundant positive charge of the lipoplexes facilitates the efficient interaction with the negatively charged components of the cell membrane, as well as the transport *via* the cell membrane.
A direct correlation was observed between the value of ξ-potential and TE, when studying the transfection in vitro. It was demonstrated that the cationic liposomes formed by lipid **2a** had the highest ζ-potential in comparison with liposomes formed by lipids **1a,b, 2b,c** and **3a,** and exhibited the highest TE with respect to HeLa, COS-7, and NIH 3T3 cells (Takeuchi et al., 1996). However the dependence of the structure of the lipid, ξ-potential and the TE was not always obvious (Kearns et al., 2008; Malaekeh-Nikouei et al., 2009).

3.3 Influence of physico-chemical parameters on the TE *in vivo*

The low transfection efficiency *in vivo* is one of factors that hinders the design of an efficient liposomal gene delivery systems. Negatively charged components of serum could interact with cationic liposome and compete with DNA for cationic liposome binding, leading to a decrease in TE. Other destructive effects of serum components attributed to its interaction with lipoplexes and early release of DNA from lipid shielding bilayer that reduces the TE. Moreover, the released nucleic acids could be recognized by Toll-like receptor expressed in B cells and dendritic cells; resulting in toxicity *via* induction of the cytokine production (Tousignant et al., 2000). Lipoplexes were reported to be generally more than 100 nm in diameter, as well as tend to self-aggregate in the blood stream, which resulting in limited passage through the vessel walls (Pouton & Seymour, 2001).
The difference between the optimal transfection parameters of *in vitro* and *in vivo* due to the profound difference of the biochemical characteristics between the cells and the organism, is the most severe problem associated with the use and practical implementation of cationic lipid or liposomes for the treatment of genetic and acquired diseases. In the case of monocationic lipids there is a single reference that the *in vivo* result corresponded to the *in*

vitro one (Ding et al., 2008) and positively charged lipoplexes are significantly more effective than negatively charged ones. Other investigations revealed that the best TE *in vivo* corresponds to a low N/P ratio, small size of lipoplexes, and negative ξ-potential (Hattori et al., 2007; Gao & Hui, 2001). It is noteworthy when considering polycationic lipids, that lipoplexes that have a total negative charge and small size (200–300 nm) are optimal for both *in vitro* and *in vivo* transfection. (Stewart et al., 2001).

It was revealed that the efficient gene delivery by polycationic lipids (Cooper et al., 1998) *in vivo* requires the cationic liposome systems, which are able to bind DNA more tightly than for the *in vitro* delivery. In comparison to CDAN(**18d**)/DOPE and DC-Chol(**1a**)/DOPE liposomes, CTAP(**18g**)/DOPE was able to neutralize, condense and encapsulate nucleic acids into lipoplex particles with a high efficiency. SDS stimulated the DNA release from a lipoplex and revealed the structure-activity relationships between the TE and lipid shielding of DNA (Bajaj et al., 2008c). Low shielding could facilitate the release of DNA and its hydrolysis within the cells. Certainly, this characteristic could be expected to be useful *in vivo,* given the greater complexity of the extracellular environment *in vivo* as compared to *in vitro*. Caminiti and co-workers demonstrated that both unstable and lipoplexes that are too stable, result in a strong and poor DNA release respectively, and exhibit a low transfection efficiency (Caracciolo et al., 2007).

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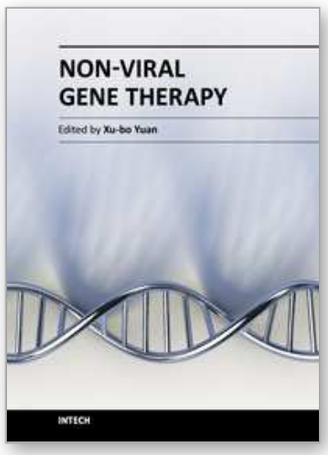
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